

***In vitro* metabolism studies to inform physiologically-based pharmacokinetic modelling of mefloquine, ritonavir and proguanil**

Cleavon K. Cloete

Supervisor: Dr Mathew Njoroge

Department of Chemistry, University of Cape Town

Co-Supervisor: Prof Kelly Chibale

Department of Chemistry, University of Cape Town

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Declaration

I hereby declare that this thesis is my own original work produced from research undertaken under supervision and has not been presented for a degree in this university or another university elsewhere for the award of any other degree.

Dedication

To my mother:

Zaundelia Sheree Cloete

Who passed on during the writing of this thesis. You were my rock and guiding light. Everything I am, I am because of you. You are the best in me and everything I have achieved is because of you. I love and miss you and I know that you are proud of me.

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Abstract

Clinically observed variability in drug exposure has various negative implications, ranging from an increased possibility of adverse drug reactions to treatment failure. A key contributor to this variability is the contribution of drug metabolism, substantially mediated by the cytochrome P450 (CYP) enzyme superfamily. Thus, a valuable step in assessing the contribution of drug metabolism to variability in drug exposure is the measurement of the kinetics of CYP-mediated metabolism. However, this *in vitro* data needs to be translated to whole body *in vivo* clearance, which is also impacted by the physicochemical properties of a drug and how the drug interacts with physiological environments in the body. One method of doing so is through the use of physiologically based pharmacokinetic (PBPK) modelling. By combining *in vitro* data with available knowledge on the physicochemical properties of a drug PBPK modelling provides a mechanistic understanding of drug exposure.

As such, the *in vitro* metabolic characteristics of 3 commonly used drugs, namely mefloquine, proguanil and ritonavir, were measured. This was done by measuring the microsomal metabolism of these drugs in human liver microsomes, then determining the fraction of the drug metabolized by CYP (fm,CYP) isoforms and confirming this metabolic pattern using recombinant CYP enzymes.

In the case of mefloquine, it was found that the drug was metabolized mainly by CYP3A and CYP1A2. While mefloquine metabolism by CYP3A is widely reported, this is the first report of its metabolism by CYP1A2. Proguanil was found to be metabolized by a number of enzymes, namely CYP1A2, CYP2D6, CYP2C19 and CYP3A. This confirms reported data for CYP2C19 and CYP3A, and provides new data for CYP1A2 and CYP2D6. Lastly, ritonavir was found to be metabolized by CYP2D6 and CYP3A.

These determined fm,CYPs were then used, along with available data from literature, to develop PBPK models for mefloquine, proguanil and cycloguanil as a metabolite of proguanil using a middle-out approach with the use of Simcyp modelling software. These models were verified by comparing the pharmacokinetic (PK) profiles they simulated to various PK profiles found in literature. The verified models were then applied to various scenarios of interest.

In the case of mefloquine, these applications showed that *in vitro* data does not accurately scale to *in vivo* clearance which excludes the possibility of doing a bottom-up model for this compound. The verified PBPK model built using this data demonstrates the expected drug-drug interaction between mefloquine and a CYP1A2 inhibitor, fluvoxamine. The model also

shows the limited effect of population differences on mefloquine PK, including the reported finding that polymorphisms of CYP3A5 do not affect mefloquine PK. Disease state may therefore have a bigger effect on the variability in mefloquine PK than variability in metabolism does.

The model application for proguanil focussed on the potential effects of various CYP genotypes on proguanil metabolism given the number of polymorphic CYPs found to be involved in its metabolism. The effect of CYP2C19 polymorphisms on proguanil and cycloguanil exposure could be clearly demonstrated. Variability in metabolism by other CYPs does not have as big an impact on proguanil exposure, likely because of their lower contributions to the metabolism of the drug.

Finally, a previously developed and verified ritonavir model was used, in conjunction with the mefloquine model developed and verified here, to explore the use of ritonavir as an inducer and inhibitor of CYP3A activity. This showed the limited ability of ritonavir to induce mefloquine metabolism, with it being a more capable inhibitor. The ability of ritonavir to induce CYP1A2 activity was also explored using the available model by optimizing its CYP1A2 induction capabilities through parameter optimization. This showed the importance of taking this induction into account when conducting DDI studies between ritonavir and substrates of CYP1A2.

Overall, this work addresses gaps in the understanding of the *in vitro* metabolism of mefloquine, proguanil and ritonavir, and demonstrates how this data can be combined with PBPK modelling to understand and predict PK variability.

List of Abbreviations

°C	Degrees Celsius
µg	Microgram
µL	Microliter
µM	Micromolar
ADAM	Advanced Dissolution Absorption and Metabolism
ACN	Acetonitrile
ADH	Alcohol Dehydrogenase
ATV/r	Atazanavir/ritonavir
AUC	Area under the curve
B/P ratio	Blood to plasma concentration ratio
CYP	Cytochrome P450
cDNA	Complimentary DNA
CE	Collision energy
CI	Confidence Interval
CL_{int}	Intrinsic clearance
CL_{iv}	Intravenous clearance
C_{max}	Highest concentration of drug in blood
CL_r	Renal Clearance
CPPGL	Cytosolic protein per gram liver
CPB	4-chlorophenylbiguanide
DNA	Deoxyribonucleic Acid
DDI	Drug-drug interaction
DMSO	Dimethyl sulfoxide
DP	Declustering Potential
EM	Extensive metabolizers
EP	Entrance potential
EPI	Enhanced product ion
ESI	Electrospray ionization
F_a	Fraction available from dosage
FDA	US Food and Drug Administration
FIA	Flow injection analysis
FMO	Flavin-Containing Monooxygenase

List of Abbreviations

fm,CYP	Fraction of substrate metabolized by cytochrome P450 enzyme
f_u	Fraction unbound in plasma
$f_{u(gut)}$	Unbound fraction in enterocytes
$f_{u(inc)}$	Fraction unbound <i>in vitro</i>
HIV	Human Immunodeficiency Virus
HLM	Human liver microsomes
HPGL	Hepatocellularity per gram liver
HV	Healthy Volunteers
IM	Intermediate metabolizers
Ind _{Max}	Maximal fold induction
ISEF	Intersystem extrapolation factor
ISTD	Internal Standard
IVIVE	<i>in vitro-in vivo</i> extrapolation
k_a	Absorption rate constant
k_{dep}	Substrate depletion constant
K_i	Inhibition constant
k_{inact}	Inactivation rate constant
K_M	Michaelis constant
KTZ	Ketoconazole
L/kg	Liters per kilogram
LC-NMR	Liquid Chromatography Nuclear Magnetic Resonance
LC-MS/MS	Liquid Chromatography tandem mass spectrometry
LPV/r	Lopinavir/ritonavir
MD	Multiple Dose
MeOH	Methanol
MFQ	Mefloquine
MgCl ₂	Magnesium chloride
min	Minutes
mg	Milligram
mg/L	Milligram per liter
mg/L·h	Milligram per liter hours
mg/mL	Milligram per milliliter
mL	Milliliter

List of Abbreviations

mM	Millimolar
MIST	Metabolite in safety testing
MPPGL	microsomal protein per gram liver
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSS	Metabolite stock solution
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	<i>N</i> -acetyl transferase enzyme
NCA	Non-compartmental analysis
ND	Not determined
nM	Nanomolar
NR	Not reported
PB	Phosphate Buffer
PBPK	Physiologically based pharmacokinetic
$P_{\text{eff,man}}$	Effective permeability in man
PK	Pharmacokinetic
pK_a	Acid dissociation constant
PM	Poor Metabolizer
pmol	Picomole
pmol/mL	Picomoles per milliliter
pmol/mg/min	Picomoles per milligram protein per min
pmol/mg/pmol	Picomoles per milligram protein per picomole enzyme
Q_{gut}	Nominal flow in gut model
RAF	Relative activity factor
rcf	Relative centrifugal force
RIF	Rifampicin
rpm	Revolutions per minute
RTV	Ritonavir
rCYP	Recombinant cytochrome P450 enzyme
RTT	Reverse Translational Tool
S9PPGL	S9 protein per gram liver
SD	Single Dose

List of Abbreviations

SNP	Single nucleotide polymorphisms
TB	Tuberculosis
UGT	Uridine 5'-diphospho-glucuronosyltransferases
UM	Ultra-rapid metabolizers
V_{ss}	Volume of distribution at steady-state
V_{max}	<i>In vitro</i> maximum enzyme velocity
WHO	World Health Organization
WS	Working solution

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Chapter 1 Introduction and Literature Review

The success of a drug *in vivo* is not only dependent on its efficacy, but also on the extent and duration of its exposure. The pharmacokinetic (PK) properties of a drug are critical to this and, in turn, also play a significant role in drug failure when they are unsatisfactory.¹ As such, an early analysis of these properties provides a great source of useful information towards reducing the chances of drug failure in the early development stages. While there are many factors that influence PK, drug metabolism is one of the primary sources of variability in PK profiles.

1.1 Drug Metabolism

Metabolism can be defined as the biotransformation of a compound so that it can be removed from the body. In humans, this biotransformation is carried out by a wide variety of drug metabolizing enzymes, found primarily in the liver but also in various other organs including the kidneys, lungs and intestines.² Drug metabolism has traditionally been classified as occurring in phases, namely phase I and phase II.

Phase I drug metabolism involves functionalization reactions such as oxidation, reduction, or hydrolysis in which a functional group is created or unmasked on the compound.³ This increased substrate hydrophilicity contributes to the removal of the compound from the body. These groups can also serve as sites for conjugation reactions in phase II.

Conjugation reactions, which make up phase II metabolism, are characterized by the addition of polar endogenous groups, such as glucuronic acid, to the compound. These reactions result in the formation of a covalent linkage between a functional group on the substrate or one introduced to the substrate through a phase I reaction.³ In contrast to phase I reactions, which typically only result in a slight increase in substrate hydrophilicity, phase II reactions greatly increase the water solubility of the substrate.⁴ Figure 1.1 below illustrates a variety of phase I and phase II reactions.

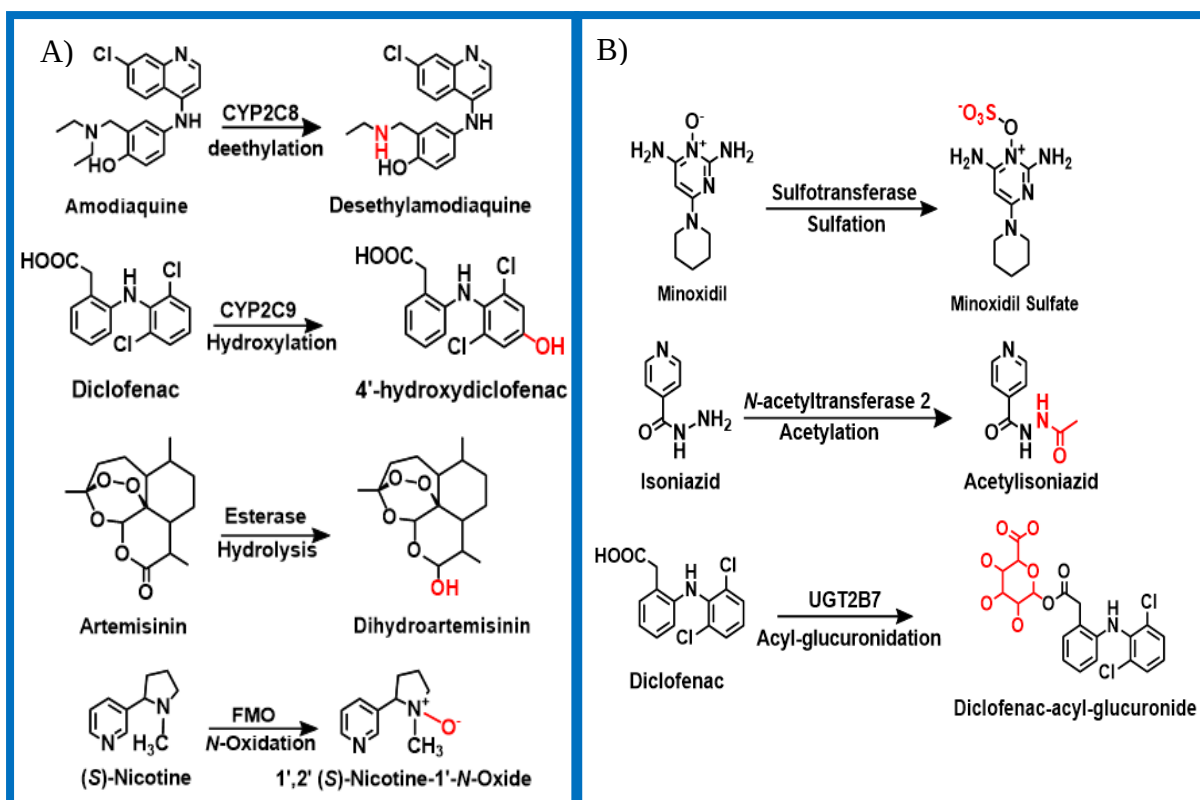


Figure 1.1: Common phase I (A), and phase II (B) reactions

Despite phase II reactions often involving the product formed from phase I metabolism, it can also occur directly on the substrate. It should also be noted that a drug needn't undergo both phase I and II metabolism for it to be removed. Undergoing one of the two is often enough for many drugs.⁴ In addition, hydrophilic drugs, such as atenolol, metformin and ampicillin, can be excreted from the body directly without undergoing any form of drug metabolism.⁵

Hydrophilic drugs, on the other hand, can undergo excretion directly, without the need for metabolic changes to their molecular structures.

1.1.1 Drug metabolising enzymes

Phase I and II metabolism reactions are mediated by a variety of enzymes in the body. Some of these enzymes, as well as which of the two phases of metabolism they carry out, are captured in Table 1.1 below.

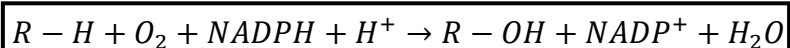
Table 1.1: Summary of different drug metabolising enzymes

	Enzyme	Role in metabolism	Example of substrate
Phase I	Cytochrome P450 (CYP)	Mixed-function oxidation enzymes largely responsible for the oxidation or reduction of xenobiotics. ^{2,6}	Dextromethorphan
	Flavin-Containing Monooxygenase (FMO)	Oxidative species with functions complementary to CYPs, although with narrowed range of substrates. ²	Voriconazole
	Aldehyde Oxidase and Xanthine Oxidoreductase	Molybdenum containing involved in the oxidation of several aldehydes and heterocyclic species. ⁶	Methotrexate, Allopurinol
	Alcohol Dehydrogenases (ADH)	Has a broad specificity for primary and secondary alcohols. Main enzyme involved in the metabolism of ethanol in humans. ⁶	Ethambutol
	Esterases	A large and heterogeneous family of enzymes involved in the cleavage of esters in xenobiotics. ²	Aspirin
Phase II	UDP-Glucuronosyltransferases	Conjugative enzyme that adds glucuronic acid to xenobiotic on various functional groups such as alcohols, amines and phenols among others. ²	Morphine
	Sulfotransferases	Enzymes that carry out sulfonation reactions where 3'-phosphoadenosine-5'-phosphosulphate is used as a cofactor. These take place on C- and N- alcohols as well as -NH side chains to yield O- and N- sulfates. ²	Acetaminophen
	N-Acetyl Transferases	A group of enzymes that acetylate xenobiotics with the use of acetyl-CoA as a cofactor. The acetyl group is moved from the acetyl-CoA to form an acetylated enzyme intermediate, after which the substrate is acetylated and the CoA is released. ⁴	Isoniazid
	Glutathione-S-Transferases	Conjugative enzymes that catalyse the reaction of glutathione, the cofactor used in this reaction, with electrophiles to form glutathione conjugates by activating the thiol group of glutathione into the more reactive thiolate anion within their active site. ²	Cisplatin

However, the main enzyme family involved in phase I drug metabolism are the cytochrome P450s (CYP).⁷

1.1.2 Cytochrome P450s

Cytochrome P450s are responsible for the oxidative metabolism of approximately 90% of clinically used medications.⁷ They are heme-thiolate proteins which, at their active sites, contain a prosthetic ferroporphyrin IX ring containing a reduced iron (Fe) atom at its centre which is bound to four nitrogen atoms.⁸ They have two free coordination sites for bonding to a conserved cysteine residue from the surrounding protein chain and to substrate atoms wherein they catalyse the chemical addition of a single oxygen atom to a wide variety of endo- and exogenous substrates in a reaction simplified and described below:⁸



These enzymes are grouped into families, subfamilies, and isoforms according to amino acid sequence homology. Families are designated by a number, subfamilies by a letter, and individual enzymes by a number as well.^{9,10} Despite there being as many as 57 functional human CYP genes,¹¹ it has been found that three families, namely CYP1, 2, and 3, are the main contributors to human oxidative metabolism.⁹ While good agreement can be found when assessing the abundances of CYPs in the liver, these abundances are characterized by large interindividual differences.¹² In addition, there are several genetic and non-genetic factors that influence this variability; and by extension the outcome of clinical therapies.¹³

As previously stated, these enzymes are also responsible for over 90% of the oxidative metabolism of drugs in humans with the relative activity determined to be, in approximation: CYP1A2 (4%), CYP2A6 (2%), CYP2C9 (10%), CYP2C19 (2%), CYP2E1 (2%), CYP2D6 (30%), and CYP3A (50%). The relative contributions of CYP enzymes to the metabolism of clinically used drugs is illustrated in Figure 1.2.¹⁴

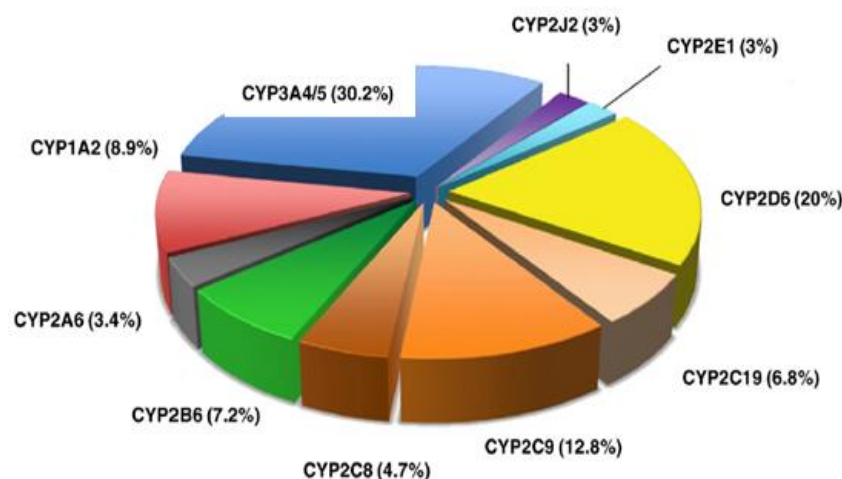


Figure 1.2: Fraction of clinically used drugs metabolized by CYP isoforms. Adapted from and used with permission. (Zanger and Schwab, 2013)

CYPs are found in nearly all tissues in the body. A few organs in which they are found in considerable expression levels are the intestines, lungs, kidneys, skin, heart and brain.¹⁵ However, the liver has the highest expression of CYP and other drug metabolizing enzymes.⁴

1.1.3 Role of metabolites in drug therapy

Drugs that undergo metabolism are subject to a variety of fates. These include the formation of inactive metabolites, which are devoid of any pharmacological or toxicological activity, the formation of active metabolites, which retain pharmacological activity at the same site of action as the parent drug, and the formation of toxic metabolites, which are chemically reactive species that can be short-lived but are characterised by their toxicological activity.^{16,17}

1.1.3.1 Inactive metabolites

Inactive metabolites are characterized by their lack of toxicological or pharmacological activity. They are usually less potent than the parent drug from which they were formed and are often more readily cleared from the body, into the bile, urine, and faeces, because of the metabolism they underwent. These metabolites are also usually chemically stable.

For most drugs, metabolism results in the formation of inactive metabolites and thus acts as a clearance process, reducing pharmacological activity over time.^{17,18} As an example, the metabolism of paracetamol, a widely used analgesic, at therapeutic doses results in the formation of several inactive metabolites, such as acetaminophen sulfuric acid and acetaminophen glucuronic acid.¹⁹ These are then readily excreted via the kidneys in urine.¹⁹

1.1.3.2 Active metabolites

The formation and study of active metabolites is an important aspect within drug discovery. Indeed, the use of prodrugs, which is an approach where drug metabolism is used to convert pharmacologically inactive or less active compounds into more pharmacologically active metabolites, is an area of study within drug discovery. These pharmacologically active metabolites are often developed as drugs after their discovery. Examples of active metabolites that were developed as drugs include atorvastatin (Lipitor®),²⁰ and cetirizine (Zyrtec®), with the latter being developed from the clinically used hydroxyzine (Atarax®). The structures of these drugs are seen in Figure 1.3 below.²¹

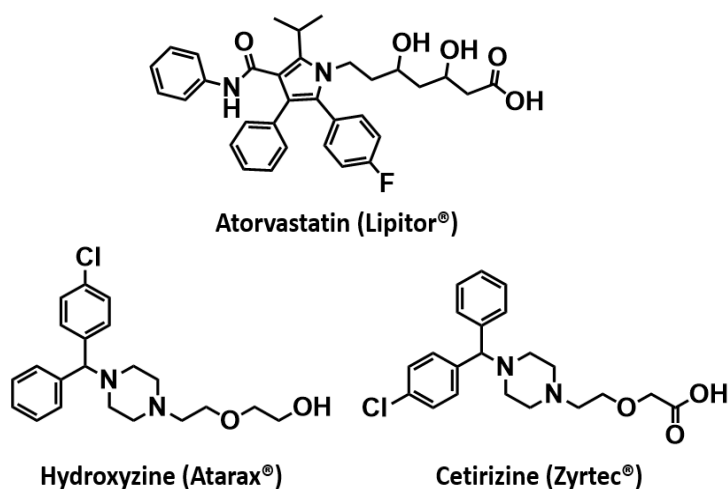


Figure 1.3: Chemical structures of atorvastatin, hydroxyzine and cetirizine.

Active metabolites may also have improved pharmacological or PK and safety profiles relative to the parent compound.^{17,18} An example of this is the metabolism of the opioid codeine. *O*-demethylation of codeine leads to the formation of morphine, a more potent analgesic.^{17,18} Further, glucuronidation of morphine leads to the formation of morphine-6-glucuronide, which is even more potent.¹⁷ It should be noted that morphine-3-glucuronide, another potential product of morphine glucuronidation, is inactive. This series of reactions is illustrated in Figure 1.4 below.

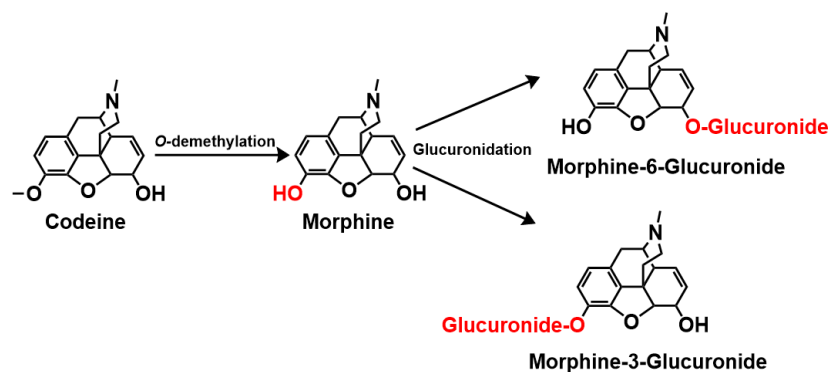


Figure 1.4: Overview of codeine metabolism which results in multiple active metabolites.

1.1.3.3 Toxic metabolites

Drug metabolism can also lead to the formation of chemically unstable reactive metabolites that have the ability to interfere with cellular functions or certain cellular macromolecules such as proteins and nucleic acids.^{16,22} This in turn may lead to carcinogenicity, cellular necrosis and teratogenicity.¹⁶

A wide variety of drug metabolizing enzymes can generate toxic metabolites with CYPs playing a key role. Given the important role they play in human oxidative metabolism, and the reactivity of the oxygen intermediate that is formed as part of this metabolism, they are able to form various reactive species.²² These metabolites have relatively short half-lives and are too short lived to be detected in plasma although their presence can be inferred from *in vitro* and animal studies.

As is the case with active metabolites and prodrugs, the formation of chemically reactive metabolites through drug metabolism does not necessarily lead to toxicity. Often, conversion of a reactive metabolite, which may be toxic, is followed swiftly by the conversion of the said metabolite into an inactive form, in this way avoiding toxicity for clinically used drugs. A good example of this would again be paracetamol. As part of its metabolism it is converted to the reactive metabolite *N*-acetyl-*p*-benzoquinone imine by CYP2E1.¹⁹ It is then immediately converted to an inactive form by conjugation to a glutathione, which eliminates hepatotoxicity at therapeutic doses of this drug.¹⁹ The major metabolic and detoxification pathways of paracetamol are illustrated in Figure 1.5 below.²³

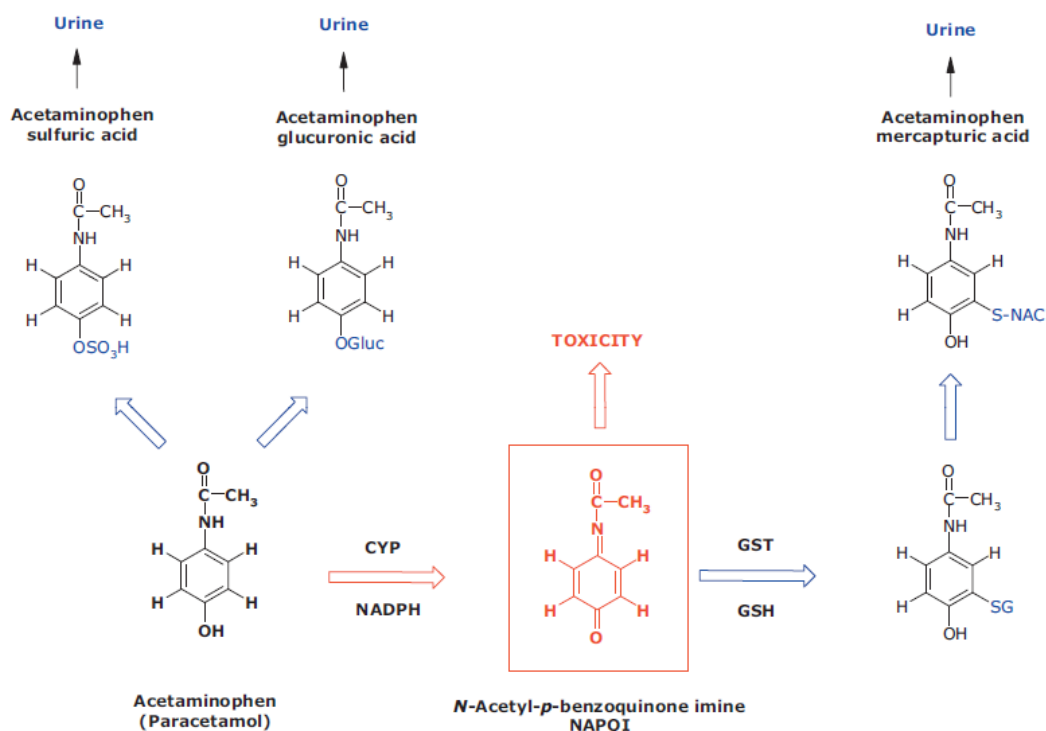


Figure 1.5: Schematic representation of the major elimination and detoxification pathways of paracetamol. Adapted from and used with permission (Tsikas et al, 2011)

However, when paracetamol is taken above therapeutic doses, *N*-acetyl-*p*-benzoquinone imine is formed at levels that saturate the available glutathione *S*-transferase enzymes, overwhelming the detoxification process, leading to hepatotoxicity.²² As a result, not only is using the correct doses of this particular drug of great importance, but understanding various factors which lead to variability in drug metabolism is essential to both drug efficacy and safety.

The study of metabolites is an important regulatory requirement for new chemical entities prior to their registration and marketing.^{24,25} The US Food and Drug Administration (FDA) has released metabolite in safety testing (MIST) guidance documents on when and how to characterize the safety of drug metabolites in humans. A safety assessment is required when a metabolite generated in humans exceeds 10% of total drug-related systemic exposure at steady state in humans receiving the therapeutic dose of the drug.²⁶ This is characterized by monitoring drug related exposure in plasma, although C_{max} can be used if it is deemed more relevant.²⁶ This assessment includes non-clinical characterization prior to the conduct of large scale clinical trials, normally phase 3 trials.^{24,26} Safety should also be assessed for all major human metabolites in at least one species used to study general toxicity, reproductive toxicity, and carcinogenicity studies, respectively.^{25,26}

1.1.4 Interindividual Variation in metabolism

The effects of variability in drug metabolism can be wide ranging. These effects range from treatment failure to an increase in likelihood of adverse drug reactions. There are various factors that could lead to variation in drug metabolism between individuals. Two that have been well studied are the effects of drug-drug interactions (DDI) and pharmacogenetics.

1.1.4.1 Effect of drug-drug interactions on drug metabolism

The concomitant usage of drugs plays a major role in drug metabolism, especially metabolism that is mediated by specific drug metabolizing enzymes. One of the widely studied remedies, due to its wide-ranging effects when co-administered with drugs that are substrates of CYP3A4, is St John's wort. This herbal remedy is a potent inducer of CYP3A; thus, co-administration with a CYP3A4 substrate can lead to treatment failure, or drug toxicity if the co-administered drug is a prodrug or produces active metabolites, due to increased rates of metabolism.²⁷ A notable example of this is the concomitant use of St John's wort and the HIV protease inhibitors where this combination leads to reduced drug exposure as a result of the induction effect of St John's wort.^{27,28}

Rifampicin, a major drug in the treatment of tuberculosis, is a potent inducer of drug metabolism. It has been shown to induce a number of drug metabolising enzymes including CYP2A6, several members of the CYP2C family, CYP3A4, as well as non-CYP enzymes such as the uridine 5'-diphospho-glucuronosyltransferases (UGT) and flavin-monooxygenases.²⁹ However, this induction is a rather selective effect and varies depending on a range of variables including the drug involved as well as factors intrinsic to the patient.²⁹

Carbamazepine, an anticonvulsant drug, treatment has also been linked to a decrease in exposure of drugs eliminated predominantly by glucoronidation due to its induction of UGTs.³⁰ The plasma exposure of dolutegravir, an anti-HIV drug, was reduced in subjects receiving co-administration of carbamazepine as compared to individuals not receiving the anticonvulsant.³¹ This finding was later supported by a therapeutic drug monitoring study which showed a substantially lower dolutegravir C_{min} values in patients being treated with carbamazepine.³²

The co-administration of drugs can also have advantageous effects. HIV protease inhibitors are a prime example of this. Ritonavir, a well-known HIV protease inhibitor, considerably reduces the CYP3A-mediated first pass metabolism of other HIV protease inhibitors and significantly increases their levels in plasma.^{28,33} This approach forms the basis for the combination therapies prescribed for HIV treatment.²⁸

This approach has also been used more recently. Specifically, for the treatment of the severe acute respiratory syndrome coronavirus 2 virus, the causative agent behind the recent covid-19 pandemic. One of the few approved treatments available for the treatment of the covid-19 disease is Paxlovid®, which consists of nirmatrelvir tablets co-packaged with ritonavir tablets.³⁴ Since nirmatrelvir is predominantly metabolized by CYP3A4, ritonavir serves as an effective PK enhancer, reducing the metabolism and increasing the exposure of nirmatrelvir.

1.1.4.2 Effect of genetic polymorphisms on drug metabolism

Variations in how drug metabolic enzymes are encoded at the genetic level have the potential to significantly influence how drug metabolism differs between individuals. The most common type of genetic polymorphisms in metabolic enzymes are single nucleotide polymorphisms (SNP), which come about as a result of a variation in the nucleotide base at a single position in the DNA encoding for the enzyme.³⁵ The effects these SNPs can have on the phenotypic expression of metabolic enzymes range from complete loss of function to producing an enzyme that is far more active than its normal variant.

The terms used to refer to these phenotypically different individuals are; poor metabolizers (PM), which refers to individuals who carry genes which yield reduced enzyme activity; intermediate metabolizers, individuals that carry genes resulting in impaired metabolic ability; extensive metabolizers, carriers of functional genes, usually constituting a predominant portion of individuals; and the ultra-rapid metabolizers, individuals who, due to a SNP, have an increased number of functional gene copies, or a SNP that results in increased activity or expression of an enzyme.^{35,36}

Multiple CYPs have been shown to be polymorphic. However the most extensively studied of all CYP polymorphisms are those that are present in CYP2D6.³⁷ Polymorphisms arising from this enzyme contain the broadest possible range of genetic variants; from null alleles to several fold gene amplification.³⁶

The most common variant of this enzyme is the CYP2D6*4 null variants, which all have the same genetic mutation leading to little to no protein being detected in the liver.³⁶ The aggregate *4 allele occurrence among Caucasians in particular is approximately 20 to 25%, making it responsible for roughly 70 to 90% of previously determined PMs.³⁸

The major clinical effect of these null alleles is the greater likelihood of adverse reactions among people who are PMs. Several opioid drugs such as codeine, oxycodone, and tramadol,

are metabolically activated by CYP2D6, with the conversion of codeine to the pharmacologically active morphine being the most widely recognised. Due to the need for this conversion to a pharmacologically active compound, genotype has been shown to play a major role in the efficacy and safety of these prodrugs.^{39,40} When CYP2D6 PMs are treated with codeine, there is little to no pharmacological effect due to lower morphine exposure.⁴⁰ The potential effects of genetic polymorphisms on drug metabolism, and thus exposure, are illustrated in Figure 1.6 below.

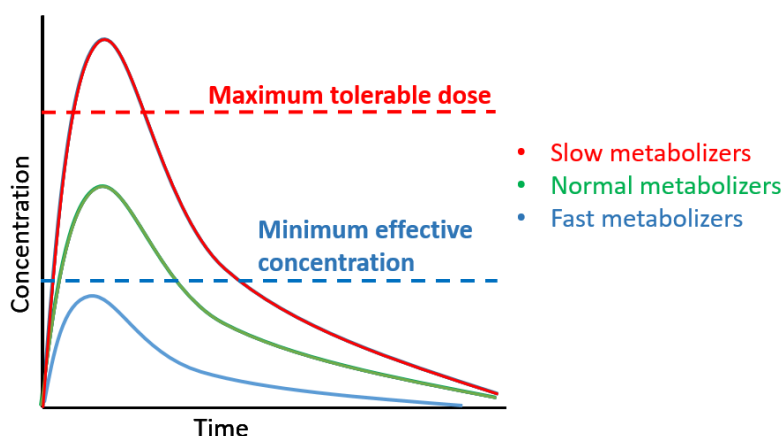


Figure 1.6: Illustration of the effect of variability in drug metabolism in drug exposure

The figure above shows the potential safety and efficacy effects of variability in drug metabolism where the drugs do not have pharmacologically active metabolites. Individuals who are fast metabolizers of a drug, as seen in blue, could have drug concentration levels which are below the minimum effective concentration which could lead to treatment failure. This failure could have significant consequences as in the example of antibiotic treatment. When antibiotic drug levels linger at sub-therapeutic levels, not only is a patient not cured, but this could lead to the selection of antibiotic resistant mutants of a communicable disease.^{41–43}

In contrast, individuals who are slow metabolizers of a drug, as seen in red, could have unwanted drug accumulation potentially resulting in toxicity. This toxicity could be an intensification of a drugs therapeutic action, or unrelated damage caused to tissues or organ systems.⁴ Over accumulation of some antipsychotic drugs leads to confusion and drowsiness, while excessive levels of the anti-HIV drug efavirenz causes central nervous system side effects.⁴⁴

1.2 Preclinical drug metabolism studies

The previous sections illustrate the importance of drug metabolism in PK and, by extension, in the efficacy and safety of drug therapy. Drug metabolism studies are therefore an important

component of drug discovery and development. There are several types of studies with each one providing critical information on the properties of a drug at varying stages of the drug development pathway. An overview of what type of drug metabolism studies are done during different phases of drug discovery and development is summarized and illustrated in Figure 1.7 below.⁴⁵

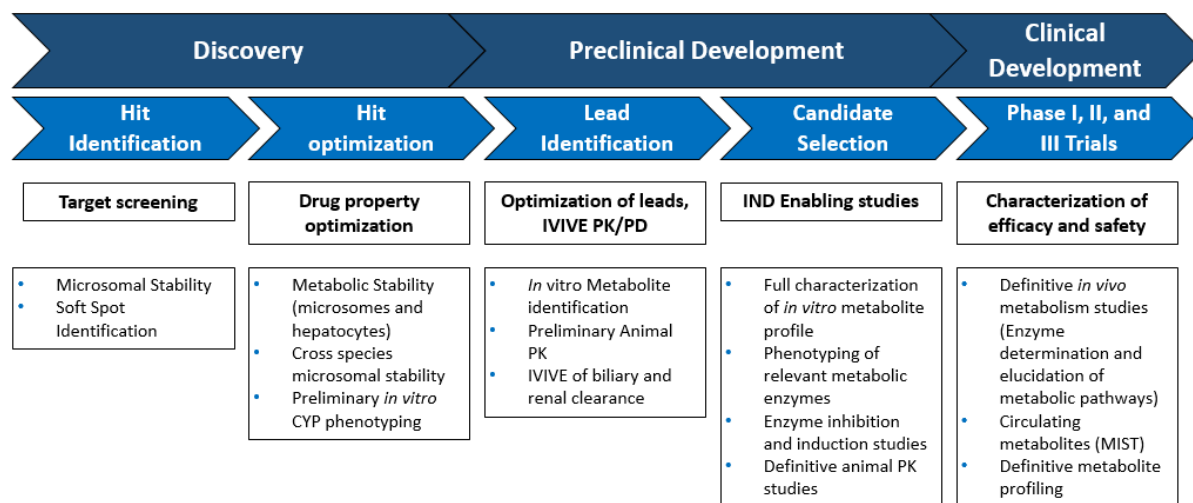


Figure 1.7: Overview of drug metabolism studies in drug discovery. Adapted from frontage labs (Ref 44).

Initially, metabolic stability investigations are undertaken in an appropriate metabolic system to identify potential “soft-spots”, regions of the drug that are most susceptible to metabolism, as well as establishing how quickly the investigated drug is cleared.⁴⁶ This data allows researchers to identify whether a structure, and potential locations on a structure, needs to be changed to improve its drug properties.

In addition, these studies allow for the preliminary identification of metabolites when the products of these incubations are analysed using LC-MS/MS or LC-NMR.⁴⁶ Definitive characterization of metabolite structure *in vitro* is only done later in the drug development pathway and is often followed by the synthesis of relevant metabolites for MIST investigation, if necessary, as referenced in section 1.1.3. Full characterization of the metabolic pathways of an investigated drug, with the use of metabolites, is done *in vivo* once safety has been established.^{25,26}

Once general metabolic stability has been established and metabolites have been identified, it then becomes possible to determine which enzymes are responsible for the metabolism of the investigated drug in a process called reaction phenotyping. This is a set of experiments that are undertaken to determine the specific enzymes that participate in the metabolism of a drug.⁴ In general, three methods are available to conduct CYP reaction phenotyping studies. These are

the incubation of the drug of interest with HLM in the presence of specific chemical inhibitors or monoclonal antibodies, incubation with individual human cDNA-expressed recombinant CYP enzymes, and incubation with a panel of HLM prepared from individual donors which have been characterized for the activities of individual CYPs, known as correlation analysis.² These studies allow for the prediction of DDIs, identification of potential sources of interindividual variability such as pharmacogenetics, and provides an understanding of the implications of tissue-specific metabolism since different enzymes are present in different tissues at various levels of expression.⁴⁶

Animal PK studies are also carried out at various stages of the drug discovery pathway as a way to boost confidence in *in vitro* data through *in vitro-in vivo* extrapolation (IVIVE).^{2,47} These studies, as well as the other *in vitro* studies mentioned above, can and are often done concurrently with the data from all these studies being used to drive the development of a drug.

In the early stages of drug development, *in vitro* drug metabolism investigations provide crucial information on the metabolic profile of a drug which are to be expected in humans. Since the liver is the main site of drug metabolism, liver fractions are used to study *in vitro* drug metabolism. There are a variety of tools used to investigate drug metabolism *in vitro* including the use of hepatocytes, subcellular fractions, as well as recombinant enzymes.

1.2.1 Hepatocytes

Hepatocyte refers to the cell of the main parenchymal tissue of the liver and makes up approximately 80% of the liver cell mass.⁴⁸ These cells play a major role in various liver functions, including drug metabolism. They also contain a wide range of drug metabolizing enzymes; including enzymes that conduct both phase I and II metabolism.⁴⁹ As a result, hepatocytes, in suspension or culture, are routinely used in drug discovery to measure drug metabolism *in vitro*.

When used as an *in vitro* tool, hepatocytes are isolated from whole liver or liver tissue by the process of collagenase digestion.^{48,50} As part of this process the liver material is chemically disrupted, after which a solution containing collagenase is added as a means of separating the hepatocytes from the liver stroma; cells that act as connective tissue and support parenchymal cells.⁵⁰ This procedure creates a suspension of hepatocytes which can be cultured and maintained over a period ranging from days to a few weeks. A comprehensive characterization of the processes carried out by hepatocytes, such as phase I and II enzyme activities and glucose

metabolism, is imperative to ensure that the functions of interest are maintained during culture.⁵¹

Hepatocytes which have been freshly isolated, but are not immediately used, can also be cryopreserved. Unfortunately, these cannot be cultured and are sensitive to damage after freezing and thawing as well as during the cryopreservation process.⁵² This damage is associated with a loss in activity, which affects the quality of data obtained from such material.^{50,53,54}

1.2.2 Subcellular fractions

Subcellular fractions of the liver used in *in vitro* metabolism studies include the microsomal, cytosolic, and S9 fractions. In Figure 1.8 below is illustrated the process of repeated ultracentrifugation of homogenized liver by which various subcellular fractions are obtained.⁵⁵

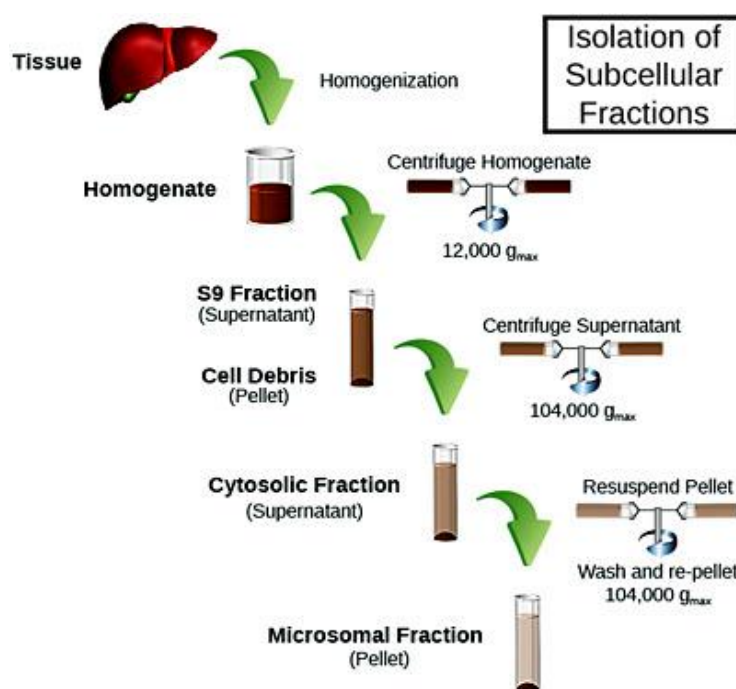


Figure 1.8: Illustration of the generation of subcellular fractions of the liver for use in *in vitro* drug metabolism studies

Human liver microsomes (HLM) contain high concentrations of many drug metabolizing enzymes, importantly including high concentrations of CYP enzymes.⁵⁶ The S9 fraction contains a combination, in comparatively low concentrations, of cytosolic and microsomal enzymes. The cytosolic fraction, in contrast, contains many water soluble drug metabolizing enzymes, such as aldehyde oxidase, that are not found in other liver fractions in high concentrations.⁵⁷

Considering that each of these fractions contain different concentrations of a variety of drug metabolizing enzymes, they each serve an important and unique function within drug discovery.⁵⁷ Selecting the correct one to use for any study is thus a function of the specific enzymatic reaction to be monitored or measured.⁵⁴ HLM would be more appropriate than either the S9 or cytosolic fractions when measurements of CYP activity are to be undertaken, while the cytosolic fraction would be more appropriate for investigations of aldehyde oxidase activity, for example.

This specificity does have its drawbacks since, compared to hepatocytes, no single fraction can uncover all the potential metabolic pathways that might exist for an investigated drug. On the other hand, the ability of subcellular fractions to be stored for extended periods of time at -80°C as well as maintain activity through multiple freeze-thaw cycles is a significant advantage over hepatocytes and greatly improves the cost and ease of use of these fractions.⁵⁴

1.2.3 Recombinant Enzymes

Individual recombinant enzymes, which have been cloned and heterologously expressed in various cell lines, are widely available from commercial vendors. These enzymes are a valuable tool in evaluating the intrinsic ability of each individual isoform to metabolize the studied drug.⁵⁸ These enzymes form an important part of enzyme phenotyping studies which seek to elucidate the involvement of specific enzymes in the metabolism of studied drugs. Moreover, like with subcellular fractions, recombinant enzymes can be stored for extended periods of time at -80°C.⁴⁹

One of the drawbacks of employing this *in vitro* tool is that the enzyme levels are not always physiologically relevant.⁴⁹ As a result, other tools should be used prior to the conduct of studies using recombinant enzymes to confirm their involvement. For example, recombinant CYP enzymes (rCYP) that are capable of metabolizing a drug when incubated with drug in the absence of other CYPs, show little to no ability to metabolize the drug in microsomal metabolism when the other CYPs are present due to differences in substrate affinity.⁵⁸ In addition, scaling factors are also required for accurate estimation of physiologically significant metabolic rates from recombinant enzyme data.⁵⁹

1.3 *In vitro-in vivo* extrapolation

In vitro kinetic metabolism data ultimately needs to be scaled up to *in vivo* clearance to understand its impact on pharmacokinetics. One of the most prominent ways of understanding the kinetics of *in vitro* drug metabolism is the Michaelis-Menten equation. This method

describes enzymatic reactions resulting from reversible binding at a single saturable site. Under the assumptions of the model, the reaction rates increase rapidly at low substrate concentrations but reach a maximum due to saturation at higher concentrations.⁶⁰ This results in a typical hyperbolic profile, shown in Figure 1.9 below, characterised by V_{max} , the maximal rate of the reaction, and K_M , the Michaelis-Menten constant which is the substrate concentration where the velocity of the reaction is half V_{max} . The Michaelis-Menten equation is shown below:

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad (1)$$

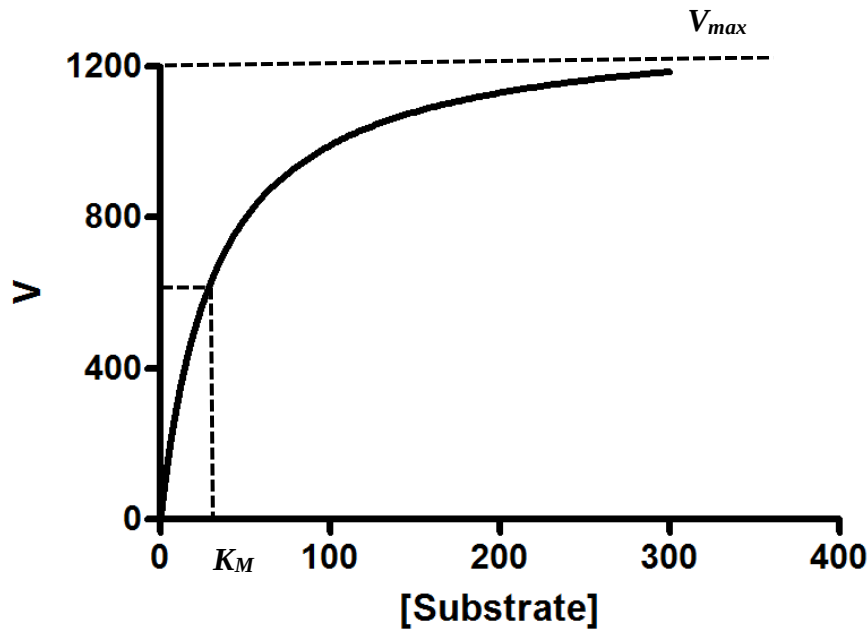


Figure 1.9: A typical example of Michaelis-Menten kinetics substrate saturation plot

V_{max} and K_M from this model can be used to determine *in vitro* intrinsic clearance ($CL_{int, in vitro}$) using the equation below:

$$CL_{int, in vitro} = \frac{V_{max}}{K_M + S} \quad (2)$$

Under linear conditions, which are critical for the measurement of enzyme kinetics, substrate concentration S is lower than K_M . Consequently, $CL_{int, in vitro}$ of a studied drug is usually measured using the following equation:

$$CL_{int, in vitro} = \frac{V_{max}}{K_M} \quad (3)$$

In vitro intrinsic clearance is an important optimization parameter in early drug discovery and development. It can also be scaled to organ clearance and then whole-body clearance to understand the contribution of metabolism to pharmacokinetics.

The first part of this method is to scale up the $CL_{int,in vitro}$ measured in liver fractions to an intrinsic clearance per gram liver ($CL_{int,gram liver}$). Since the $CL_{int,in vitro}$ measured in *in vitro* assays is expressed as “per milligram of protein” when subcellular fractions are used and “per number of viable cells” for hepatocyte assays, this clearance can be scaled up to $CL_{int,gram liver}$ with the use of microsomal protein per gram liver (MPPGL) when HLM are used, S9 protein per gram liver (S9PPGL) when the S9 fraction is used, cytosolic protein per gram liver (CPPGL) when the cytosolic fraction is used, and hepatocellularity per gram liver (HPGL) when hepatocytes are used, respectively.⁴⁹ There is a wealth of knowledge in literature for each of these scaling factors.^{61–69} Scaling up $CL_{int,in vitro}$ to $CL_{int,gram liver}$ with these scaling factors yields the following equation:

$$CL_{int,gram liver} = CL_{int,in vitro} \times \text{MPPGL or S9PPGL or CPPGL or HPGL} \quad (4)$$

Scaling up $CL_{int,gram liver}$ to $CL_{int,in vivo}$ can then be done by factoring in the weight of the liver as a fraction of total body weight. This then yields the following equation:⁶⁹

$$CL_{int,in vivo} = CL_{int,gram liver} \times \frac{\text{liver weight}}{\text{body weight}} \quad (5)$$

The overall process is summarized in the equation and below:

$$CL_{int,in vivo} = CL_{int,in vitro} \times (\text{MPPGL or S9PPGL or CPPGL or HPGL}) \times \frac{\text{liver weight}}{\text{body weight}} \quad (6)$$

Recombinant enzymes are also routinely used in drug metabolism studies. Appropriate scaling factors are also needed to accurately estimate an *in vivo* metabolic rate from rCYP data. Using a relative activity factor (RAF) is one scaling factor that can be used to do this. This is done using relevant probe substrates to correct for differences in expression levels and enzyme activities between microsomes and recombinant enzymes.^{49,70} This correction is done using the following equation:

$$RAF = \frac{CL_{int,HLM}}{CL_{int,rCYP}} \quad (7)$$

Where $CL_{int,HLM}$ and $CL_{int,rCYP}$ refer to the intrinsic clearance measured using HLM and rCYPs respectively.

Another method for scaling recombinant enzyme data to *in vivo* metabolic data is by using an intersystem extrapolation factor (ISEF). In addition to accounting for the enzyme expression and protein level differences between HLM and rCYPs, the ISEF method also accounts for differences in specific activity between rCYP heterologous expression systems and HLM by

taking into account enzyme abundance in HLM.⁷⁰ ISEF can be determined using probe substrates of the investigated enzyme using the following equation:⁷¹

$$CL_{int} ISEF = \frac{CL_{int,HLM}}{CL_{int,rCYP} \times CYP \text{ Abundance in HLM}} \quad (8)$$

The ISEF or RAF measured using probe substrates can then be used to scale $CL_{int,rCYP}$ of the studied drug to an $CL_{int,in vitro}$ using the following equation:

$$CL_{int,in vitro} = CL_{int,rCYP} \times ISEF \text{ or RAF} \quad (9)$$

The $CL_{int,in vitro}$ obtained from equation (9) can then be scaled up to $CL_{int,in vivo}$ using equation (6) along with MPPGL as the scaling factor when working with CYPs.

However, it is noteworthy that the use of IVIVE to scale *in vitro* data to *in vivo* outcomes does have its drawbacks. Over the years, obtaining exact predictions has been a challenge, with a general trend of underpredictions by various investigators.⁷² This approach works best only for the prediction of hepatic clearance and does not consider a variety of important biological parameters, such as the permeability of a drug or uptake into and blood flow of organs other than the liver, which are important in understanding and predicting *in vivo* PK. Modern computational modelling does account for these parameters and is a powerful tool in *in vivo* PK predictions.

1.4 Physiologically Based Pharmacokinetic Modelling

The origins of physiologically based pharmacokinetic (PBPK) modelling can be traced back to work performed by Teorell in 1937.^{73,74} Despite its early origins, PBPK modelling, as it is understood in the modern sense, did not develop for some time after Teorell published his work. This is likely due to the work yielding equations that were too complex to solve mathematically at that time.⁷⁵ Instead, what we now consider classical PK analysis developed and was implemented over time with these analyses employing simple empirical and compartmental kinetic models.^{75,76}

PBPK modelling, in its current form, refers to a mathematical modelling technique that combines information of a drug or new chemical entity, such as its physicochemical properties, with prior knowledge of species physiology and biology to achieve a mechanistic understanding of the drug within a biological system.^{77,78} These models are constructed with reference to the different compartments in the body, corresponding to different physiological organs.⁷⁸ These organs are, linked by the blood circulatory system, namely the arterial and

venous blood compartments, with each compartment being characterised by a tissue volume and blood flow rate that resembles that of the species being modelled.^{77,78} This enables the estimation of the drug concentration-time profile prior to clinical administration. The type of data used to construct PBPK models are illustrated in Figure 1.10 below.⁷⁷

The complexity of PBPK models vary from full PBPK models, where all organs and tissues are regarded as separate individual compartments, to minimal PBPK models where tissues with similar perfusion kinetics are grouped together to simplify the model.⁷⁹ Due to the increased complexity and the increased number of parameters tested, full PBPK models generally describe clinical data better and confer more confidence in model accuracy as well.⁷⁹ Despite this, minimal PBPK models have their uses with the reduced complexity of this model type making it ideal for mechanistic simulations in compartments of interest.⁷⁹

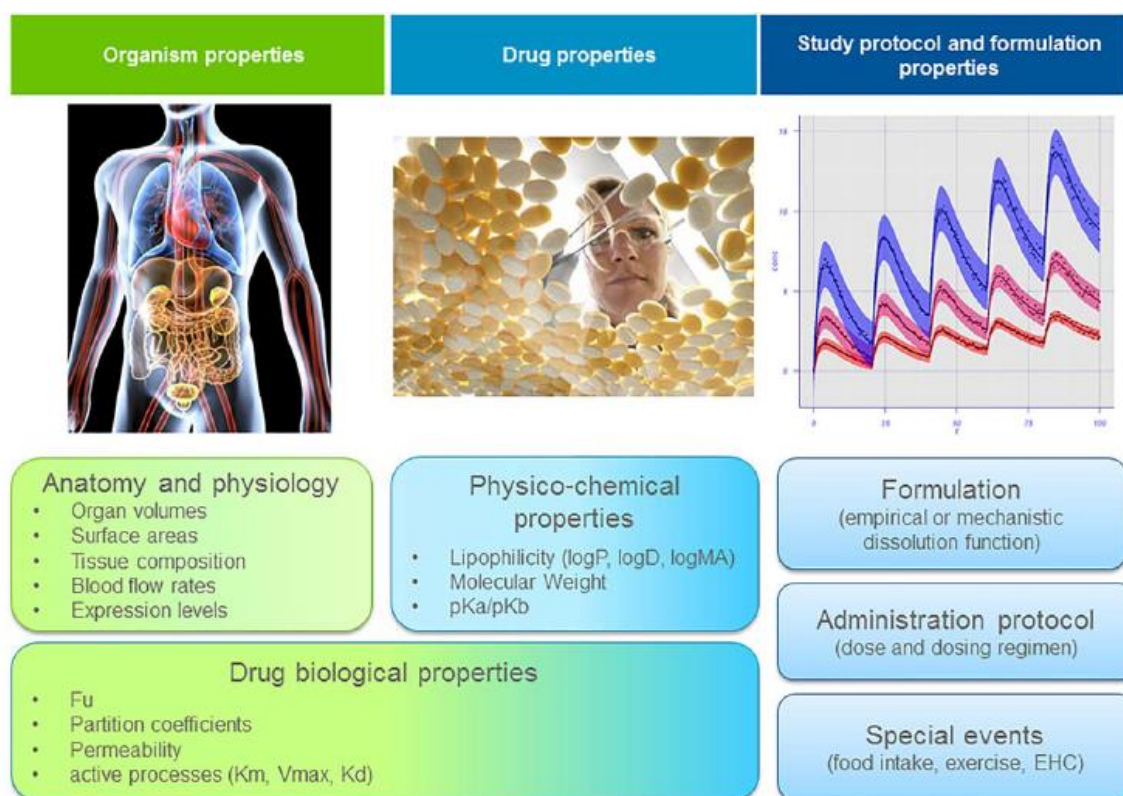


Figure 1.10: General representation of the parameters used to build PBPK models. Some components may be optional depending on the modelling approach used. Adapted from and used with permission (Kuepfer et al, 2016)

1.4.1 Approaches to PBPK modelling

There exists three main approaches to PBPK modelling and simulation; the top-down approach, wherein models are built predominantly with the use of observed *in vivo* data, the bottom-up approach, where models are built by utilizing prior knowledge of the physiological characteristics of the human body with measured *in vitro* information being used as the input

data, and the middle-out approach, which combines the top-down and bottom-up approaches and allows for the use of the available *in vivo* and *in vitro* information.^{80,81}

1.4.1.1 Top-down

Used more often in early attempts at PK modelling, the top-down modelling approach aims to determine the characteristics of a drug within a physiological system with the use of measured *in vivo* data.⁸⁰ As a result, this approach can be used to identify covariates which affect the PK profile of an investigated drug as a means to ascertain ways to improve it. In this way, the use of this approach can assist in evaluating and optimizing a drug candidate during clinical trials which in turn can result in improvements in the speed and cost of drug development.⁸²

However, this approach requires PK data from clinical studies in human, or animal, subjects and is thus not suitable for use in early drug discovery.⁸³ Moreover, this approach does not make full use of broader knowledge of species physiology or drug specific information which limits its utility as a predictive tool.^{80,82}

1.4.1.2 Bottom-up

In contrast to top-down, the more modern bottom-up approach, often referred to as the systems approach, makes use of drug and species data to provide a mechanistic understanding of the fate of a drug within the body. The physiological data used to construct these models have been acquired from a variety of multidisciplinary sources, with the compound specific data being determined with the use of *in vitro* assays or predicted using *in silico* tools.^{78,79,81,84} Species-dependent parameters, also referred to as system-dependent parameters, include glomerular filtration, blood flow, organ volumes, plasma protein concentrations and enzyme expression.^{75,78,79,82} Drug-dependent parameters include molecular weight, pK_a , solubility, permeability, lipophilicity ($\text{Log}D$), volume of distribution, drug binding to various proteins in the body, the partitioning of the drug into plasma, *in vitro* data on the metabolism of the drug and intrinsic clearance (CL_{int}).^{78,79} The drug and system parameters are independent of each other and are integrated mathematically using differential equations.^{79,84} As an example, tissue-plasma partition coefficients are calculated based on the physicochemical properties of the studied drug (drug-dependent parameter) and the composition of tissue the drug is partitioning into (system-dependent parameter).⁸⁴

By making use of such a wide array of parameters, the bottom-up approach allows for the prediction of plasma-concentration time profiles and, as a result, can assist in the identification of potential sources of inter-individual variability in these profiles prior to the conduct of

clinical studies.^{83,84} The use of *in vitro* data and *in silico* predictions of important physiological parameters also means that this approach can be used in the initial stages of the drug development process allowing for its use as a tool to guide drug development much earlier.^{80,83}

While the bottom-up approach has generally been shown to be effective in predicting *in vivo* PK, especially when the underlying PK mechanisms of an investigated drug are well understood, it does have its drawbacks.^{85,86} In situations where *in vitro* data acquisition has not been adequate, which can often be the case for older drugs where there is a paucity of data due to data not being gathered during development or not reported in literature, or the available data does not accurately predict the *in vivo* PK profile of the studied drug, bottom-up modelling loses its value.^{78,79,83} In these instances, *in vivo* data, by the process of parameter optimization, can be used to refine the model to fit clinical outcomes.^{79,83,87,88} As stated above, this combination of the top-down and bottom-up approaches is termed the middle-out approach.

1.4.1.3 Middle-out

As previously mentioned, the middle-out approach incorporates data from both *in vitro* assays and *in silico* predictions, as well *in vivo* studies. In this way this approach integrates the strengths of both previously discussed methodologies. As a consequence of this, middle-out PBPK models can do more than explain observed data but can be used to investigate novel scenarios of interest which have not been previously tested.⁸⁹

A commonly used example of this approach is the retrograde PBPK model. When this approach is employed the *in vivo* clearance determined clinically is scaled back to *in vitro* intrinsic clearance by assigning portions of the *in vivo* clearance to each clearance pathway associated with the drug.^{84,89}

Despite its usefulness, and the wealth of data it can incorporate, the middle-out approach also has its limitations. These limitations often arise due to some parameters being fixed, and others being estimated as described above. Indeed, the predictive ability of the model can be limited due to issues related to parameter identifiability.^{85,87} This arises specifically as a result of the large number of variables this modelling approach can make use of and can make it difficult to elucidate which parameter is responsible for the lack of correspondence between clinical data and the model output.^{83,85,87}

Figure 1.11 below illustrates the separated top-down and bottom-up approaches in comparison to the middle-out approach which incorporates both methods.⁸⁹ Despite the clear separation seen in the below figure, the distinction between each of these modelling approaches can often

be blurred. Bottom-up models rarely make use of clinical data, while top-down models also often infer mechanistic understandings of the fitted parameters under investigation.⁸⁹

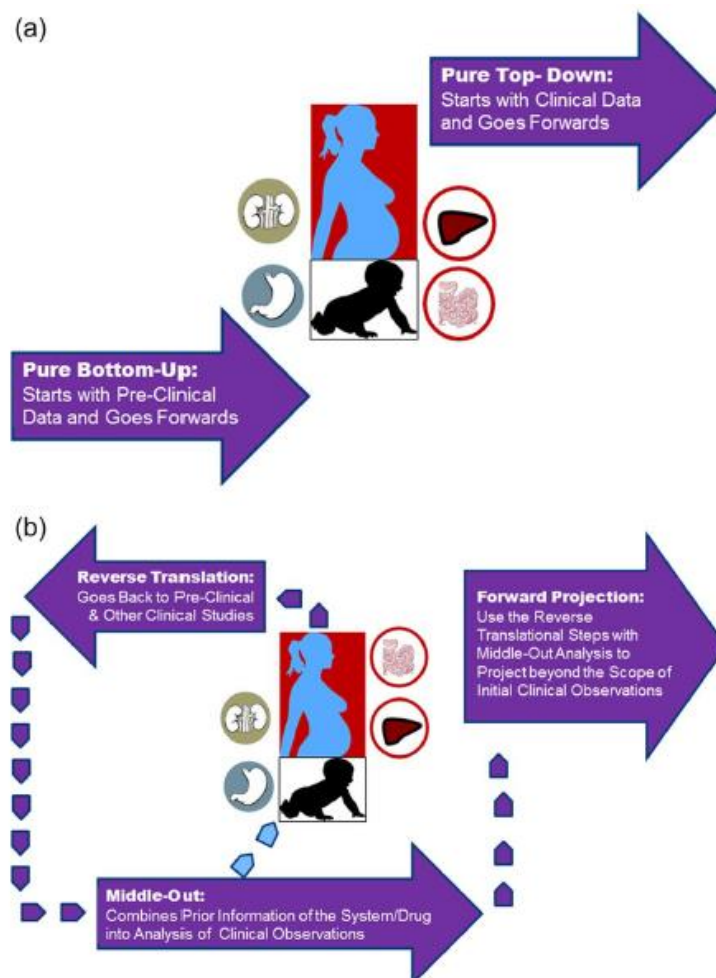


Figure 1.11: Illustration of the differences between bottom-up and top-down approaches in PBPK modelling (a), in contrast to the middle-out approach which combines the two (b). Adapted from and used with permission (Rostami-Hodjegan, 2018)

1.4.2 Applications of PBPK modelling within drug discovery

Physiologically based pharmacokinetic modelling has multiple uses within the context of drug discovery and development. These include the prediction and simulation of drug-drug interactions (DDI), mechanistic *in vitro-in vivo* extrapolation (IVIVE) of drug clearance, and full drug concentration time profiles.

1.4.2.1 Predicting full drug concentration time profiles

The concentration time profile for a drug or new chemical entity can be predicted using PBPK based simulations. This is usually done by inputting the known characteristics of a drug, such as its various physicochemical properties and its metabolic profile, into the prediction software

and subsequently comparing this data to *in vivo* data for model verification. These known characteristics can be measured, obtained from literature, or predicted *in silico*.

An example of this is the work done by Nguyen *et al.*,⁹⁰ who conducted a study during which they predicted the exposure of midazolam metabolites using the Simcyp simulator. They built this model by measuring various metabolic parameters, such as the enzyme kinetic parameters of specific metabolic reactions, as well accruing data on the physicochemical properties of midazolam and its investigated metabolites from various literature sources, and predicting others *in silico*. Once the model was constructed they compared their model predictions to a multitude of *in vivo* results from several different sources. In doing so they found that the data they predicted using the Simcyp software, constructed using the data sources, was in agreement with the observed clinical data. The predicted overall exposure of both the parent and metabolites fell within ranges observed in literature and the model correctly predicted the ratios of the formation of different metabolites using *in vitro* data as well.⁹⁰ This work is illustrated in Figure 1.12 below.⁹⁰

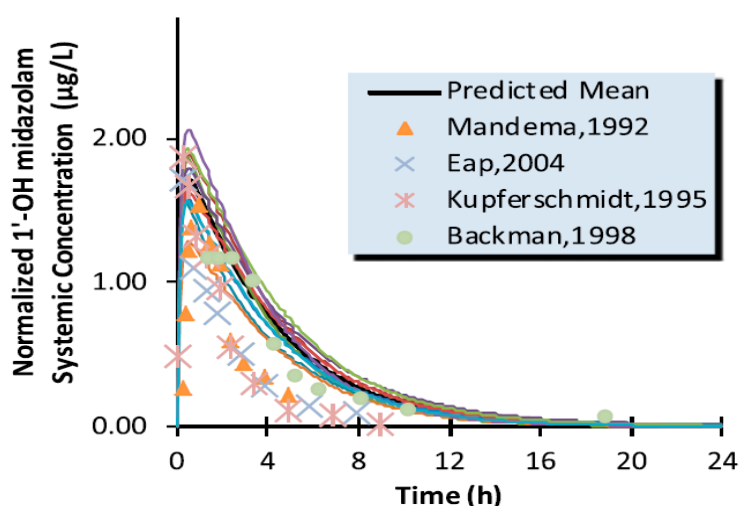


Figure 1.12: Simulated (continuous line) and observed (points) human oral plasma concentration-time profiles for 1'-hydroxymidazolam from the work of Nguyen *et al.* Adapted from and used with permission (Nguyen *et al.*, 2016)

Much like with predicting full drug concentration time profiles, the prediction of drug clearance can also be achieved using PBPK modelling. As clearance is a function of exposure and is involved in other aspects of PK, the prediction of clearance has many uses within a PBPK modelling context.

The work done by T'jollyn *et al* also shows the flexibility of modern PBPK software.⁹¹ In an attempt to more accurately predict tramadol clearance, they recreated the study design of the clinical research data they used to verify model accuracy within their PBPK model. This was

achieved by mimicking the reference populations in terms of the male-to-female ratio, age range, and CYP2D6 metabolizer status.⁹¹

They further specialised their model by selecting a distribution model that considered the physicochemical properties of tramadol. Since tramadol is almost completely ionized at pH 7.4, a distribution model was selected which takes into account both a tissue's acidic phospholipid fraction and the extent of ionization of a compound at the pH of the compartment concerned.^{91,92} Using this approach, they constructed models to predict tramadol clearance *in vivo* based on different *in vitro* systems, namely from HLM and rCYP enzymes.⁹¹

1.4.2.2 Prediction of the effects of pharmacogenetics

The knowledge available in literature on genetic polymorphisms in drug metabolizing enzymes has also been incorporated into PBPK modelling software. This information is then used to predict the effects potential polymorphisms can have on the exposure of drugs. This is especially useful for drugs that have narrow therapeutic windows.

For example, by incorporating differing rates of enzyme expression and activity as a result of polymorphisms in genes encoding for the expression of CYP2C9, Dickinson *et al* were able to investigate the potential effects these could have on *S*-warfarin metabolism.⁹³ This then allowed them to assess the effect the genotypes had on clinical response in published pharmacogenetic studies. Investigations such as this, especially for a drug like warfarin, can inform the improvement in dosage regimens.

Another way PBPK modelling can be used as a tool to measure the effects of pharmacogenetics on drug therapy is through ethnic sensitivity studies (ethnobridging studies) where the PK profile of drugs are predicted in different populations. The work done by Inoue *et al* is a great example of this.⁹⁴ As a means of comparing the fate of various drugs in the Caucasian and Asian populations, they made use of the Simcyp simulator as well as various physiological data on these populations, including the level of expression and activity of different CYP enzymes as a result of genetic polymorphisms. In doing so, they were able to accurately predict differences in the PK profile of the investigated drugs between these populations and were then able to identify the potential sources of this variability as well as areas where more physiological data was required.⁹⁴

The work done by Adiwidjaja *et al.* provides a good illustration of ethnic sensitivity studies.⁹⁵ They sought to obtain an optimum dosage regimen for imatinib administration in different populations. Thus, they built an imatinib PBPK model using the Simcyp simulator, integrating

in vitro drug metabolism and clinical PK data. Their model accounted for the ethnic differences in body size as well as the abundance of drug metabolizing enzymes involved in imatinib disposition.

Their model was able to accurately predict the PK profiles of patients of various ethnic groups receiving imatinib treatment. Moreover, their model suggested a similar dose of imatinib for European, Japanese and Chinese patients would provide a desirable range of plasma concentrations for imatinib treatment while African patients may need a higher initial dose to achieve the targeted drug levels. This is illustrated in Figure 1.13 below.⁹⁵

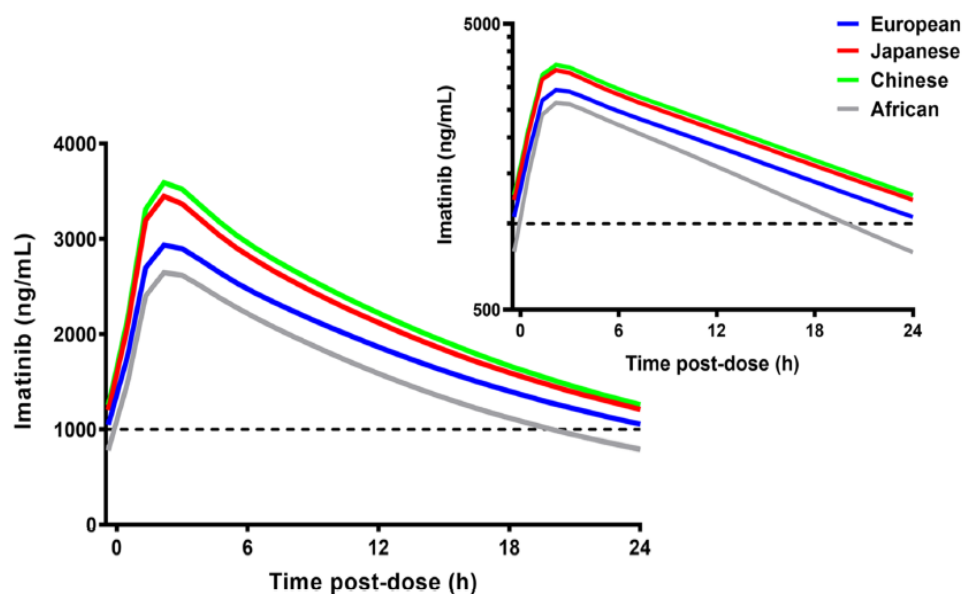


Figure 1.13: Illustration of ethnic sensitivity study carried out by Adiwidjaja *et al* showing the similar levels of imatinib plasma concentrations in Europeans, Japanese and Chinese as well as the slightly lower levels in Africans which necessitates a slightly higher initial dose to achieve desirable drug levels. Log transformed data shown in top right of figure. Adapted from and used with permission (Adiwidjaja *et al*, 2022)

A similar investigation was recently carried out by Ezuruike *et al*.⁹⁶ Through the use of PBPK modelling, they were able to take into account a variety of physiological characteristics which defined and separated a Caucasian and Japanese population and then explored the impact of these ethnic difference on human PK. This type of study can help inform the adoption of a drug from one region to another.⁹⁶

1.4.2.3 Assessing DDI

The assessment of DDI, particularly the ability of a drug to inhibit a CYP enzyme which is responsible for the metabolism of another drug, is an important step in drug development. This is highlighted by the continued issuing of guidance on the matter by international drug regulatory bodies.^{97,98} As such, the ability to predict the potential interaction between drugs

prior to clinical administration is of great importance with PBPK playing a prominent role in this undertaking over the years.

As with the prediction of clearance and full drug concentration time profiles, the building of a PBPK model to predict potential DDIs starts with the acquisition of data on the specific drugs under investigation from either literature, *in silico* prediction, or in-house measurement. The parameters that need to be investigated that are unique to the measurement of DDIs is the inhibition potency, either in the form of an inhibition constant (K_i) for reversible inhibitors or an inactivation rate constant (k_{inact}/K_i) for time dependent inhibitors, of a compound, along with its relevant enzyme kinetics.^{99,100} The measurement of the DDI is then done by predicting the drug concentration time profile of the affected drug in the presence and absence of the potential inhibitory compound and compared to relevant clinical data for model verification.^{99,100}

It should be noted that regulatory bodies, such as the FDA and European Medicines Agency, now accept DDI modelling work to support drug registration and labelling.^{98,101–104} Indeed, PBPK modelling work in general is increasingly being used to bolster the case for drug registration for new chemical entities with regulatory bodies.^{83,98,102,103,105} This serves to outline the growing importance of PBPK modelling in drug discovery.

1.5 Summary and project rationale

As this discussion shows, drug metabolism is an important factor in PK and consequently can be a major contributor to the variability in drug exposure that is observed clinically. Investigations on how differences in metabolism relate to pharmacokinetic variability are often performed *in vivo* during clinical development or therapeutic use of the compound. However, this process is costly and requires drugs to have been evaluated sufficiently for administration to human subjects. The use of *in vitro* methods is thus useful to generate data on what drug exposure might look like *in vivo*. Variability arising from CYP enzymes due to a variety of factors, such as genetic polymorphisms, could also have a significant clinical effect; individuals with a lower metabolic rate have higher plasma levels of a drug due to an inability to clear it leading to an increased risk of adverse effects, while an individual with a faster metabolic rate having lower plasma levels than required for effective treatment, which could lead to treatment failure.

While *in vitro* data is valuable, especially in the earlier stages of drug discovery, it alone does not provide adequate elucidation of potential sources of variability in drug exposure. Thus, there is a need to build on the information garnered during *in vitro* studies to attain a more

comprehensive understanding of drug PK. By combining *in vitro* data with available knowledge on the PK of a drug, PBPK modelling provides a mechanistic understanding of drug exposure. This allows for the explanation of why an individual or group is a PM of a specific drug by being able to point to reduced activity seen in *in vitro* data for a specific drug enzyme combination. Moreover, if a new mutation is found in an enzyme involved in the metabolism of a drug, PBPK modelling allows for the prediction of its impact on PK by studying its enzyme kinetics *in vitro*, which is a more cost and time effective way of conducting these studies.

As part of this project, three clinically used drugs – mefloquine, proguanil and ritonavir – will be used to demonstrate how *in vitro* metabolism data can be combined with PBPK modelling to understand clinical PK. The mechanistic nature of this type of work will also be demonstrated by using these models to investigate novel scenarios of interest for these drugs in the hopes of better understanding and predicting clinical outcomes. Since drugs selected are routinely used in treatment of diseases prevalent in Africa, this study comes with a more specific long-term goal of attempting to provide better optimized treatment programmes for these diseases as well as being a potential springboard to more in-depth research done in the African population.

1.6 Aims and objectives

1.6.1 Study Aims

To investigate the *in vitro* metabolism of selected compounds using human liver microsomes and recombinant enzyme systems and to build PBPK models to predict the influence of *in vitro* phenotypes on *in vivo* pharmacokinetics

1.6.2 Objectives

1. To investigate the metabolism of selected drugs in human liver microsomes and recombinant CYP enzymes
2. To use *in vitro* metabolism data to build PBPK models to predict *in vivo* pharmacokinetics
3. Apply developed and verified models to scenarios of interest to predict the influence of the *in vitro* metabolism phenotype on *in vivo* pharmacokinetics

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Chapter 2 Mefloquine

2.1 Chapter 2 Overview

In this chapter, data from *in vitro* metabolism experiments of mefloquine is used to inform PBPK modelling. Enzyme kinetics data is measured in HLM and chemical inhibition, as well as recombinant enzymes are used to determine fm,CYP. PBPK models are built and validated in Simcyp. The verified model is then applied to various scenarios including ethnic sensitivity and various DDIs.

2.2 General Introduction: Mefloquine

Mefloquine is a 4-quinoline methanol antimalarial developed by the Walter Reed Army institute as part of the U.S Army Antimalarial Drug Development project in the late 1960s.¹ A collaboration between the U.S Army, Hoffmann-La Roche, as a manufacturer, and the World Health Organization (WHO) was formed in 1976 after which mefloquine, under the trade name Lariam, was first introduced to the market in 1984 and was made available to European travellers in 1985.² It was subsequently approved by the FDA in 1989.³

Mefloquine has four stereoisomers but is prescribed as a hydrochloride salt of its two erythro-enantiomers namely; (9R,10S)- (+)- α -piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol-hydrochloride ((+)-erythro-mefloquine) and (9S,10R)-(-)- α -piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol ((-)-erythro-mefloquine).⁴ The chemical structures of mefloquine and its four stereoisomers are shown in Figure 2.1 below.

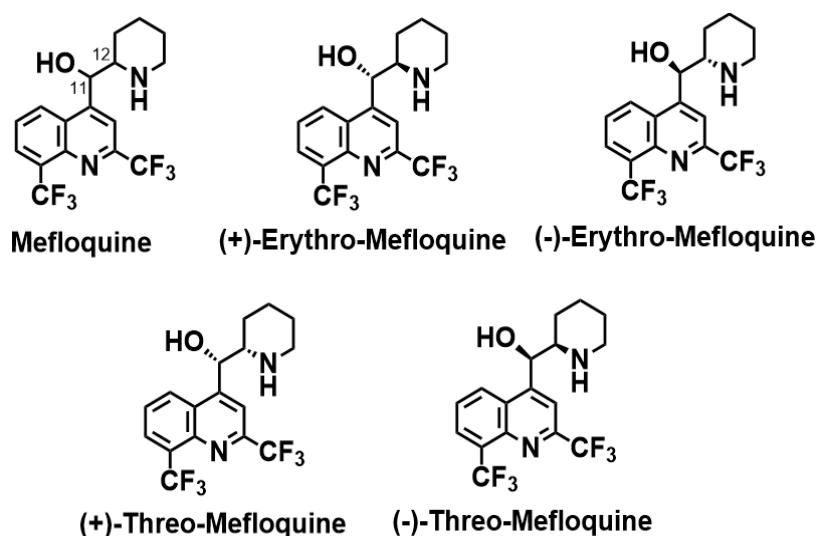


Figure 2.1: Chemical structures of mefloquine and its four stereoisomers

Mefloquine hydrochloride is orally administered and is used as a first-line agent for prophylaxis as well as a treatment, along with artesunate, for malaria infections.⁵ It is reasonably well

tolerated, however, there is a risk of adverse reactions. The most common adverse reactions reported for mefloquine are nausea, vomiting, diarrhoea as well as neurologic side-effects including dizziness, loss of balance and abnormal dreams. The neurologic effects resulted in a black box warning for mefloquine by the FDA.^{5,6}

No suitable intravenous formulation of mefloquine exists since intravenous injection of mefloquine causes severe irritation at the injection site and, consequently, absolute oral bioavailability has not been measured.^{7,8} However, Looareesuwan *et al.* studied the bioavailability of mefloquine hydrochloride tablets compared to a deuterium labelled mefloquine hydrochloride solution.⁸ They reported the relative bioavailability of mefloquine tablets to be 87% in Thai patients with acute malarial infection and 89% in healthy Swiss Caucasian volunteers.

Mefloquine is extensively distributed into body tissues, where it concentrates in infected erythrocytes.^{9,10} Estimates for apparent oral volume of distribution show large variability and range from 13.3 to 40.9 L/Kg.¹¹

Clearance of mefloquine is slow. However, this varies widely with the reported elimination half-life of mefloquine ranging from 14-41 days.¹¹ Metabolism of mefloquine takes place primarily in the liver, where it is metabolized into its main metabolite; a carboxylic acid metabolite 2-8-bis-trifluoromethyl-4-quinoline carboxylic acid (Carboxymefloquine).^{4,12} An alcohol metabolite of mefloquine, which is formed in extremely low quantities, has also been identified. Neither the alcohol metabolite nor carboxymefloquine show any antimalarial activity.^{12,13} The chemical structures of both these metabolites are illustrated in Figure 2.2. Urinary excretion of the alcohol metabolite could not be measured as a result of its limited presence while the urinary excretion of unchanged mefloquine and carboxymefloquine was measured as 9% and 4%, respectively.¹³ The majority of the drug is thought to be excreted in feces.¹⁰

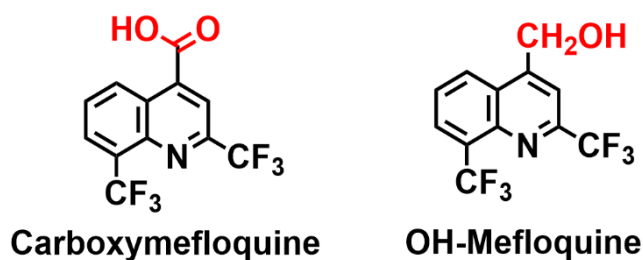


Figure 2.2: Mefloquine metabolites

Mefloquine was chosen as a study compound since data relating to its *in vitro* metabolism was more than a decade old; when techniques to investigate these properties were not as advanced.

Moreover, a wealth of clinical data was available from which a PBPK model could be developed and verified in multiple populations.

Available data does show that mefloquine is metabolized slowly in microsomes from a variety of species.^{14,15} Carboxymefloquine has also been shown to be a major *in vivo* and *in vitro* metabolite.^{14–17} Additionally, CYP3A4 is thought to play a major role in hepatic metabolism of mefloquine, with both inhibition and induction studies suggesting this.^{14–16,18,19} Tentative data on auto-inhibition of mefloquine metabolism has also been found.¹⁴ In contrast, the enzymes that contribute to mefloquine metabolism are not fully understood. Previous work, both *in vitro* and *in vivo*, shows the involvement of CYP3A4, with the fraction metabolized by CYP3A4 (fm,CYP_{3A4}) being determined as 0.47 *in vivo*, and 0.41 *in vitro*.^{19,20} The remaining contributions by other CYPs remains unaccounted for. Moreover, interactions between mefloquine and other clinically administered drugs, other than those directly involved in CYP3A metabolism, have not been extensively studied. A prominent example is that of the anti-HIV drug efavirenz. While a potential interaction between efavirenz and mefloquine has been suggested as a result of the induction effect of efavirenz on CYP3A, a clinical study to investigate this interaction has not been undertaken.^{21,22}

While PBPK models of mefloquine do exist, they do not explore ethnic sensitivity.^{23–25} They also do not explore employing differing levels of mefloquine metabolism in explaining clinical variability. Indeed, different models make use of different metabolism profiles for mefloquine, chiefly the fm,CYP_{3A4}. Some models use a value for fm,CYP_{3A4} of 50%, without knowing what constitutes the rest, while others use a value of 95%.²³ This gap in knowledge may result in an incomplete understanding of the DDIs with mefloquine.

In this work, *in vitro* bioanalytical methods were developed to determine the fraction metabolized by CYP (fm,CYP) of mefloquine for use in a middle-out approach to constructing a PBPK model for mefloquine as a means to determine potential sources of variability in mefloquine PK.

2.3 Chromatographic development and analysis

Mefloquine was infused on an AB Sciex 4500Q LC-MS/MS in electrospray negative mode. The spectra of the deprotonated molecular ions as well as their fragments are illustrated in Figure 2.3.

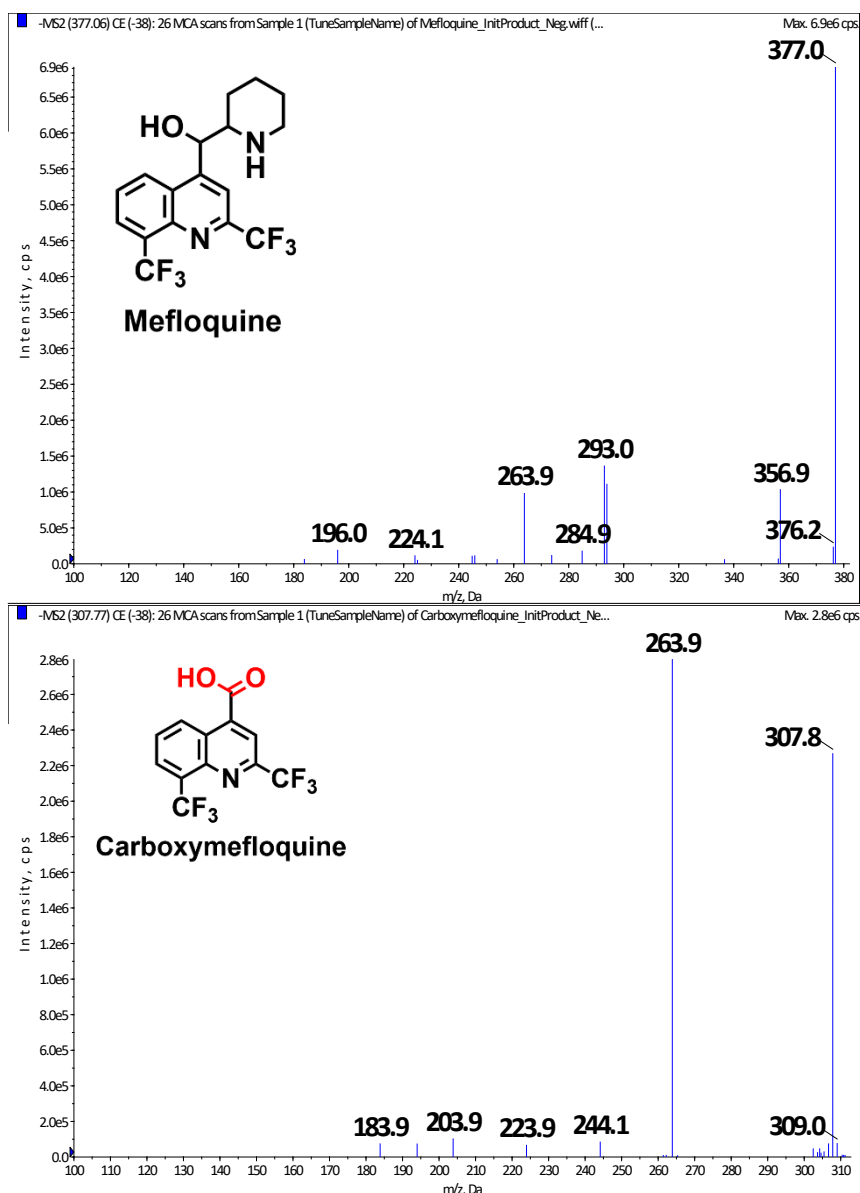


Figure 2.3: Initial product ion mass spectrum of A) Mefloquine and B) Carboxymefloquine, in negative mode, showing the $[M-H]$ ions at m/z 377 and 308 respectively and their product ion at 264

The product ions shown here were selected during chromatographic development as they showed the best sensitivity and reproducible chromatography. These transitions are also reported by other literature on mefloquine LC-MS/MS methods.^{15,26,27} Table 2.1 below summarizes the quantifier and qualifier ions for each infused compound.

Table 2.1: Summary of the quantifier and qualifier ions for each infused compound

	Mefloquine	Carboxymefloquine
Q1 [M-H]	377	308
Q3 [M-H]	264	264

Since the method developed to analyse mefloquine would need to distinguish between the substrate and metabolite, and given that the substrate and metabolite generate similar transitions due to in-source fragmentation, baseline separation of the two was necessary to minimize cross-talk.²⁸ This separation was achieved with the use of gradient chromatography in conjunction with a reversed phase chromatographic column. The final analytical method is summarised in Table 2.2 with the chromatography illustrated in Figure 2.4. Phenacetin was used as the internal standard (ISTD) for the developed method. It was chosen as it has similar extraction and ionization properties as the analytes under investigation.

Table 2.2: Summary of the analytical conditions for the developed method

Mefloquine	
Analytical Conditions	
Internal Standard	Phenacetin
Analyte	Carboxymefloquine
Column (Dimensions)	Gemini NX-C18 (2.0 x 50 mm; 5 µm)
Injection volume (µL)	5
Gradient Program %B (min)	10(0) → 95(2.5) → 95(3.5) → 10(3.6) → 10(5)
Flow rate (µL/min) ^a	1000

^aFlow split 1:2 into MS

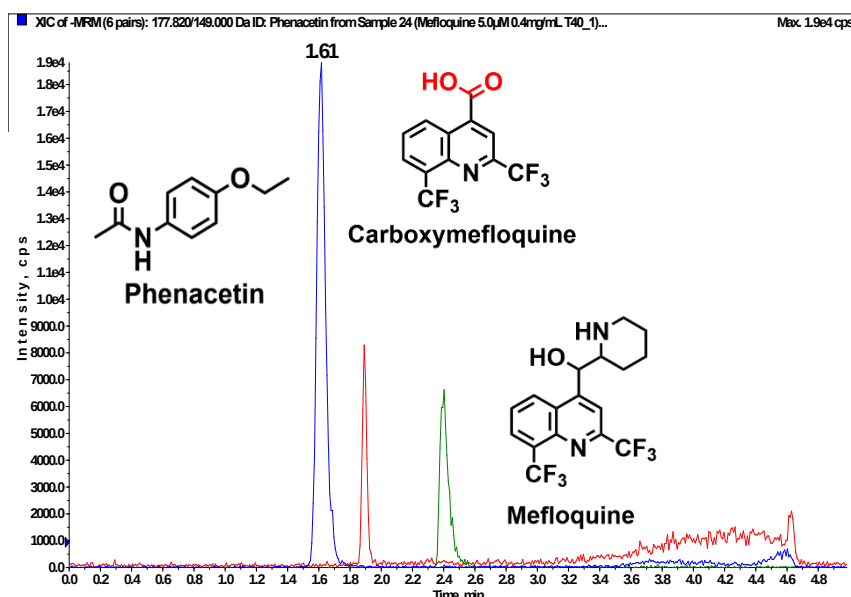


Figure 2.4: Representative chromatogram for mefloquine (green), carboxymefloquine (red) and phenacetin (blue)

2.3.1 Flow injection analyses

Upon obtaining optimal chromatographic conditions, an on-column flow injection analysis (FIA) was carried out to optimise the MS source parameters, such as curtain gas, ion spray voltage, and source temperature, as well as compound parameters to ensure optimal response

and sensitivity from the MS for this reaction. The resulting optimized MS/MS parameters are summarized in Table 2.3 below.

Table 2.3: Summary of the optimized MS conditions for mefloquine and carboxymefloquine

	Mefloquine	Carboxymefloquine
Q1 (m/z)	377	308
Q3 (m/z)	264	264
Source Temp. °C	525	525
Curtain Gas	40	40
Ion Source Gas 1	25	25
Ion Source Gas 2	41	41
Ion Spray Voltage	-4500	-4500
CE (eV)	-40	-25
EP	-4	-4
DP	-62	-25

This method was partially validated to ensure reproducibility. The results of this partial validation are included in the appendix.

2.3.2 Sample extraction

Protein precipitation is readily used as a sample extraction method when enzyme kinetics or metabolic stability studies are undertaken using liver microsomes or recombinant enzymes.^{29,30} As such, this was chosen as the extraction method for the methods developed here.

2.4 Development of biological method

Optimisation of the incubation conditions requires an assessment of reaction linearity with respect to microsomal protein and incubation time.^{31,32} It has been previously reported that mefloquine metabolism was linear for 3 hours up to a protein concentration of 5 mg/mL microsomal protein.¹⁷ This initial incubation was also done to test whether or not the substrate depletion approach was viable since literature suggested mefloquine microsomal metabolism would be slow. Linearity was assessed by plotting the log transformed percentage mefloquine remaining against time, to determine whether or not first order kinetics was being followed,³³ against time. These experiments were performed at two substrate concentrations, in duplicate. The range of conditions evaluated is summarised in Table 2.4 below.

Table 2.4: Incubation conditions used to test linearity of mefloquine kinetics

Substrate (Conc. µM)	Microsomal Protein (mg/mL)	Duration (min)
Mefloquine (0.5,5)	0.2; 0.4; 0.8; 1.2	60

The results of this linearity determination is summarized in Figure 2.5 below.

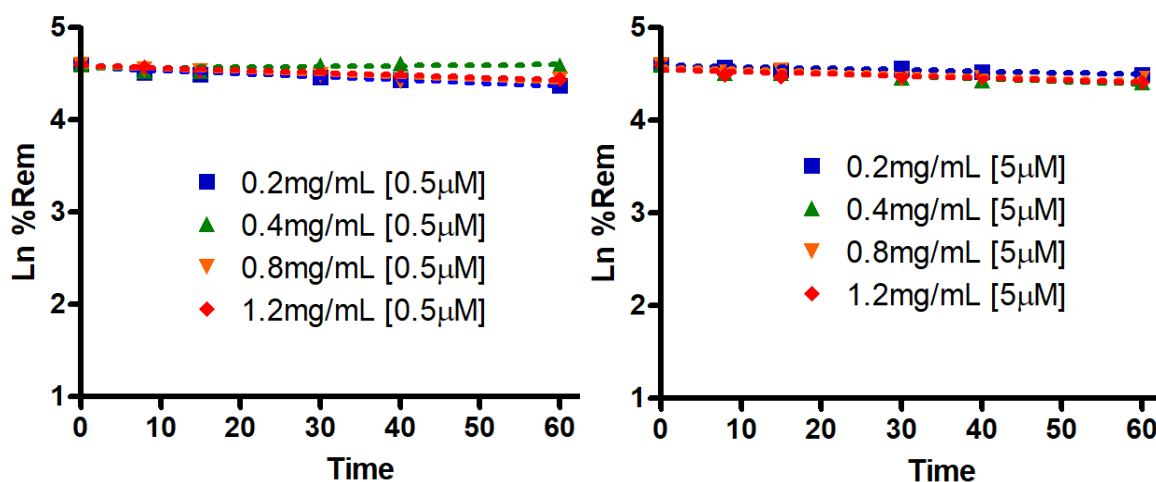


Figure 2.5: Summary of linearity determination data for mefloquine

The measured microsomal metabolism of mefloquine was slow, with mefloquine percentage remaining after 60 minutes ranging between 79-89%. There was also no statistical difference between the different incubation conditions at either substrate concentration. This illustrated that the substrate depletion approach would not be suitable to measure mefloquine kinetics since it is recommended that substrate depletion should reach a minimum of 80%.³³

It is known from literature that carboxymefloquine is the major metabolite resulting from mefloquine metabolism both *in vitro* and *in vivo*.^{17,34} As a result carboxymefloquine formation was also monitored during these analyses. The linearity of carboxymefloquine formation is plotted in Figure 2.6 below. Carboxymefloquine formation was below the limit of quantitation at the 0.5 μM substrate concentration.

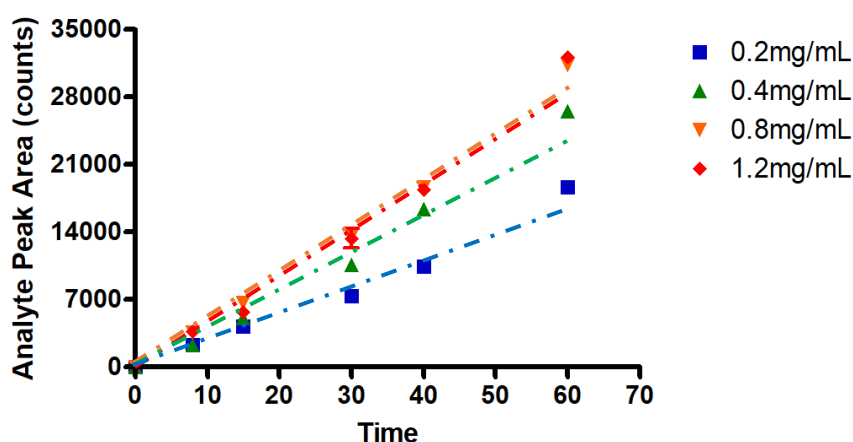


Figure 2.6: Linearity of carboxymefloquine formation at a 5 μM substrate concentration

Carboxymefloquine formation appeared linear for the duration of the incubation under all incubation conditions. Given that substrate depletion was not below 80% and that carboxymefloquine is the main metabolite formed via microsomal metabolism of mefloquine, along with the fact that carboxymefloquine formation could be measured using the developed method, the metabolite formation approach was selected to measure mefloquine kinetics.

As a means of determining carboxymefloquine linearity over time, three independent mefloquine incubations were done at three substrate concentrations. Given the need to keep protein concentrations low and incubation times short,³⁵ and that there was little difference in carboxymefloquine formation in the 0.4 mg/mL, 0.8 mg/mL and 1.2mg/mL incubations, a protein concentration of 0.4mg/mL for 40 minutes, were selected as the incubation conditions. The results of the linearity determination are summarized in Figure 2.7 below.

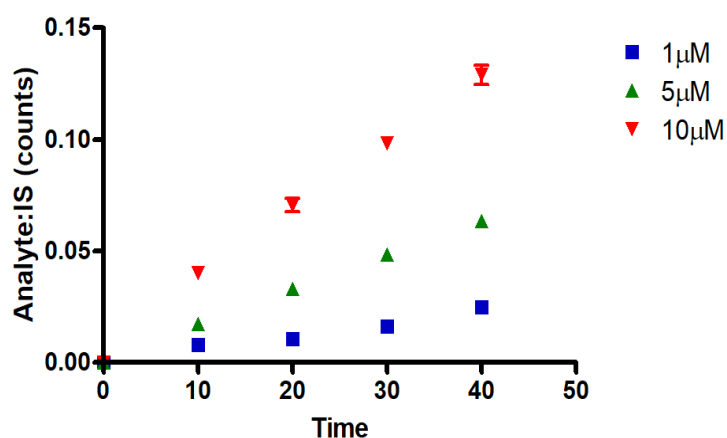


Figure 2.7: Linearity of carboxymefloquine formation

Carboxymefloquine formation was linear for the duration of the incubation at all substrate concentrations. A variety of incubation conditions as well as standard curve ranges were tested, including an attempt to replicate the work done by Bangchang *et al.*,¹⁷ however these did not provide an accurate determination of kinetics. In the case of the work done by Bangchang *et al.*¹⁷ specifically, kinetics could not be measured at the 400-800 μM incubation conditions, the upper limit of their substrate concentration range; likely due to mefloquine crashing out of solution. Turbidity was noted when the microsomal mixture was added to these substrate concentrations.

The incubation conditions were refined based on the collected data with the final incubation conditions selected for the determination of mefloquine kinetics summarised in Table 2.5.

Table 2.5: Refined incubation parameters for the determination of mefloquine kinetics

Parameter	Conditions tested
Protein Concentration (mg/mL)	0.4
Time (min)	0; 10, 20, 30, 40
Substrate Concentration Range (μM)	5-250
Standard Curve Range (nM)	0.5-1500

A representative substrate saturation plot for an incubation carried out under these conditions is shown in Figure 2.8 below.

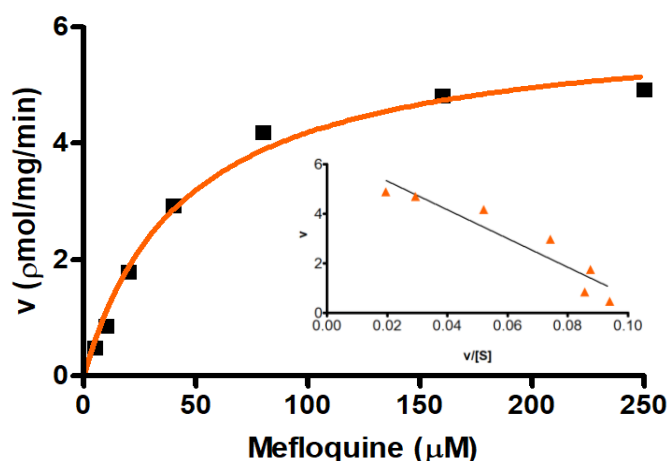


Figure 2.8: Substrate saturation curve for the determination of mefloquine kinetics using the refined incubation parameters. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics

The measured kinetic parameters from these incubations is summarized in Table 2.6 below. Data fitting was carried out using GraphPad v4 software.

Table 2.6: Summary of measured enzyme kinetics for mefloquine. Data gathered from two independent incubations

Kinetic Parameters	Mean	95% CI
K_M (μM)	47.2	39.1-55.3
V_{max} (pmol/mg/min)	7.11	6.70-7.53

CI = Confidence Interval

2.5 Measurement of fm,CYP

With the above data as a guide, the measurement of the fm,CYP for mefloquine was undertaken using the chemical inhibition method corrected for CYP inhibitor cross reactivity, as previously described by Njuguna *et al.*³⁶ As part of this process an incubation is done using different CYP inhibitors, corresponding to the six major isoforms; namely CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, along with the compound of interest at a concentration lower than its K_M in HLM. This is done to ensure that the substrate does not saturate any

enzyme involved in its metabolism, thereby improving accuracy of inhibition results.³¹ The incubation conditions to measure mefloquine fm,CYP are shown in Table 2.7 below.

Table 2.7: Incubation conditions for the measurement of mefloquine fm,CYP

Parameter	Conditions tested
Protein Concentration (mg/mL)	0.4
Time (min)	0; 10, 20, 30, 40
Standard Curve Range (nM)	0.5-1500
K_M (μ M)	47.2
Substrate Concentration (μ M)	10

The chemical inhibitor-CYP enzyme combinations used are shown in Table 2.8.

Table 2.8: Summary of chemical inhibition conditions for the fm,CYP investigation

Enzyme	Inhibitor	Concentration	Type of inhibition
CYP1A2	Furafylline	10 μ M	Mechanistic
CYP2C8	Montelukast	2 μ M	Competitive
CYP2C9	Sulfaphenazole	5 μ M	Competitive
CYP2C19	Ticlopidine	10 μ M	Mechanistic
CYP2D6	Quinidine	2 μ M	Competitive
CYP3A	Ketoconazole	1 μ M	Competitive

The HLM in buffer pre-mix was pre-incubated with NADPH along with the mechanistic inhibitors, for 20 minutes before the incubation was started by the addition of mefloquine. The competitive inhibitors were added to the incubation plate at the same time as the compound and the incubation was carried as described above. In Figure 2.9 below are the results of the chemical inhibitor incubations.

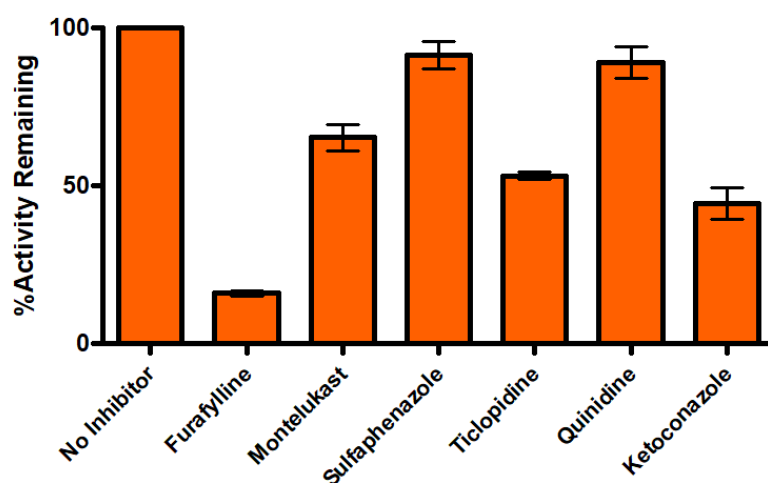


Figure 2.9: Chemical inhibition results showing the percentage enzyme activity remaining after the addition of specific inhibitors to the HLM incubation

Mefloquine fm,CYP was calculated using the rate of metabolite formation k as shown in the equation below.

$$\left[1 - \left(\frac{k \text{ in presence of inhibitor}}{k \text{ with no inhibitor present}}\right)\right] * 100 \quad (10)$$

The fm,CYP for mefloquine calculated using this approach are shown in Table 2.9 below.

Table 2.9: fm,CYP for mefloquine of the six major CYP isoforms. Data gathered from two independent incubations

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	84.8	16.7	9.50	22.2	4.90	42.8

From the data in Table 2.9, CYP1A2 and CYP3A4 appear to be the major isoforms involved in the *in vitro* microsomal metabolism of mefloquine with moderate contributions from CYP2C8 and CYP2C19 and minor contributions from the other CYPs. However, inhibitors can potentially play a role in the inhibition of more than one enzyme.³⁷ Therefore, correcting for this cross-reactivity is necessary to more accurately calculate fm,CYP. This was done using the cross-reactivity matrix, pictured in Figure 2.10 below, established by Njuguna *et al*³⁶.

$$\begin{array}{l}
 \text{CYP1A2} \quad \text{CYP2C8} \quad \text{CYP2C9} \quad \text{CYP2C19} \quad \text{CYP2D6} \quad \text{CYP3A} \\
 \begin{array}{l}
 \text{Furafylline (10 } \mu\text{M)} \\
 \text{Montelukast (2 } \mu\text{M)} \\
 \text{Sulfaphenazole (5 } \mu\text{M)} \\
 \text{Ticlopidine (10 } \mu\text{M)} \\
 \text{Quinidine (2 } \mu\text{M)} \\
 \text{Ketoconazole (1 } \mu\text{M)}
 \end{array}
 \begin{pmatrix}
 0.89 & 0.14 & 0.063 & 0.076 & 0.16 & 0.0055 \\
 0.0082 & 0.87 & 0.49 & 0.039 & 0.091 & 0.34 \\
 0.13 & 0.10 & 0.89 & 0.11 & 0.074 & 0.080 \\
 0.67 & 0.21 & 0.19 & 0.87 & 0.54 & 0.00 \\
 0.00 & 0.090 & 0.041 & 0.16 & 0.89 & 0.029 \\
 0.12 & 0.45 & 0.27 & 0.00 & 0.092 & 0.95
 \end{pmatrix}
 \cdot
 \begin{pmatrix}
 a \\
 b \\
 c \\
 d \\
 e \\
 f
 \end{pmatrix}
 =
 \begin{pmatrix}
 p \\
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 u
 \end{pmatrix}
 \end{array}$$

Figure 2.10: Adaptation of the cross-reactivity matrix established by Njuguna *et al*.

Once the calculated fm,CYP values were substituted into the matrix equation the corrected fm,CYP values, seen in Table 2.10, were obtained.

Table 2.10: Corrected fm,CYP values for mefloquine of the six major CYP isoforms

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	65.3	5.97	0	0	9.85	18.9

The data shows contributions from four of the six isoforms tested. This is of great interest since only the contribution of CYP3A has previously been reported.

To account for differences in the methodology to obtain enzyme kinetic data, the cross-reactivity matrix established by Njuguna *et al.*³⁶ was re-measured using the methodology established here. As a result, the incubation conditions were revised with a protein concentration of 0.1mg/mL for a duration of 60 minutes being used for CYP1A2 and CYP2C19 inhibition, and a protein concentration of 0.03mg/mL for a duration of 15 minutes used for the remaining CYP isoforms. This updated cross-reactivity matrix is seen in Table 2.11.

Table 2.11: Updated cross-reactivity matrix for fm,CYP correction

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A
Furafylline (10 µM)	1.00	0.135	0.0633	0.0757	0.0026	0.055
Montelukast (2 µM)	0.008	1.00	0.645	0.0393	0.250	0.918
Sulfaphenazole (5 µM)	0.126	0.10	0.948	0.111	0.0742	0.0802
Ticlopidine (10 µM)	0.668	0.214	0.189	0.868	0.495	0.00
Quinidine (2 µM)	0.00	0.155	0.003	0.162	1.00	0.079
Ketoconazole (1 µM)	0.117	0.156	0.2128	0.00	0.064	1.00

Chemical inhibition, as well as fm,CYP, of mefloquine was also re-evaluated for all CYP isoforms using the incubation conditions used for CYP1A2 inhibition to more accurately measure furafylline inhibition while also maintaining adequate metabolism to meet the turnover requirements set out by Njuguna *et al.*³⁶, being 70-80% consumption of substrate at the end of the incubation period. The revised fm,CYP data are summarised in Table 2.12 below.

Table 2.12: Updated fm,CYP values for mefloquine. Data gathered from two independent incubations

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	84.8	16.7	9.50	22.2	4.90	42.8
Corrected fm,CYP	65.4	0	1.13	0	9.07	24.4

2.6 Recombinant enzyme studies

To confirm the involvement of the aforementioned enzymes in the conversion of mefloquine to carboxymefloquine, mefloquine was incubated with recombinant CYP (rCYP) enzymes generated from baculovirus transfected cell strains. The conditions used mirrored those used during the determination of mefloquine fm,CYP with only the protein source differing. These conditions are summarised in Table 2.13 below.

Table 2.13: Incubation conditions for the determination of mefloquine metabolism by rCYPs

Parameter	Conditions tested
Enzyme Concentration (pmol/mL)	50
Time (min)	0; 10; 20; 30; 40
Standard Curve Range (nM)	0.5-1500
K_M (μ M)	47.2
Substrate Concentration (μ M)	10

Shown in Figure 2.11 below are the results of the rCYP incubations. Although fm,CYP was not determined for CYP3A5, due to the difficulty in establishing HLM kinetic experiments as a result of a lack of a truly specific substrate and inhibitor, the ability of CYP3A5 to convert mefloquine to carboxymefloquine was tested due to the involvement of CYP3A4 in mefloquine metabolism. This was done since CYP3A4 and CYP3A5 often overlap in the compounds they are able to metabolize.

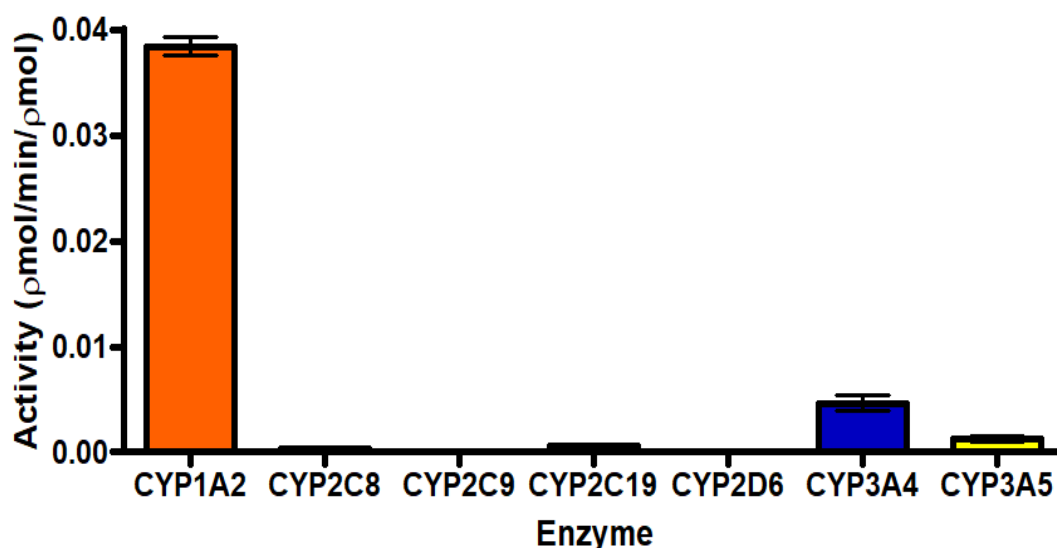


Figure 2.11: Results from the rCYP incubations showing the enzyme activity of tested CYP enzymes in converting mefloquine to carboxymefloquine.

The data in the above Figure confirms the metabolic pattern determined during the fm,CYP studies discussed in the preceding section. With this pattern confirmed, full kinetics studies using rCYPs were undertaken to characterise the reactions between mefloquine and the enzymes responsible for its *in vitro* metabolism. The conditions used mirrored those used during the determination of mefloquine enzyme kinetics in HLM with only the protein source differing. These conditions are summarised in Table 2.14 below.

Table 2.14: Incubation parameters for the determination of mefloquine enzyme kinetics

Parameter	Conditions tested
Enzyme Concentration (pmol/mL)	50
Time (min)	0; 10, 20, 30, 40
Substrate Concentration Range (μM)	5-250
Standard Curve Range (nM)	0.5-1500

In Figures 2.12-2.14 are substrate saturation plots for the determination of mefloquine CYP1A2, CYP3A4 and CYP3A5 kinetics.

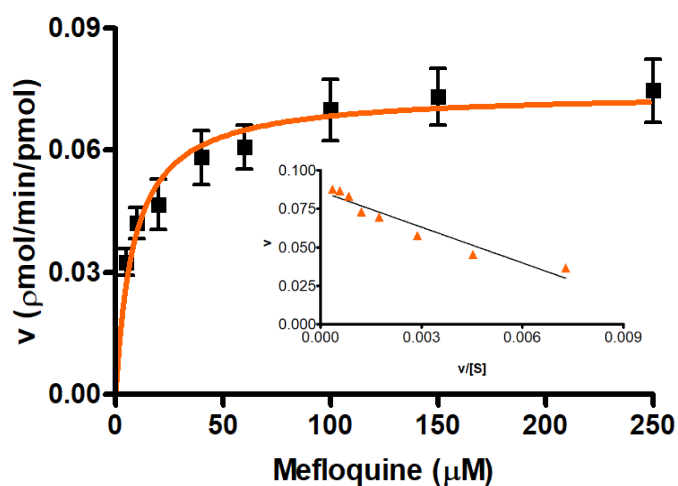


Figure 2.12: Substrate saturation curve for the determination of mefloquine CYP1A2 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics. Data gathered from two independent incubations

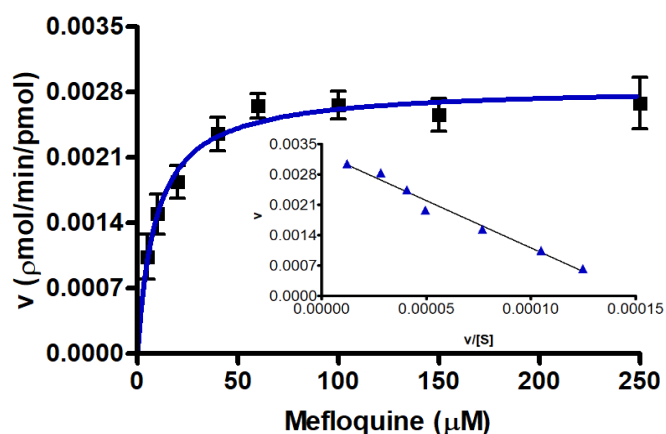


Figure 2.13: Substrate saturation curve for the determination of mefloquine CYP3A4 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics. Data gathered from two independent incubations

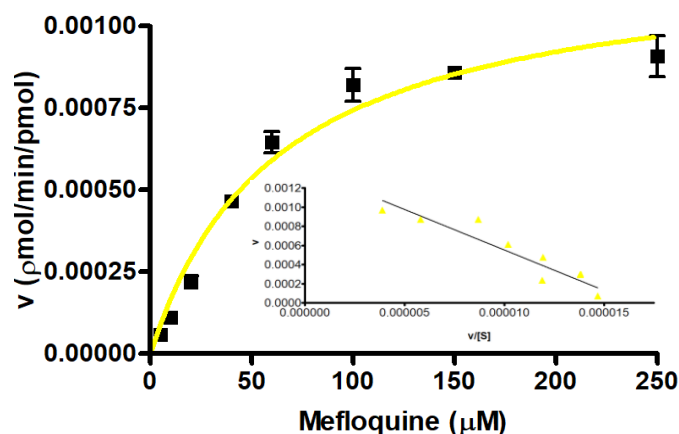


Figure 2.14: Substrate saturation curve for the determination of mefloquine CYP3A5 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics. Data gathered from two independent incubations

In Table 2.15 is summarised the enzyme kinetics parameters determined for the conversion of mefloquine to carboxymefloquine by the enzymes previously determined to be involved in its *in vitro* metabolism.

Table 2.15: Enzyme kinetic parameters for the conversion of mefloquine to carboxymefloquine via specific rCYP isoforms. Data gathered from two independent incubations

Enzyme	K_M (μM) [95%CI]	$V_{\max}$ (pmol/min/pmol) [95%CI]
CYP1A2	8.51 [7.10-9.92]	0.0742 [0.0718-0.0767]
CYP3A4	24.0 [20.9-27.2]	0.00347 [0.00333-0.00361]
CYP3A5	63.0 [43.1-83.0]	0.00121 [0.00106-0.00136]

The conversion of mefloquine to carboxymefloquine by CYP1A2 was shown to be a low K_M reaction, whereas this same reaction mediated by CYP3A4 and CYP3A5 was shown to be more moderate. This reaction was also shown to occur at a more than 20-fold faster rate than when mediated by CYP1A2 as compared to either CYP3A enzyme. However, it's important to note that due to low CYP1A2 K_M , only one mefloquine concentration was below K_M , which suggests that CYP1A2 K_M may be underestimated.

The *in vitro* metabolic profile for mefloquine was subsequently used to inform the development of PBPK models to predict mefloquine exposure.

2.7 Mefloquine PBPK Modelling

PBPK models were developed using the population-based PBPK software Simcyp (Simcyp Ltd, a Certara company, Sheffield, UK; version 21). The development strategy for this model followed established best practice.³⁸ This process entailed model development and optimization, followed by model verification and lastly, the application of the model to scenarios of interest. An overview of this process is illustrated in Figure 2.15 below.

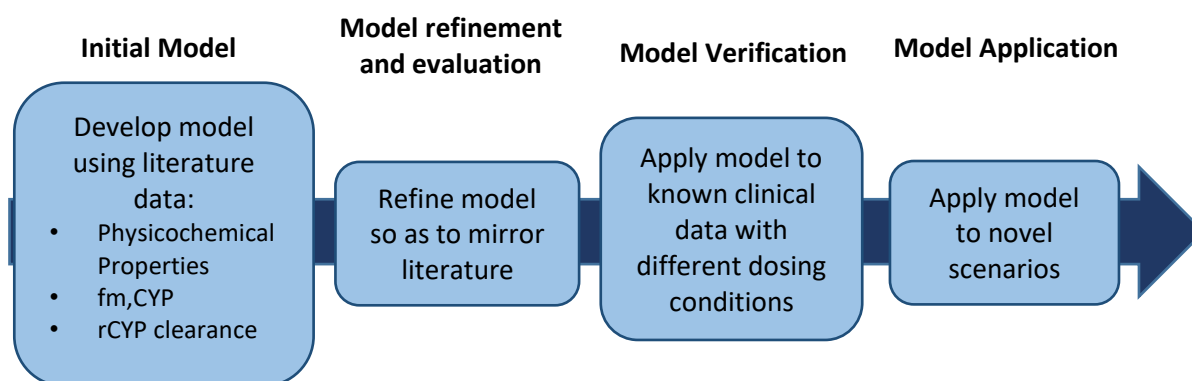


Figure 2.15: Overview of PBPK modelling process

2.7.1 Development of Mefloquine model

Mefloquine drug input parameters are listed in Tables 2.16-2.18 below.

Table 2.16: Input parameter values used to simulate the pharmacokinetics of mefloquine

Input Parameter	Description	Value	Unit	Reference
MW	Molecular weight	379.3	g/mol	Johnson <i>et al.</i> 2019 ²³
Log $D_{7.4}$	Octanol-water partition	2.7		Charman <i>et al.</i> 2020 ³⁹
Log $P_{O:W}$		3.83		Calculated from LogD in Simcyp
Compound Type	Acid, base or neutral	Base		Charman <i>et al.</i> 2020 ³⁹
pK_a	pKa in water	8.5		Charman <i>et al.</i> 2020 ³⁹
B/P ratio	Blood to plasma concentration ratio	2		Schwartz <i>et al.</i> , 1982 ³⁴
f_u	Fraction unbound in plasma	2	%	Looareesuwan <i>et al.</i> 1987 ⁸
$f_{u(inc)}$	Fraction unbound in vitro			
	Human Plasma	0.015		
	Albumax	0.27		Charman <i>et al.</i> 2020 ³⁹
	HLM	0.05		
F_a	Fraction available from dosage	0.731	%	Predicted by Simcyp
K_a	Absorption rate constant	0.401	1/h	Predicted by Simcyp
Q_{gut}	Nominal flow in gut model	6.03	L/h	Predicted by Simcyp
$P_{eff,man}$	Effective permeability in man	0.571	(10 ⁻⁴ cm/s)	Predicted based on Caco-2 Data
$f_{u(gut)}$	Unbound fraction in enterocytes	0.0006		Predicted by Simcyp
S_o	Intrinsic Solubility	0.29	mg/mL	Johnson <i>et al.</i> 2019 ²³ (Roche data files)
V_{ss}	Volume of distribution at steady-state	18	L/kg	Schwartz <i>et al.</i> , 1982 ³⁴
K_p Scalar	Scalar applied to all predicted tissue K_p values	1.08		Input into Simcyp to obtain V_{ss} of 18

Values in bold used in model

Table 2.17: Permeability input parameters, in the form of Caco-2 data, for the simulation of mefloquine pharmacokinetics.
Data sourced from Charman et al. 2020³⁹ & Katneni et al. 2018⁴⁰

Reference Compounds					
Unit P _{app} (10 ⁻⁶ cm/s)					
Compound	Description		A-B Range	B-A Range	Efflux Ratio Range
Lucifer Yellow	Minimal Marker	Permeability	<2	Not assessed	-
Propranolol	High Marker	Permeability	58	52	0.9-1.0
Rhodamine 123	P-gp efflux Marker		0.7-1.6	10-28	6-39
Atenolol			0.39		
Cimetidine			1.7		
Metoprolol			50		
Verapamil			41	45	1.1
Compounds in bold were used to predict a scalar from which Peff,man was predicted using mefloquine Caco-2 data below					
Unit P _{app} (10 ⁻⁶ cm/s)					
Compound	Description		A-B	B-A Range	Efflux Ratio
Mefloquine	-		2.1	4.7	2.2
Given mefloquine efflux ratio >2, Mean of the A-B and B-A was used i.e 3.4 with passive and active transport					

Values in bold used in model

Table 2.18: Elimination input parameters for mefloquine for the simulation of mefloquine pharmacokinetics

Input Parameter	Description	Value	Unit	Reference
CL _{iv}	Intravenous clearance	1.8	L/h	FDA Label
CL _r	Renal Clearance	9% of drug in urine i.e 0.162	L/h	Schwartz <i>et al.</i> 1987 ¹³
V _{max}	<i>In vitro</i> maximum velocity measured in HLM	7.11	pmol/mg/min	Measured
K _M	<i>In vitro</i> Michaelis-Menten constant measured in HLM	47.2	μM	Measured
HLM CL _{int}	Intrinsic clearance measured in HLM	0.151	(μL/min/mg)	Measured
CYP1A2 rCYP CYP3A4 rCYP CYP3A5 rCYP	Intrinsic clearance measured in rCYP	0.00872 0.000144 0.0000192	(μL/min/pmol)	Measured
CYP1A2 CL _{int} CYP3A4 CL _{int} CYP3A5 CL _{int}	Intrinsic clearance determined for enzyme by RTT	0.267 0.0651 0.0310	(μL/min/pmol)	Simcyp

Table 2.19. Interaction input parameters, in the form of inhibition constants, for the simulation of mefloquine pharmacokinetics

Interaction Properties				
Interaction	Description	Value	Unit	Reference
CYP2D6 (Weak Inhibitor)	K _i	10.0	μM	39

A single-dose PK study published by Schwartz *et al* was used as the basis for the development of this model.³⁴ A mefloquine paediatric PBPK model, published by Johnson *et al*,²³ was also used for model refinement. PK simulations during the development phase were thus performed using the Simcyp Healthy Volunteer population. The trial design was replicated as closely as possible, by using the same age range and number and gender of participants for example, with these virtual trials being simulated ten times in each run. The fasted state Advanced Dissolution Absorption and Metabolism (ADAM) and full PBPK model modes were employed.⁴¹

These models, in contrast to the traditionally used first order absorption model which divides the body into only two or three compartments, considers the physiological properties of various sections and subsections of the human body, as discussed in Chapter 1. As an example, the gut, which is traditionally treated as only one compartment with one uniform blood flow rate, is broken down into its constituent parts; stomach, duodenum, jejunum, ileum, cecum, and colon, with the volume as well as blood flow rate into and out of each part individually accounted for. This provides added complexity to the model which, in turn, provides greater confidence in the data generated by said model.

Initial simulations were attempted using the above-mentioned input data. Figure 2.16 below show simulations done using the measured *in vitro* metabolism data. The green line represents the simulated concentration-time profile while the orange dots represent the data obtained from Schwartz *et al*. overlayed over the simulated profiles.

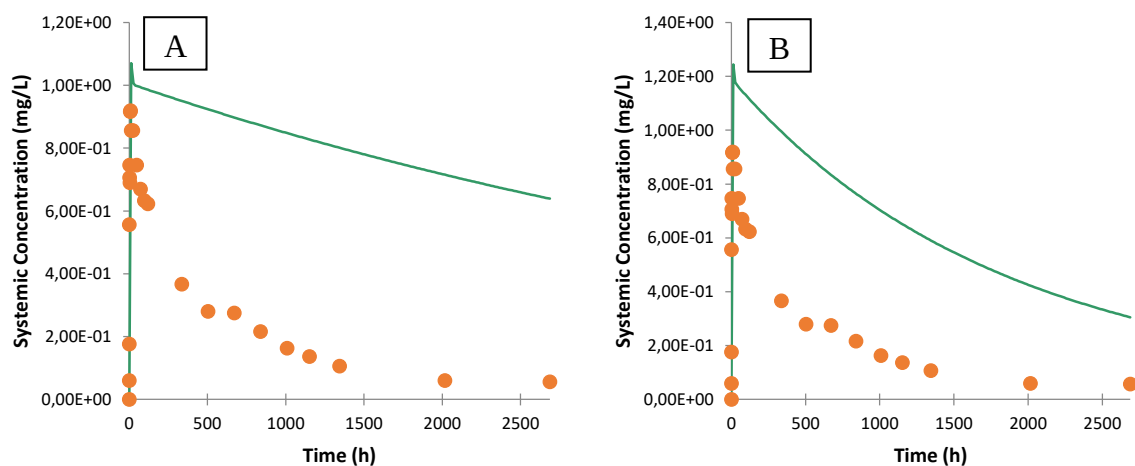


Figure 2.16: Simulated (green line) and observed (orange data points) mean plasma concentration-time profiles of mefloquine after a single oral dose of 1000 mg mefloquine with trial parameters matching that of Schwartz *et al.*, (10 trials of 3 male subjects, 42-49 yrs). (A) Model run with elimination being determined by HLM Clint (B) Model run with elimination being determined by rCYP clearance.

As can be seen, these models underpredict mefloquine clearance. To overcome this, a retrograde modelling approach was employed. When using this process, the known CL_{iv} was used to calculate mefloquine clearance by the CYP enzymes determined to be involved in its metabolism using the Reverse Translational Tool (RTT) function in the Simcyp software. As part of this approach, 50% of mefloquine hepatic clearance was assigned to CYP1A2 with the remaining 50% assigned to CYP3A. These assignments were used based on the fm,CYP determined for mefloquine, the rCYP kinetics studies showing similar activity for CYP1A2 and CYP3A4, and because, as stated before, it is known that CYP3A is an important enzyme involved in *in vivo* mefloquine clearance.^{18,19,42} Since CYP3A5 activity was shown to be approximately 30% of CYP3A4 activity, the 50% assignment of hepatic clearance to CYP3A was assigned as 35% CYP3A4 and 15% CYP3A5. Figure 2.16 below shows simulations done when the retrograde model was employed to predict mefloquine clearance parameters using the RTT. Table 2.20 shows the PK parameters obtained from the developed model as well as the observed data from Schwartz *et al.*³⁴

Table 2.20: Simulated and observed PK parameters based on the developed model and data from Schwartz *et al.*

PK Parameter	Model	Literature
C_{max} (mg/L) (5 th and 95 th)	1.09 (0.969-1.21)	0.92
AUC (mg/L·h) (5 th and 95 th)	535 (402-716)	517

Figure 2.17 below illustrates that the developed model could describe the concentration-time profile of mefloquine under the stated conditions. The simulated PK parameter mean values were within two-fold of the observed values, $C_{\max}$ (mean 1.19-fold, range 0.991 to 1.38 fold) and AUC (mean 1.03-fold, range 0.739 to 1.48-fold).

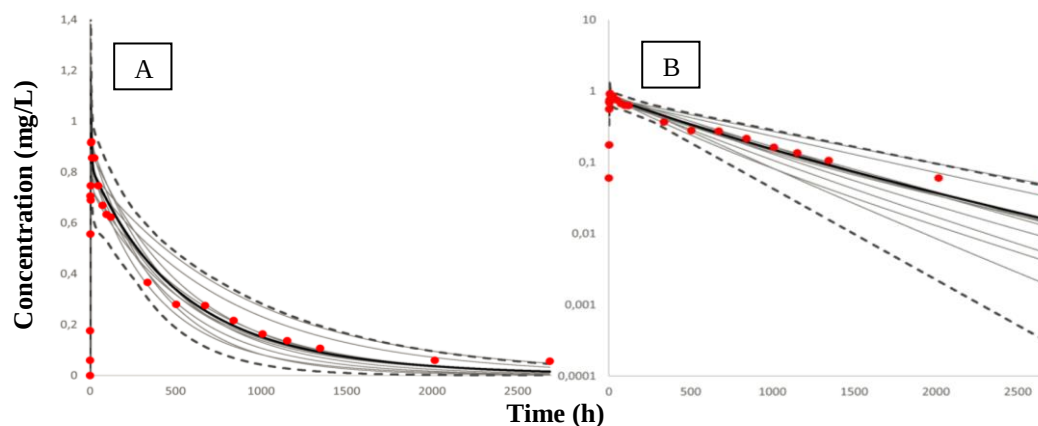


Figure 2.17: : Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after a single oral dose of 1000 mg mefloquine (A), (10 trials of 3 male subjects, 42-49 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Schwartz et al. 1982. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

It should be noted that, likely due to the duration of the study used for model development necessitated by the prolonged mefloquine exposure profile, the model shows sensitivity to the number of data points sampled. The default number of data points sampled in Simcyp is 200. However, this does not provide an accurate resolution of the data. This brings about variability in the predicted concentration-time profiles when the duration of the model is changed or a delay in when the first mefloquine dose is administered in the model; such as in the case of interaction studies where a drug with a potential DDI is administered prior to the administration of mefloquine. To overcome this, a sampling number of 3000, roughly one data point per simulated hour, was used and was seen to provide consistent model output and more accurate simulated data resolution.

2.7.2 Model Verification: Mefloquine

The model developed above was then verified by simulating mefloquine concentration-time profiles using different dosage parameters. These simulated parameters were then compared to known data in literature where their dosage parameters were used while maintaining the same compound input parameters. Other study parameters, such as proportion of females involved in the study and the age range of the participants, was also changed to more accurately represent the study being used for verification. Table 2.21 below lists the different studies, and their study details, used to verify the developed model. When a study was conducted in Thai individuals,

the Simcyp Chinese Healthy Volunteer population was used to better represent those individuals. In all other cases, the Simcyp Healthy Volunteer population was used.

Where reported PK data did not provide corresponding summary data, observed data points in reported figures were retrieved using WebPlotDigitizer v4.6.⁴³ These were then superimposed onto simulated profiles for visual predictive checks. PK data was retrieved by conducting non-compartmental analysis (NCA) of reported figures using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA) with a method based on curve stripping. In the case of the studies conducted by Khaliq *et al.*⁴², data was only reported for a single study day in the form of a figure. Data was retrieved as described here and comparison between observed and simulated data was done for only the corresponding simulated day.

Table 2.21: Summary of literature sources used in model verification

Pharmacokinetic studies used in model verification		
Study Details	Study type and description (Duration)	Reference
Thai HV 24-28 yrs 5 M	SD. 750 mg (72 days)	Boudreau <i>et al.</i> 1990 ⁴⁴
Thai HV 24-35 yrs 5 M	SD. 1500 mg (72 days)	Boudreau <i>et al.</i> 1990 ⁴⁴
Caucasian HV 23-25 yrs 6 M	SD. 250 mg (63 days)	Looareesuwan <i>et al.</i> 1987 ⁸
Brazilian HV 26-39 yrs 5 M	MD. 21 weekly 250 mg doses followed by 125 mg for one week then 250 mg in week 24 (168 days)	Mimica <i>et al.</i> 1983 ⁴⁵
Caucasian HV 19-40 yrs 6 M 6 F	MD. 250 mg dosed once daily for 3 days then once weekly for 3 weeks (30 days)	Khaliq <i>et al.</i> 2001 ⁴²
Thai HV 24-35 yrs 7 M	Interaction. 600 mg RIF was dosed once daily for 7 days prior to MFQ administration on day 7 as a 500 mg dose. RIF was then dosed once daily for 7 days post MFQ dose, then twice weekly from day 8 post MFQ dose to day 56 (57 days)	Ridtitid <i>et al.</i> 2000 ¹⁸
Thai HV 16-39 yrs 8 M	Interaction. 400 mg KTZ was dosed once daily for 5 days prior to MFQ administration on day 5 as a 500 mg dose; with KTZ dosing continuing for 5 days after MFQ dose (57 days)	Ridtitid <i>et al.</i> 2005 ¹⁹

HV=Healthy Volunteers; MFQ=Mefloquine; RTV=Ritonavir; RIF=Rifampicin; KTZ=Ketoconazole

In Figures 2.18-2.20 below are representative concentration-time profiles for model verification. These represent single dose, multiple doses, and DDI studies. These figures illustrate that the developed model could describe the concentration-time profile of mefloquine under a variety of conditions reasonably well.

Complete data sets for all the other studies used for model verification can be found in the appendices at the end of the document.

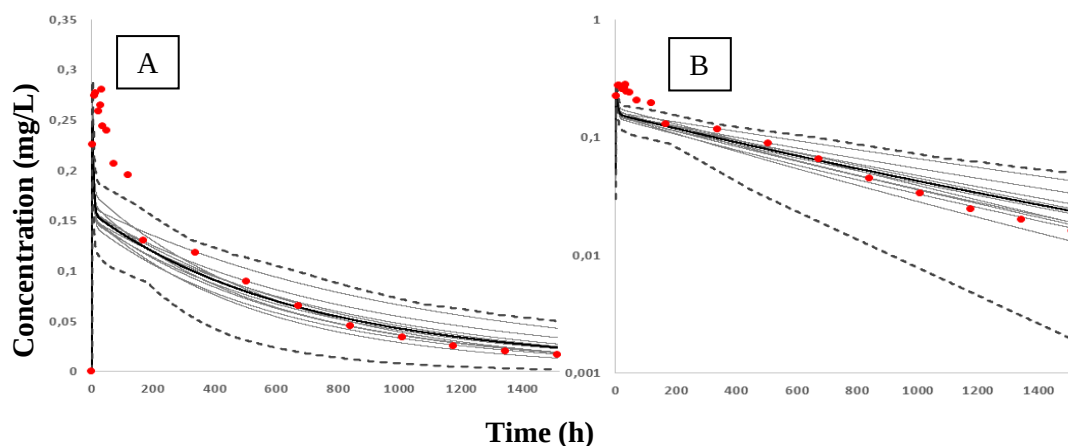


Figure 2.19: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after a single oral dose of 250 mg mefloquine (A), (10 trials of 6 male subjects, 23-25 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Looareesuwan et al., 1987. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

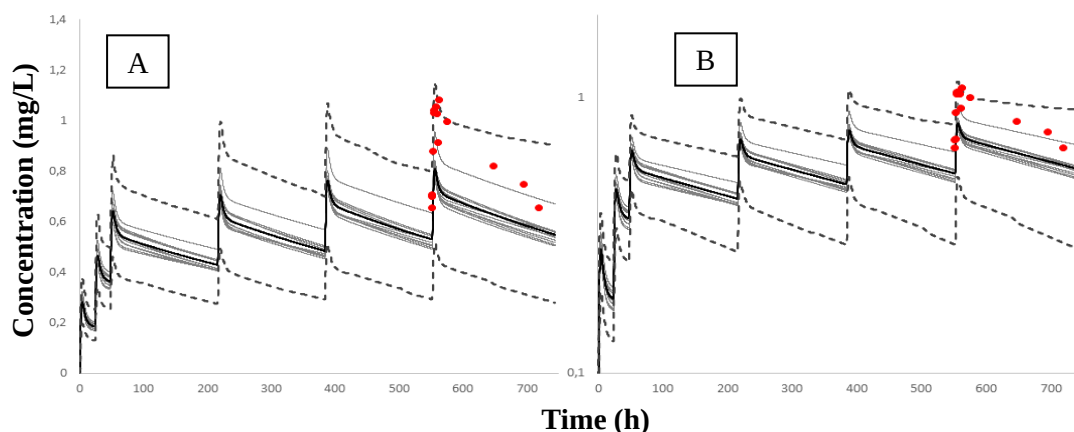


Figure 2.18: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after 3 daily doses followed by 4 weekly doses of 250 mg mefloquine (A), (10 trials of 6 male and 6 female subjects, 19-40 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Khaliq et al., 2001. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population

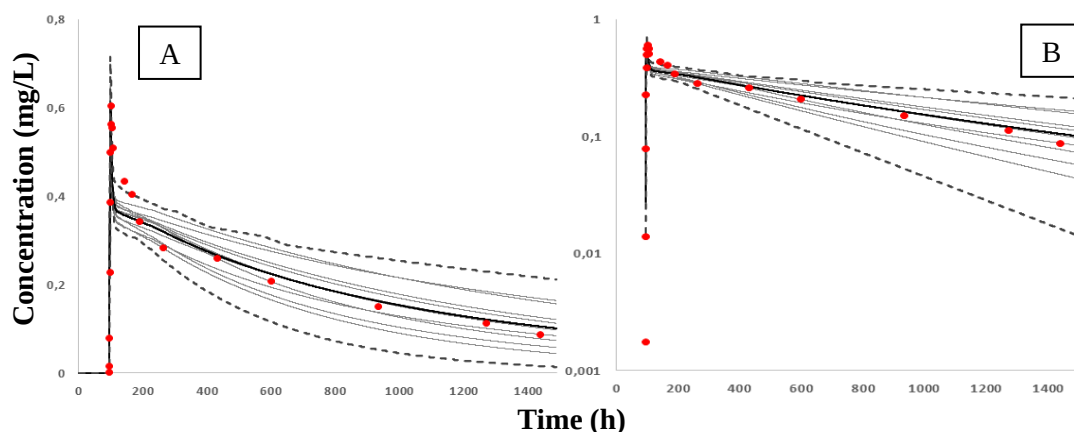


Figure 2.20: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after single oral dose of 500 mg preceded by pre-treatment with 400 mg of ketoconazole, taken once a day for 5 consecutive days before mefloquine dose and 5 consecutive days after mefloquine dose (A), (10 trials of 8 male subjects, 16-39 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Rittitid et al., 2005. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population

The observed and predicted $C_{\max}$ ratios were 0.81 vs 0.98 for the interaction with rifampicin, and 1.64 vs 1.01 for the interaction with ketoconazole. The observed and predicted AUC ratios were 0.32 vs 0.69 for the interaction with rifampicin, and 1.79 vs 1.04 for the interaction with ketoconazole. The model had difficulty in predicting the interaction ratios for the interaction with ketoconazole. This could be due to the inhibitory effect ketoconazole has on P-glycoprotein (P-gp) as others have suggested²³. However, investigating this specific interaction is outside the scope of this work and these mean fold differences between simulated and observed data for model verification were similar to previously published mefloquine PBPK models^{23,24}. In addition, the simulated PK parameter mean values were also within two-fold of the observed values for all studies used. Thus, the mefloquine model was considered verified.

Table 2.22: Comparison between reported and simulated PK parameters for the studies used for model verification

Study (Type)	PK Parameter	Model	Literature	Mean Fold Diff. (Range)
Boudreau <i>et al.</i> 1990 ⁴⁴ (SD; 750 mg)	$C_{\max}$ (mg/L) (5 th and 95 th)	0.876 (0.819-0.946)	1.18	0.742 (0.684-0.802)
	AUC (mg/L·h) (5 th and 95 th)	445 (386-554)	518	0.859 (0.707-1.00)
Boudreau <i>et al.</i> 1990 ⁴⁴ (SD; 1500 mg)	$C_{\max}$ (mg/L) (5 th and 95 th)	1.84 (1.72-1.93)	1.44	1.28 (1.18-1.35)
	AUC (mg/L·h) (5 th and 95 th)	901 (775-1090)	602	1.50 (1.20-1.68)
Looareesuwan <i>et al.</i> 1987 ⁸ (SD)	$C_{\max}$ (mg/L) (5 th and 95 th)	0.247 (0.233-0.269)	0.320*	0.771 (0.727-0.862)
	AUC (mg/L·h) (5 th and 95 th)	103 (84.9-127)	113*	0.912 (0.733-1.18)
Mimica <i>et al.</i> 1983 ⁴⁵ (MD)	$C_{\max}$ (mg/L) (5 th and 95 th)	0.873 (0.421-1.32)	0.900*	0.969 (0.639-1.11)
	AUC (mg/L·h) (5 th and 95 th)	2470 (996-4220)	2360*	1.05 (0.630-1.22)
Khaliq <i>et al.</i> 2001 ⁴² (MD)	$C_{\max}$ (mg/L) (5 th and 95 th)	0.809 (0.515-1.15)	1.08**	0.749 (0.720-0.885)
	AUC (mg/L·h) (5 th and 95 th)	122 (69.0-184)	143**	0.846 (0.824-1.01)
Riditid <i>et al.</i> 2000 ¹⁸ (Interaction; RFP)	$C_{\max}$ (mg/L) (5 th and 95 th)	0.602 (0.567-0.631)	0.696	0.864 (0.807-0.910)
	AUC (mg/L·h) (5 th and 95 th)	198 (158-249)	120	1.65 (1.29-2.21)
Riditid <i>et al.</i> 2005 ¹⁹ (Interaction; KCZ)	$C_{\max}$ (mg/L) (5 th and 95 th)	0.617 (0.548-0.658)	0.568	1.09 (0.927-1.17)
	AUC (mg/L·h) (5 th and 95 th)	285 (216-358)	286	0.996 (0.729-1.26)

* Values not reported, data obtained through non-compartmental analysis of reported figures

**Data only reported for one study day, modelling data reported for corresponding simulated day

2.7.3 Model Application: Mefloquine

Upon verification, the mefloquine model was applied to various scenarios of interest. In all model application investigations, mefloquine was administered in the model at a dosage of 1000 mg. All model application investigations were done as 10 trials of 5 male and 5 female subjects with an age range of 20-65 years.

2.7.3.1 Model Application: Ethnic sensitivity

Simcyp has an available black South African tuberculosis population file (V16R1_SouthAfricanTB_Population), with supporting information, accessed through the global health repository within the Simcyp members area.^{46,47} This population file is distinct from the healthy volunteer population files used up to this point in a variety of factors which reflect the unique physiological conditions of a black South African population group infected with tuberculosis. Specifically, physiological parameters relating to body surface area, age ranges, height and weight relationships as well as the expression and distribution of metabolic enzymes were changed. Due to a paucity of available data, organ volumes, with the exception of kidney weight, were not altered. Factors relating to blood proteins, such as α -glycoprotein and human serum albumin as well as haematocrit, were also changed.

Despite the above-mentioned population file reflecting a population group infected with tuberculosis, within the published supporting information are physiological parameters for a healthy black South African population as well. As a result, changes were made to the aforementioned population file so as to reflect a healthy black South African population, within the bounds of the available information, so as to compare mefloquine disposition within this population group to the healthy volunteers' population group, made up of data derived from Caucasians. Specifically, changes were made to parameters relating to the previously mentioned blood proteins as well as body weight.

In Figure 2.21 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 2.23 below.

Table 2.23: Comparison between verified model and model run with the modified black South African population file

	Verified Model	Black SA Pop.	C _{max} R/AUCR
C _{max} (mg/L) (5 th and 95 th)	1.22 (1.12-1.31)	1.23 (1.08-1.34)	1.01
AUC (mg/L·h) (5 th and 95 th)	552 (452-638)	565 (461-643)	1.02

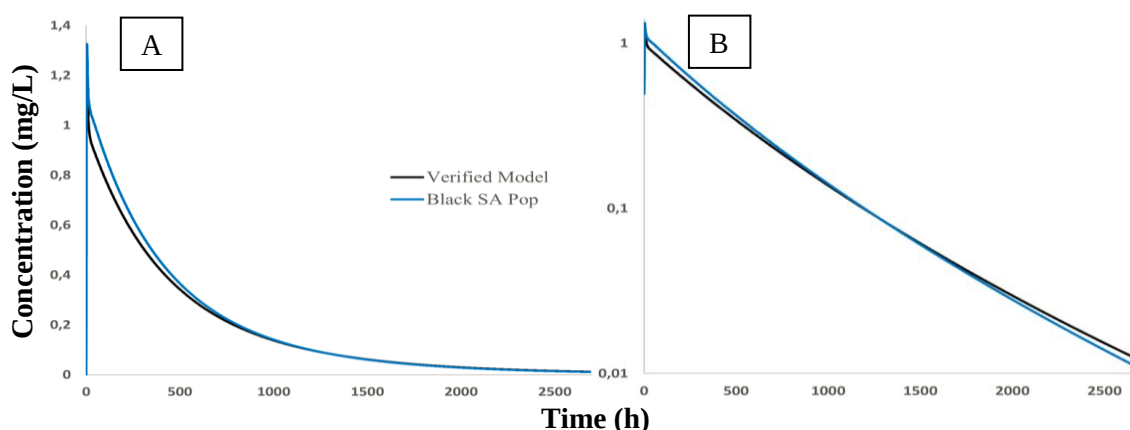


Figure 2.21: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine(A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the prediction of the effect of ethnicity of mefloquine disposition.

As seen in the above figure, the simulated PK parameters are comparable between the verified model and the representative black South African population.

The effect of CYP1A2 polymorphisms was also investigated. There is evidence of these polymorphisms having a clinical effect in the African population; a group wherein mefloquine is administered due to the high prevalence of malaria on the continent.^{48,49} Moreover, the discovery of the involvement of CYP1A2 in the *in vitro* metabolism of mefloquine in the work previously described provides impetus for this investigation.

To test the effect of CYP1A2 polymorphisms on mefloquine metabolism, changes were made to the enzyme phenotype frequencies in the Simcyp simulator to create populations with phenotype frequencies seen in Table 2.24 below. The original mefloquine model, which was run with the default frequencies, was then run with these newly created populations.

Table 2.24: Enzyme frequencies in populations used to test effect of CYP1A2 genotype on mefloquine metabolism

	Enzyme frequency in population				
	EM	PM	IM1	IM2	UM
Verified Model	1	0	0	0	0
0.5 PM CYP1A2	0.5	0.5	0	0	0
1 PM CYP1A2	0	1	0	0	0

EM=Extensive metabolizers; PM=Poor metabolizers; IM1=Intermediate metabolizers; IM2=Intermediate metabolizers; UM=Ultra-rapid metabolizers

In Figure 2.22 below are the representative concentration-time profiles for this model application. In Table 2.25 below is summarized the PK parameters for these model runs.

Table 2.25: Comparison between verified model and model run with populations with specific CYP1A2 polymorphisms.

	Verified Model	0.5 PM CYP1A2	1.0 PM CYP1A2
$C_{\max}$ (mg/L) (5 th and 95 th)	1.22 (1.12-1.31)	1.23 (1.13-1.34)	1.23 (1.14-1.34)
AUC (mg/L·h) (5 th and 95 th)	552 (452-638)	733 (600-823)	928 (828-1030)
$C_{\max}R$	-	1.01	1.01
AUCR	-	1.33	1.68

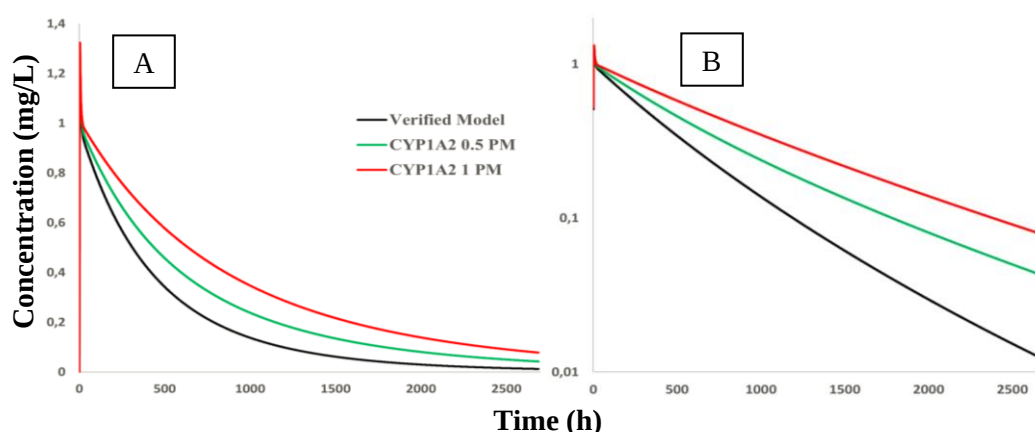


Figure 2.22: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine(A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the investigation of the effect of CYP1A2 polymorphisms on mefloquine disposition.

As expected, the simulated individuals with reduced CYP1A2 activity had higher AUCs, and as a result a higher exposure to, mefloquine.

Despite variability being observed clinically in CYP3A4 metabolism, genetic polymorphisms have not yet been found to play as big a role in this variability as compared to other CYPs.⁵⁰ As a result, polymorphisms in this enzyme were not investigated using the verified model. However, the effect of polymorphisms in CYP3A5 were investigated as a result of the presence of the reduced activity CYP3A5*3 variant in the African population wherein mefloquine is often used in malaria treatment.⁵¹

The effect of the CYP3A5*3 variant on mefloquine metabolism was investigated in a similar manner to CYP1A2 polymorphisms above. Changes were made to the enzyme phenotype frequencies in the Simcyp simulator to create populations with phenotype frequencies seen in Table 2.26 below. The original mefloquine model, which was run with the default frequencies, was then run with these newly created populations.

Table 2.26: Enzyme frequencies in populations used to test effect of CYP3A5*3 variant on mefloquine metabolism

	Enzyme frequency in population				
	EM	PM	IM1	IM2	UM
Verified Model	0.17	0.83	0	0	0
1 EM CYP3A4	1	0	0	0	0
1 PM CYP3A5	0	1	0	0	0

EM=Extensive metabolizers; PM=Poor metabolizers; IM1=Intermediate metabolizers; IM2=Intermediate metabolizers; UM=Ultra-rapid metabolizers

In Table 2.27 below is summarized the PK parameters for these model runs. In Figure 2.23 below are the representative concentration-time profiles for this model application.

Table 2.27: Comparison between verified model and model run with populations with specific CYP3A5 polymorphisms

	Verified Model	1 EM CYP3A5	1 PM CYP3A5
C_{max} (mg/L) (5 th and 95 th)	1.22 (1.12-1.31)	1.22 (1.12-1.31)	1.21 (1.13-1.29)
AUC (mg/L·h) (5 th and 95 th)	552 (452-638)	500 (405-579)	565 (450-675)
$C_{max}R$	-	1.00	0.992
AUCR	-	0.906	1.02

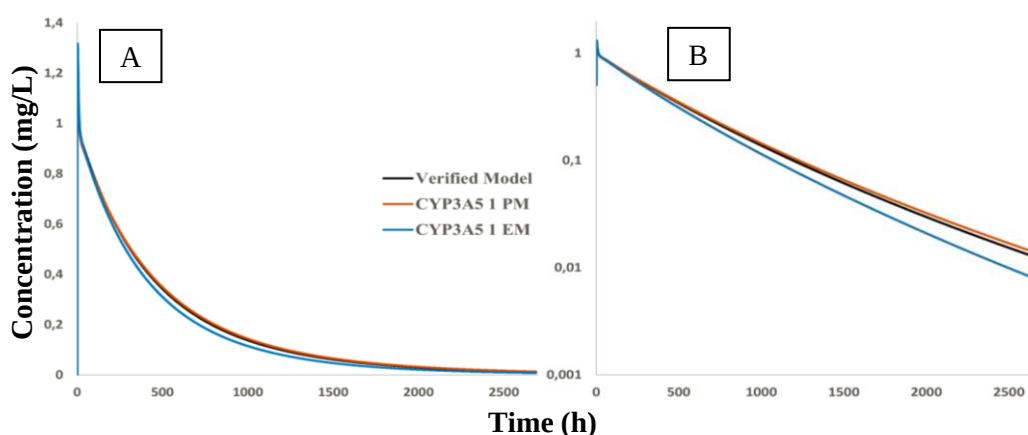


Figure 2.23: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine(A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the investigation of the effect of CYP3A5 polymorphisms on mefloquine disposition.

As illustrated in the above figure, little difference in the overall PK profile was observed between the different simulated populations.

2.7.3.2 Model Application: DDI

With the involvement of CYP1A2 in the metabolism of mefloquine being uncovered here, potential DDI between mefloquine and an inhibitor of CYP1A2, namely fluvoxamine, was explored using the verified model. The potential interaction between a CYP1A2 inhibitor as well as a CYP3A inhibitor, namely ketoconazole, was also investigated to determine whether

or not any increase in exposure was additive. This was done by running the verified model with fluvoxamine as an inhibitor on its own, ketoconazole as an inhibitor on its own, and then fluvoxamine and ketoconazole as inhibitors together. Single dose and multiple dose effects were investigated.

In the case of the single dose studies, mefloquine and the relevant inhibitor/s were dosed simultaneously on the first simulated day. In the case of the multiple dose investigations, the relevant inhibitor/s were administered once daily for 4 consecutive days prior to mefloquine administration, were then dosed simultaneously with mefloquine on the 5th simulated day, with inhibitor administration continuing for 5 days post mefloquine administration. As with all model application investigations, mefloquine was administered at a dose of 1000 mg. The inhibitors fluvoxamine and ketoconazole were administered at dosages of 100 mg and 400 mg respectively.

In Tables 2.28 and 2.29 below is summarized the PK parameters for these model runs. In Figures 2.24 and 2.25 below are the representative concentration-time profiles for this model application.

Table 2.28: Comparison of the effect of co-administration of a single dose of CYP1A2 and CYP3A inhibitors with mefloquine on mefloquine PK

Inhibitor status	C _{max} (mg/L) (5 th and 95 th)	AUC (mg/L·h) (5 th and 95 th)	C _{max} R	AUCR
No Inhibitor	1.22 (1.12-1.31)	552 (452-638)	-	-
Fluvoxamine	1.23 (1.13-1.33)	614 (519-699)	1.01	1.11
Ketoconazole	1.23 (1.13-1.32)	560 (460-647)	1.01	1.01
Both Inhibitors	1.24 (1.14-1.34)	621 (534-701)	1.02	1.13

Table 2.29: Comparison of the effect of co-administration of multiple dose of CYP1A2 and CYP3A inhibitors with mefloquine on mefloquine PK

Inhibitor status	C _{max} (mg/L) (5 th and 95 th)	AUC (mg/L·h) (5 th and 95 th)	C _{max} R	AUCR
No Inhibitor	1.22 (1.12-1.31)	552 (452-638)	-	-
Fluvoxamine	1.24 (1.14-1.34)	693 (600-790)	1.02	1.26
Ketoconazole	1.23 (1.14-1.33)	584 (483-673)	1.01	1.06
Both Inhibitors	1.24 (1.14-1.34)	727 (628-799)	1.02	1.32

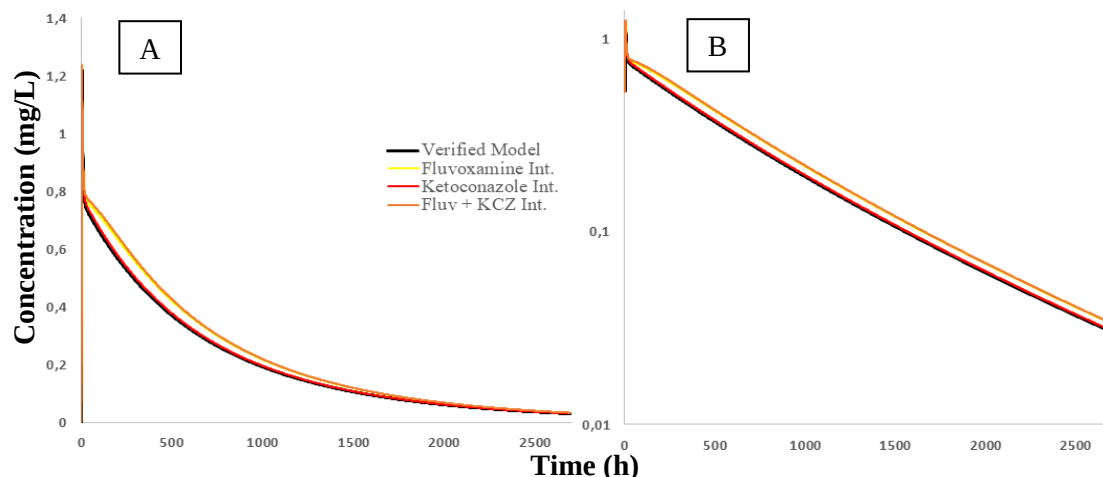


Figure 2.24: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine (A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the investigation of the effect of CYP1A2 and CYP3A inhibitors, administered together or alone as a single dose, on mefloquine PK.

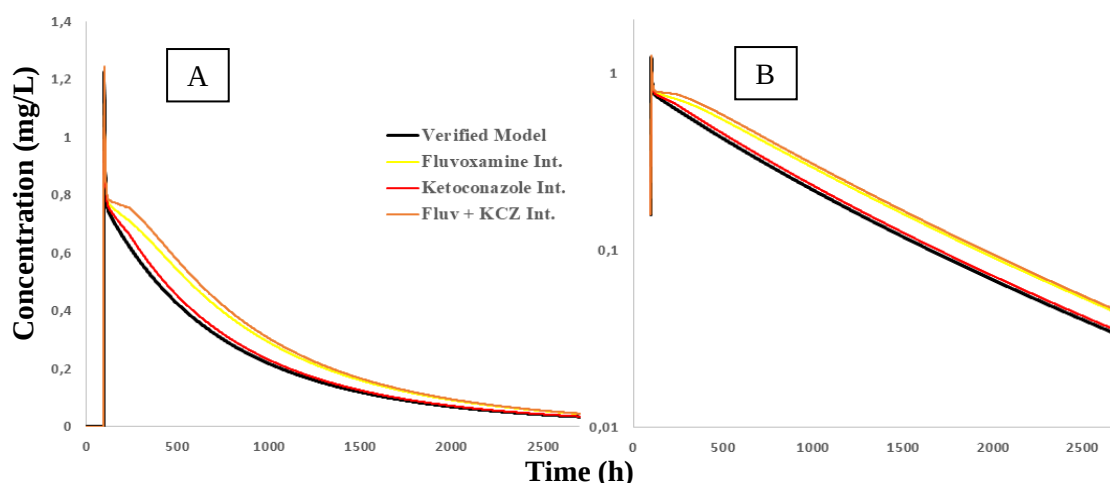


Figure 2.25: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine (A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the investigation of the effect of CYP1A2 and CYP3A inhibitors, administered together or alone over multiple doses, on mefloquine PK.

As stated previously, the interaction between efavirenz and mefloquine has not been clinically investigated despite the potential for interactions between these two medications. This is of special importance in Africa, given the prevalence of malaria and HIV co-infection. As a result, an attempt has been made here to predict the consequences of this interaction with the use of the verified model. Specifically, the effect of chronic efavirenz usage, as in the case of HIV-infected individuals, on mefloquine exposure was predicted. In this case, efavirenz was administered as a 600 mg dose once daily throughout the duration of the model, with 1000 mg mefloquine administered on the 8th simulated day to mimic conditions in chronic efavirenz use.

In Table 2.30 below is summarized the PK parameters for these model runs. In Figure 2.26 below are the representative concentration-time profiles for this model application.

Table 2.30: Comparison of the effect of efavirenz, administered as a chronic medication, on mefloquine exposure

Efavirenz Status	C _{max} (mg/L) (5 th and 95 th)	AUC (mg/L·h) (5 th and 95 th)
No Interaction	1.22 (1.12-1.31)	552 (452-638)
Efavirenz	1.21 (1.11-1.30)	323 (265-375)
C _{max} R/AUCR	0.992	0.585

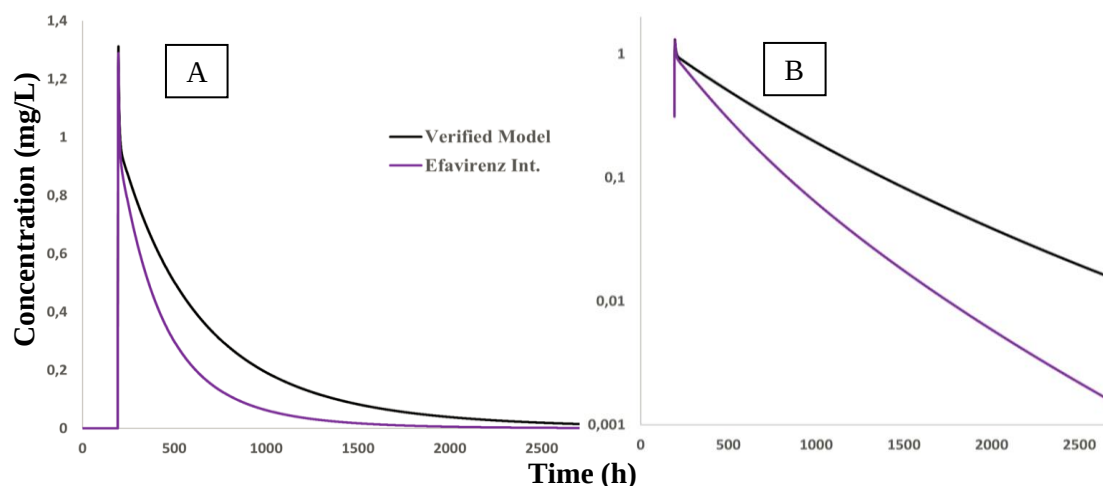


Figure 2.26: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine(A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the investigation of the effect of efavirenz, administered as a chronic medication, on mefloquine exposure.

High inter-individual variability in CYP1A2 activity and expression in human liver has been observed clinically, with these not only coming about through genetic polymorphisms, but environmental factors as well.^{52,53} Despite the role that polymorphisms might play in CYP1A2 activity, environmental factors may be more important in determining CYP1A2 expression. A key environmental factor that has been previously studied is the effect cigarette smoking has in inducing CYP1A2 expression.⁵⁴ As such, the potential effect of smoking on mefloquine PK was investigated.

The methodology for this was based on work done by Plowchalk and Rowland Yeo.⁵⁵ As part of their work they were able to model the induction effect of smoking on CYP1A2, and its consequence for drugs metabolized by CYP1A2, by adjusting CYP1A2 abundance in a model developed in Simcyp based on previously reported CYP1A2 activities reported for smokers and non-smokers for drugs metabolized by CYP1A2; namely caffeine. They assigned different CYP1A2 abundances to individuals based on their daily cigarette consumption which reflected the changes in CYP1A2 activity seen in caffeine metabolism in smokers consuming a similar number of cigarettes per day. These abundances, which were used here as well, are reflected

in Table 2.31 below. The abundance and coefficient of variation for non-smokers is the default for the Healthy Volunteers population used to develop the verified model.

Table 2.31: Hepatic CYP1A2 abundance based on daily cigarette consumption adapted from Plowchalk and Rowland Yeo⁵⁵

Cigarettes consumed	Smoking status	CYP1A2 Abundance (pmol CYP/mg protein)	%CV
0 per day	Non-smoker	52	67
1-5 per day	Light smoker	64	71
6-10 per day	Mild smoker	79	53
11-20 per day	Moderate smoker	90	46
>20 per day	Heavy smoker	94	43

In Figure 2.27 below are the representative concentration-time profiles for this model application. In Table 2.32 below is summarized the PK parameters for these model runs.

Table 2.32: Comparison of smoking-induced increases in CYP1A2 expression and its effect on mefloquine PK

Smoking status	$C_{\max}$ (mg/L) (5 th and 95 th)	AUC (mg/L·h) (5 th and 95 th)	$C_{\max}R$	AUCR
Non-smoker	1.22 (1.12-1.31)	552 (452-638)	-	-
Light smoker	1.22 (1.12-1.31)	509 (409-599)	1.00	0.922
Mild smoker	1.21 (1.12-1.31)	440 (350-520)	0.992	0.797
Moderate smoker	1.21 (1.11-1.30)	401 (318-475)	0.992	0.726
Heavy smoker	1.21 (1.11-1.30)	388 (307-459)	0.992	0.703

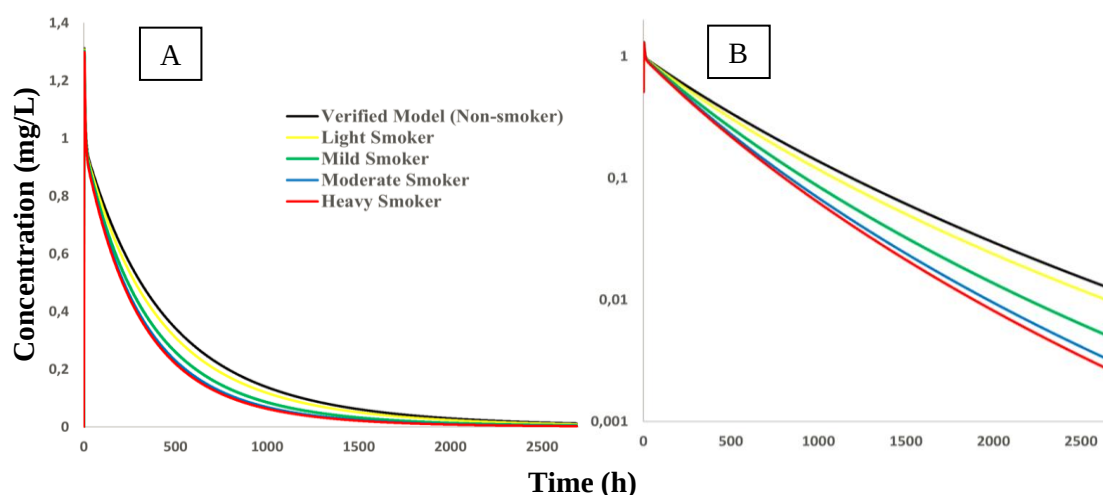


Figure 2.27: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine(A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B), for the investigation of the induction effect of smoking on CYP1A2 and its consequence for mefloquine PK.

2.8 Discussion

2.8.1 CYP1A2 is involved in the metabolism of mefloquine

The mefloquine fm,CYP investigation identified the involvement of a new CYP, namely CYP1A2, in the *in vitro* metabolism of mefloquine which has not been previously reported.

The involvement of CYP1A2 is supported by various observations in literature. A recent study in hepatocytes reported an f_m ,CYP_{3A4} of 0.41 for mefloquine, suggesting another unidentified enzyme was involved in its metabolism.²⁰ PBPK models of mefloquine with f_m ,CYP_{3A4} of 0.5 have also been successfully validated and verified using clinical data, further suggesting a role for other unidentified enzymes in the metabolism of mefloquine.²³ In addition, in the work done by Rattanapunya *et al*, the PK interactions between artesunate-mefloquine and ritonavir boosted lopinavir in healthy Thai volunteers were investigated.⁵⁶ During their work they noted an apparent decrease in mefloquine exposure in the presence of lopinavir-ritonavir which could not be explained by the inhibitory effect of lopinavir-ritonavir on the CYP3A4-mediated metabolism of mefloquine.⁵⁶ Indeed, ritonavir has previously been shown to have only a minimal effect on mefloquine PK despite its potent inhibitory activity on CYP3A4 after administration of a single 200 mg dose in healthy volunteers.⁴² This suggests the potential involvement of other enzymes in mefloquine metabolism. It has previously been shown that lopinavir-ritonavir treatment results in the potent induction of CYP2C19 and modest induction of CYP1A2 and CYP2C9 activity.⁵⁷ It stands to reason that, based on the work done by Rattanapunya *et al*, one of these enzymes is involved in mefloquine metabolism since the induction of one of these enzymes has the potential to result in the decreased mefloquine exposure that was noted.⁵⁶ The work done here suggests this enzyme is CYP1A2.

The f_m ,CYP investigation also identified CYP3A5 as a contributor to mefloquine *in vitro* metabolism. Although it could be inferred that this enzyme played a role given the overlap of substrates between this enzyme and CYP3A4; an enzyme already known to be a major contributor to mefloquine metabolism *in vitro* and *in vivo*.^{14,16} Looking forward, a wider range of substrate concentrations, specifically including a number of concentrations below 8.5 μ M, need to be employed to obtain a more accurate measurement of the K_M of CYP1A2.

2.8.2 Modelling Discussion: Mefloquine

Using the aforementioned *in vitro* metabolism data, a PBPK model was developed. The model was unable to correctly predict clinical PK when using only *in vitro* metabolism data to predict drug elimination. As a result a retrograde PBPK modelling approach using the Simcyp RTT, was used to predict drug elimination. The resulting data showed a 30.6-fold, 452-fold and 1610-fold difference between the clearance measured using rCYPs and clearance calculated using the RTT for CYP1A2, CYP3A4 and CYP3A5, respectively.

This difference excluded the possibility of using a pure bottom-up approach to mefloquine modelling. Thus, with the use of the RTT, a middle-out approach was used to develop the model which was later verified with the use of a variety of literature sources.

2.8.2.1 Model Applications: Mefloquine

Upon verification, the model was then applied to scenarios of interest. This model application showed the potential for CYP1A2 polymorphisms to have relatively minor effects on mefloquine disposition. CYP3A5 polymorphisms were shown to have even lower potential to affect mefloquine disposition. This confirmed data seen in literature which suggests that the reduced activity CYP3A5*3 genotype was associated with only a minor reduction in mefloquine clearance (14%) and only explained 1% of PK variability.⁵⁸ This could be as a result of the other enzymes involved in mefloquine metabolism clearing the drug in the place of CYP3A5.

In addition, when the model was applied to a representative black South African population, only minor changes were seen in overall mefloquine disposition. This is in agreement with earlier literature studies where only slight differences in mefloquine disposition was observed between Caucasian and African individuals.^{13,34}

While the potential for a decrease in mefloquine exposure as a result of co-administration with efavirenz has been previously suggested, this effect has not been investigated clinically.²¹ The verified model was used to simulate this clinically relevant interaction with the simulation done so as to mimic the effect chronic efavirenz administration would have on mefloquine exposure. This simulation showed a 1.71-fold reduction in mefloquine exposure, as a result of the induction by efavirenz of CYP3A4. This gives credence to previous recommendations that co-administration of mefloquine and efavirenz would result in decreased systemic mefloquine concentrations.^{21,22}

The effect of the induction effect of smoking on CYP1A2 was also investigated in light of the discovery of the involvement of this enzyme in mefloquine metabolism. Relatively minor differences were observed between non-smokers and light smokers and between moderate smokers and heavy smokers. However, a significant difference was observed between non- and light smokers and moderate and heavy smokers. This difference was most pronounced in the clearance of mefloquine where the clearance of the drug occurred more rapidly in heavier smokers in the simulated runs. This resulted in a 1.42-fold difference in AUC between heavy smokers and non-smokers; a significant difference in exposure.

A similar trend was found in an earlier literature study wherein quinine exposure, a drug structurally related to mefloquine, was increased by cigarette smoking.⁵⁹ As such, the work here suggests that non-smokers and heavy smokers might require slightly different doses of mefloquine to have the same therapeutic effect. Or at least that this effect should be explored clinically. Given that no significant relationship between cigarette smoking and blood concentrations of hydroxychloroquine, another drug structurally similar to mefloquine, were found in a previous study,⁶⁰ this might not be especially relevant for malaria patients. However, individuals travelling to malaria endemic areas and taking the commonly prescribed mefloquine as a prophylactic, may need to consider this effect if they are heavy smokers.

Although no literature sources were found that clinically measured the effect of CYP1A2 inhibitors on mefloquine PK, this interaction was also explored as part of this work. The effect of both single and multiple doses of a CYP1A2 inhibitor, namely fluvoxamine, on its own and in conjunction with a CYP3A inhibitor, namely ketoconazole, was investigated for potential DDIs. It was found that not only does the co-administration of mefloquine with a CYP1A2 inhibitor lead to increased exposure, this effect is additive with ketoconazole in both single and multiple doses in the simulations done here. This suggests that this DDI would potentially need to be investigated clinically if mefloquine is prescribed along with a CYP1A2 inhibitor.

2.8.2.2 Model limitations: Mefloquine

The PBPK model used in this work has several limitations.

First, clinical doses of mefloquine can be as high as 1500 mg which complicates the modelling of the absorption phase. It was notable during model verification that the highest difference between model and literature exposures was with the 1500 mg dose (C_{max} difference 1.3-fold, AUC difference 1.5-fold), while lower doses could be predicted more accurately. This is likely to be due to a saturation of the absorption mechanisms at high doses. However, since we did not remeasure any of the parameters used to model the absorption, such as solubility, permeability, dissolution of the mefloquine tablet in the gut among others, it is difficult to ascertain precisely why this is the case. As a result, care should be taken if this model is used to predict mefloquine disposition at doses above 1000 mg.

An additional potential limitation of the model is that the effect of food on mefloquine disposition was not examined. It has previously been shown that a high-fat breakfast increases mefloquine bioavailability by approximately 40%.⁶¹ Currently, however, neither the approved

FDA label nor the core data sheet makes any recommendation for mefloquine administration with food. As a result, the fasted state was assumed in Simcyp for all model runs.

Lastly, the model does not capture the effect of malaria infection on mefloquine PK, which appears to have a greater effect than ethnicity on mefloquine disposition.¹¹ Malaria results in multiple physiological changes which can impact drug disposition, such as differences in hematocrit due to hemolysis and differences in α_1 -acid glycoprotein. Population PK modelling suggests a link between temperature at admission, a marker of malaria severity, and the volume of distribution of mefloquine.^{62,63} This impact is further complicated by the time course of malaria infection after mefloquine treatment as a result of the prolonged exposure of the drug. Namely, after mefloquine treatment, the parasite load of malaria patients decreases and the physiological differences between patients and healthy volunteers diminish as the physiological state of the patients return to what is seen in healthy individuals. As a result, in the early portion of mefloquine treatment, drug exposure is better characterized by patient physiology than healthy physiology whereas the opposite is true in the latter portions of mefloquine exposure. This would likely mean that parameters related to volume of distribution, such as absorption, will differ between patients and healthy volunteers whereas mefloquine clearance is less affected by disease state as patients would have returned to a physiological state mirroring healthy individuals by the time the PK profile of the drug reaches the elimination phase.⁶⁴

2.9 Chapter 2: Conclusions

In this chapter, data from *in vitro* metabolism experiments were used to inform PBPK modelling of mefloquine. *In vitro* metabolism experiments confirmed the previously unreported contribution of CYP1A2 to mefloquine metabolism. A middle-out PBPK model of mefloquine was successfully used to simulate exposures across different clinical studies.

This model also showed the potential for inhibitors of CYP1A2 to increase mefloquine exposure, suggesting that care should be taken if a CYP1A2 inhibitor is co-prescribed with mefloquine. The PBPK model also suggests a DDI with efavirenz, which might be co-prescribed with mefloquine in HIV patients with malaria. Lastly, the PBPK model confirmed the observation that mefloquine exposure is not affected by polymorphisms in CYP3A5 or by ethnic differences in Caucasian and black South African populations. Malaria infection may be a more important contributor to the variability seen in the pharmacokinetics of mefloquine.

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Chapter 3 Proguanil

3.1 Chapter 3 Overview

In this chapter, data from *in vitro* metabolism experiments is used to inform PBPK modelling of proguanil PK. The *in vitro* metabolism data was then used in a PBPK model of proguanil and cycloguanil built in Simcyp, and the model was verified using clinical data. This model was then used to investigate scenarios of interest including ethnic sensitivity and various DDIs.

3.2 General Introduction: Proguanil

Proguanil is an arylbiguanide that was found to be the most active of a series of compounds of this class which were screened for antimalarial activity in the 1940s.¹ This discovery was quickly followed by clinical studies which showed proguanil to be both efficacious and non-toxic and its use as an antimalarial soon followed.^{2,3} Proguanil has relatively low *in vitro* antiparasmodial activity (*P. falciparum* 3D7 IC₅₀ = 46.2 μ M [48h], 0.486 μ M [72h], 0.113 μ M [96h]), with the antimalarial effect of proguanil administration resulting from its cyclization to its active dihydrotriazine metabolite, cycloguanil (*P. falciparum* 3D7 IC₅₀ = 11.2 nM [48h], 5.02 nM [72h], 0.511 nM [96h]).^{4,5} Proguanil is also metabolized to an inactive, secondary metabolite 4-chlorophenylbiguanide (CPB). The chemical structures of proguanil, cycloguanil and CPB are shown in Figure 3.1 below.

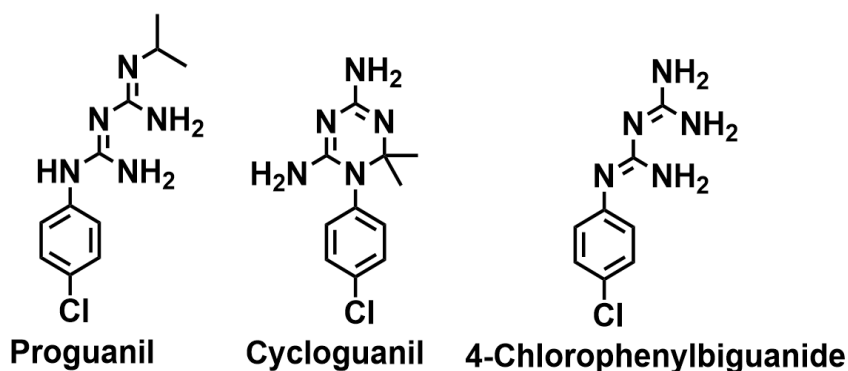


Figure 3.1: Chemical structures of proguanil and its two metabolites

Cycloguanil is a potent inhibitor of dihydrofolate reductase, a plasmodial enzyme critical to the parasite's viability due to its role in folate biosynthesis. The development and proliferation of the 4-aminoquinoline antimalarials, exemplified by chloroquine, as well as another dihydrofolate reductase inhibitor antimalarial in pyrimethamine, limited the clinical use of proguanil.^{6,7} However, the rise of plasmodial resistance to these drugs, particularly chloroquine, gave rise to fresh interest in proguanil.⁷ This ultimately led to the development of

Malarone®, a combination of proguanil and atovaquone in the 1990s.⁸ The need to combine these two drugs to form an effective antimalarial arose as a result of resistance to proguanil treatment and the lack of antimalarial activity and recrudescence observed with atovaquone treatment.⁸⁻¹⁰ After attempts at enhancing the efficacy of atovaquone with a range of antimalarials, it was found that proguanil was able to significantly improve the ability of atovaquone to collapse the mitochondrial trans-membrane potential; thereby improving its antimalarial effect.^{10,11} While the exact nature of this effect is not fully understood, the enhancement of atovaquone as an antimalarial is as a result of synergy with proguanil, not cycloguanil, indicating that this synergy arises as a result of mechanisms other than dihydrofolate reductase inhibition.¹¹

Proguanil is absorbed rapidly and extensively with the C_{max} reached in 3-5 hours.^{6,8,10,12-16} This absorption is unaffected by food intake.¹⁷ Proguanil steady-state conditions mirror those of single dose administration with absolute bioavailability thought to be as high as 60%.^{13-15,17} Proguanil has a volume of distribution at steady-state ranging from 15.4-49.3 L/kg.^{14,15} Both proguanil and CPB are concentrated in erythrocytes while cycloguanil is not.^{6,10,13} The PK of proguanil appears to be unaffected by either disease state or atovaquone co-administration.¹⁸

Proguanil is metabolized to cycloguanil and CPB both *in vitro* and *in vivo*.^{19,20} Conversion of proguanil to these metabolites accounts for approximately 30% of total proguanil clearance *in vivo*, with the rest cleared via other routes; including renally.^{19,21} The metabolism to cycloguanil is of clinical significance with a correlation being found between proguanil/cycloguanil ratio and breakthrough parasitaemia; suggesting that impaired cycloguanil formation is a contributing risk factor in prophylaxis failure.²²

The *in vitro* microsomal metabolism of proguanil has previously been shown to be a low V_{max} , high K_M reaction (K_M range 90-250 μ M, V_{max} <20 μ mol/mg/min).^{19,20,23} Several CYP enzymes are thought to be involved in proguanil metabolism with contributions from CYP2C19 and CYP3A4 being confirmed and the involvement of CYP1A2 being suggested but not definitively characterized.²³⁻²⁵ Collier *et al.*²³ were also not able to completely inhibit the formation of cycloguanil *in vitro* with the inhibition of CYP1A2, CYP2C19 and CYP3A4; indicating the potential involvement of another enzyme in proguanil metabolism.

Additionally, CYP2C19 has been shown to be the main enzyme involved in proguanil metabolism and subsequently its conversion to cycloguanil and CPB.^{21,22,26-28} Moreover, *in vitro* and *in vivo* inhibition of proguanil metabolism has been observed as a result of co-

administration with fluvoxamine and omeprazole; two known CYP2C19 inhibitors.^{19–21,29} The effect of inhibiting CYP1A2, in the case of fluvoxamine, and CYP3A4, in the case of omeprazole, on proguanil metabolism were also investigated but were found to be limited *in vitro*, in the case of CYP1A2, and both *in vitro* and *in vivo* in the case of CYP3A4.^{19,20}

Investigations into the effect of the CYP2C19 poor metabolizer phenotype on proguanil metabolism have also been conducted. A similar proguanil absorption profile was observed between extensive and poor metabolizers of CYP2C19.^{12,13,21} However, the cycloguanil PK differed depending on the CYP2C19 phenotype. A higher cycloguanil C_{max} and exposure was observed for CYP2C19 extensive metabolizers while poor metabolizers developed sub-therapeutic levels of cycloguanil on chronic administration of proguanil.^{12,13} Additionally, fluvoxamine was shown to inhibit both cycloguanil and CPB formation in CYP2C19 extensive metabolizers but not in poor metabolizers; clearly indicating that the aforementioned inhibitory effect of fluvoxamine on proguanil metabolism was as a result of the inhibition of CYP2C19.²¹

Proguanil was chosen as a study drug since, despite its *in vitro* metabolism being reasonably well characterized, certain questions still remained; specifically the involvement of CYP1A2, which was suggested as a potential contributor but not confirmed with the use of recombinantly expressed CYP1A2.²³ Additionally, clinical data was available from which a PBPK model could be developed and verified. Large inter-individual differences in various proguanil PK parameters have been previously reported.^{14,15} However, the role of the proguanil metabolism phenotype has not been well understood, likely in part due to full characterization of this phenotype not being definitively done. As such, *in vitro* bioanalytical methods were developed to determine the f_m , CYP of proguanil, and by extension a full characterization of its *in vitro* metabolism phenotype, for use in a middle-out approach to constructing a PBPK model for this drug as a means to determine the role of the metabolism phenotype on the variability seen in proguanil and cycloguanil exposure.

3.3 Chromatographic development and analysis

Proguanil was infused on an AB Sciex 4500Q LC-MS/MS in electrospray positive mode which provided good sensitivity. The spectra of the protonated and deprotonated molecular ions as well as their fragments are illustrated in Figure 3.2.

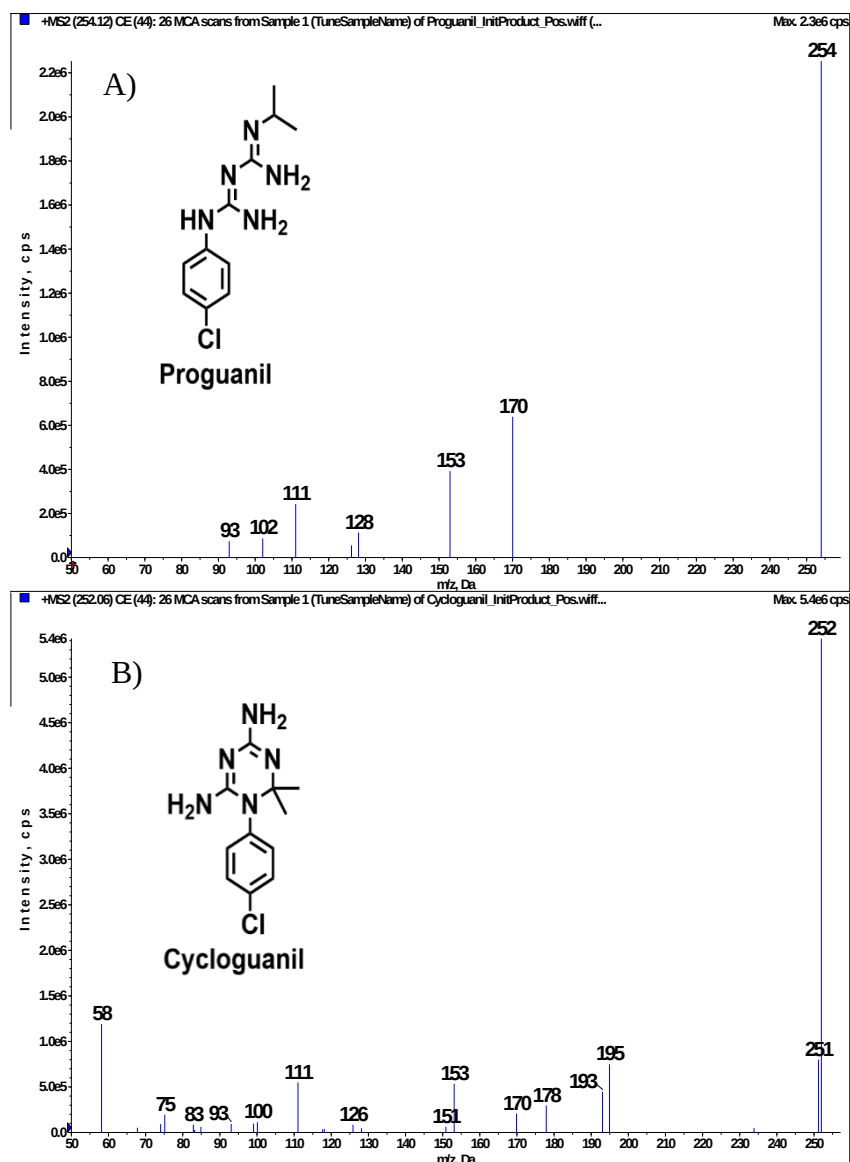


Figure 3.2: Initial product ion mass spectrum of A) Proguanil and B) Cycloguanil, in positive mode, showing the $[M+H]^+$ ions at m/z 254 and 252 and their product ions at 128 and 195, respectively

Table 3.1 below summarizes the quantifier and qualifier ions for each infused compound. These were selected on the basis of sensitivity, selectivity and reproducibility in LC-MS/MS analyses.

Table 3.1: Summary of the quantifier and ions for each infused compound

	Proguanil	Cycloguanil
Q1 $[M+H]^+$	254	252
Q3 $[M+H]^a$	128	195

Since the method developed to analyse proguanil would need to distinguish between the substrate and metabolite and, given that the substrate and metabolite generate similar transitions due to in-source fragmentation, base line separation of the two was necessary to minimize cross-talk.

This separation was achieved with the use of gradient chromatography in conjunction with a reversed phase chromatographic column. The final analytical method is summarised in Table 3.2 with the chromatography illustrated in Figure 3.3. Carbamazepine was used as the ISTD for the developed method. It was chosen as it has similar extraction and ionization properties as the analytes under investigation.

Table 3.2: Summary of the analytical conditions for the developed method

Proguanil	
Analytical Conditions	
Analyte	Proguanil
Column (Dimensions)	Hydro-RP (2.0 x 50 mm; 2.5 μ m)
Injection volume (μ L)	5
Gradient Program %B (min)	2(0) $\rightarrow$ 95(2.5) $\rightarrow$ 95(3.5) $\rightarrow$ 2(3.6) $\rightarrow$ 2(5.5)
Flow rate (μ L/min) ^a	800

^aFlow split 1:2 into MS

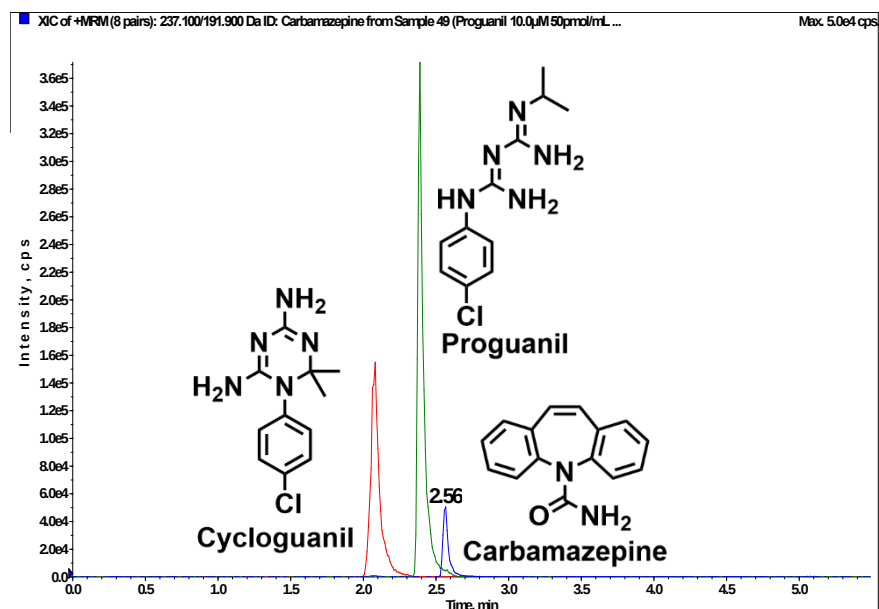


Figure 3.3: Representative chromatogram for proguanil (green), cycloguanil (red) and carbamazepine (blue).

3.3.1 Flow injection analyses

Upon obtaining optimal chromatographic conditions, an on-column flow injection analysis (FIA) was carried out to optimise the MS source parameters, such as curtain gas, ion spray

voltage, and source temperature, as well as compound parameters to ensure optimal response and sensitivity from the MS for this reaction. The resulting optimized MS/MS parameters are summarized in Table 3.3 below.

Table 3.3: Summary of the optimized MS conditions for proguanil and cycloguanil

	Proguanil	Cycloguanil
Q1 (m/z)	254	252
Q3 (m/z)	128	195
Source Temp. °C	550	550
Curtain Gas	40	40
Ion Source Gas 1	30	30
Ion Source Gas 2	30	30
Ion Spray Voltage	4500	4500
CE (eV)	35	25
EP	10	10
DP	81	76

3.3.2 Sample extraction

Protein precipitation was chosen as the extraction method for the methods developed here as described in Chapter 2.

3.4 Development of biological method

Optimisation of the incubation conditions requires an assessment of reaction linearity with respect to microsomal protein and incubation time; as described in Chapter 2. It has been previously reported that proguanil metabolism was linear for 1 hour up to a protein concentration of 1 mg/mL microsomal protein.²³ The range of conditions evaluated is summarised in Table 3.4 below.

Table 3.4: Incubation conditions used to test linearity of proguanil kinetics

Substrate (Conc. µM)	Microsomal Protein (mg/ml)	Duration (min)
Proguanil (50,500)	0.1; 0.2; 0.4; 0.6	60

The results of this linearity determination is summarized in Figure 3.4 below.

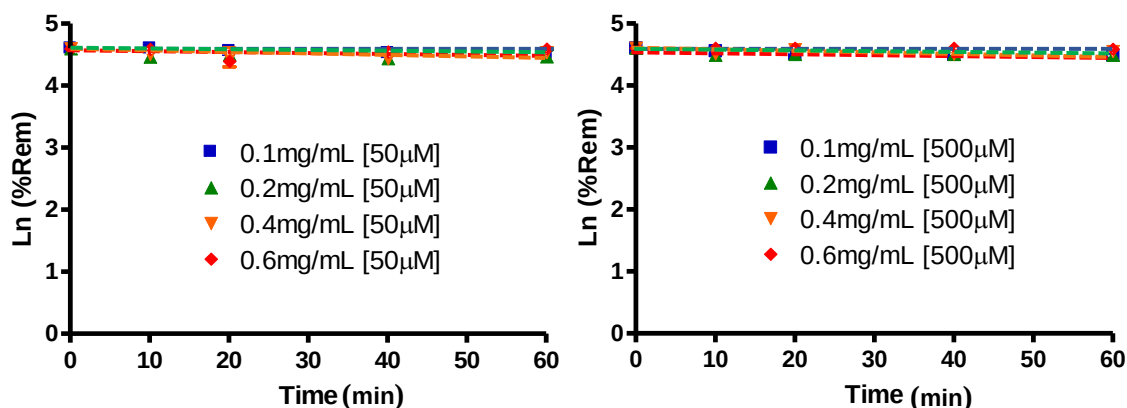


Figure 3.4: Summary of linearity determination data for proguanil

The measured microsomal metabolism of proguanil was slow, with 81-89% proguanil remaining after a 60 minute incubation. There was also no statistical difference between the different incubation conditions at either substrate concentration. This illustrated that the substrate depletion approach would not be suitable for measuring proguanil kinetics since it is recommended that substrate depletion should reach a minimum of 80%.³⁰

It is known from literature that cycloguanil is the major metabolite resulting from proguanil metabolism both *in vitro* and *in vivo*.^{19,20} Cycloguanil formation was therefore monitored during these analyses. The linearity of its formation is plotted in Figure 3.5 below.

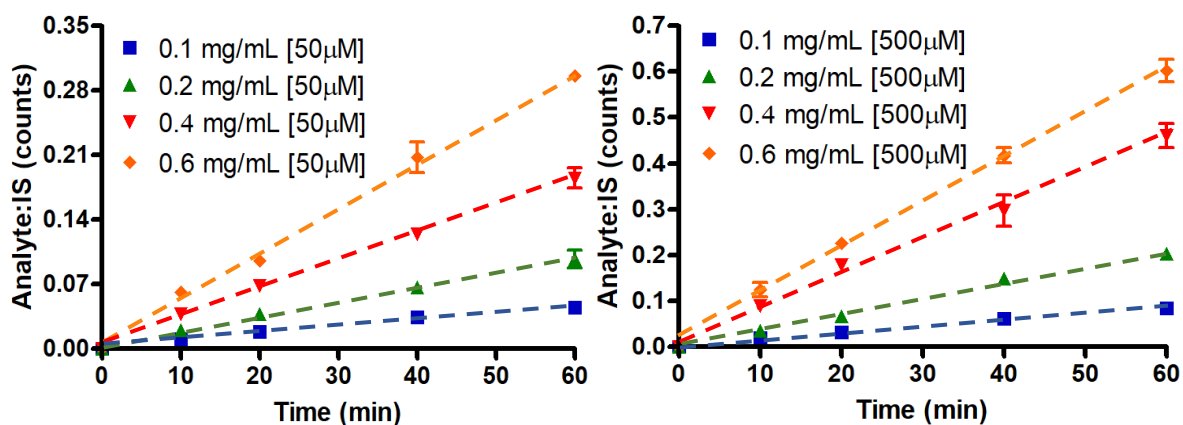


Figure 3.5: Linearity of cycloguanil formation at tested conditions

Cycloguanil formation appeared linear for the duration of the incubation at all incubation conditions. Given that substrate depletion was not below 80% and that cycloguanil is the main metabolite formed via microsomal metabolism of proguanil, along with the fact that cycloguanil formation could be measured using the developed method, the metabolite formation approach was selected to measure proguanil kinetics. The other proguanil

metabolite, CPB, was formed at levels that were close to or below the limit of quantitation and was therefore not included as part of this work.

As a means of determining cycloguanil linearity over time, microsomal protein concentrations between 0.2 and 0.6 mg/mL were evaluated. A microsomal protein concentration of 0.4 mg/mL for a duration of 60 minutes was selected as these conditions provided the lowest protein concentration at which both linearity and substrate consumption could be satisfactorily established at both low and high substrate concentrations.^{31,32} The results of the linearity determination are summarized in Figure 3.6 below, with these incubation conditions summarised in Table 3.5.

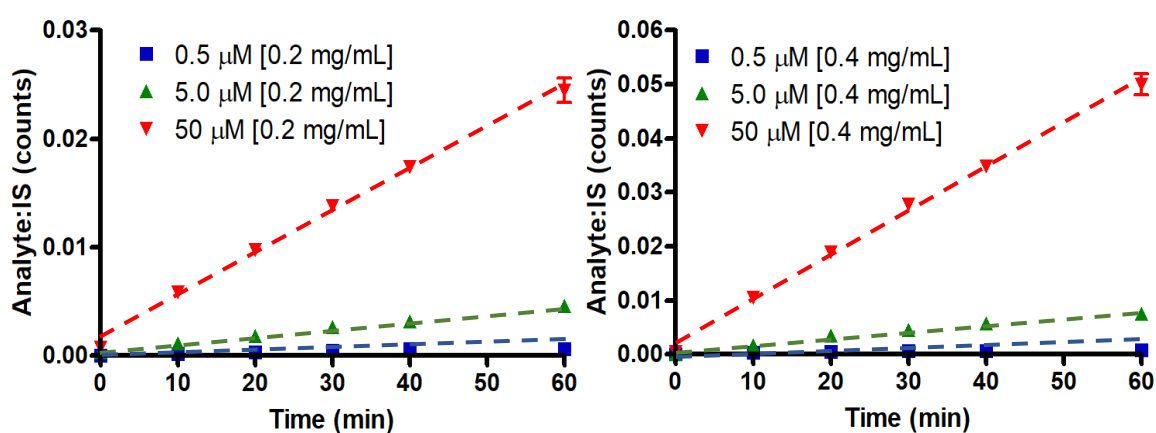


Figure 3.6: Linearity of cycloguanil formation at tested conditions

Table 3.5: Incubation parameters for the determination of proguanil kinetics

Parameter	Conditions tested
Protein Concentration (mg/mL)	0.4
Time (min)	0; 15, 30, 45, 60
Substrate Concentration Range (µM)	5-300
Standard Curve Range (nM)	1-1500

This incubation was done in duplicate over two independent incubations. The kinetics data obtained from this incubation is summarised in Figure 3.7.

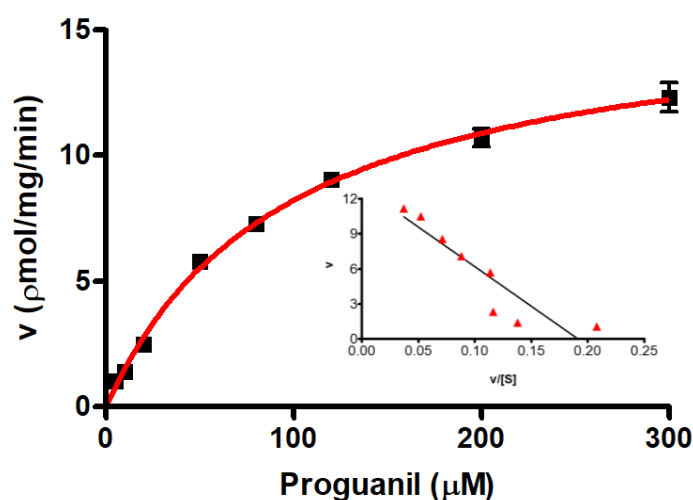


Figure 3.7: Substrate saturation curve for the determination of proguanil kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics

The selected incubation conditions could accurately measure proguanil kinetics. The shape of the Eadie-Hofstee plot suggests more than one enzyme is involved in the conversion of proguanil to cycloguanil. The measured kinetic parameters from these incubations are summarized in Table 3.6 below.

Table 3.6: Summary of measured enzyme kinetics for proguanil. Data gathered from two independent incubations

Kinetic Parameters	Mean	95% CI
K_M (μM)	96.6	90.6-103
V_{max} (pmol/mg/min)	16.2	15.8-16.6

CI = Confidence Interval

3.5 Measurement of fm,CYP

The measurement of proguanil fm,CYP was undertaken as in Chapter 2. The incubation conditions to measure proguanil fm,CYP are shown in Table 3.7 below. To note, a substrate concentration of 0.1 mg/mL was chosen to avoid similar issues arising with the determination of proguanil fm,CYP_{1A2} as was the case with mefloquine.

Table 3.7: Incubation conditions for the measurement of proguanil fm,CYP

Parameter	Conditions tested
Protein Concentration (mg/mL)	0.1
Time (min)	0; 15, 30, 45, 60
Standard Curve Range (nM)	1-1500
K_M (μM)	96.6
Substrate Concentration (μM)	10

The HLM in buffer pre-mix was pre-incubated with NADPH along with the mechanistic inhibitors, for 20 minutes before the incubation was started by the addition of proguanil. The competitive inhibitors were added to the incubation plate at the same time as the compound and the incubation was carried as described above. In Figure 3.8 below are the results of the chemical inhibitor incubations.

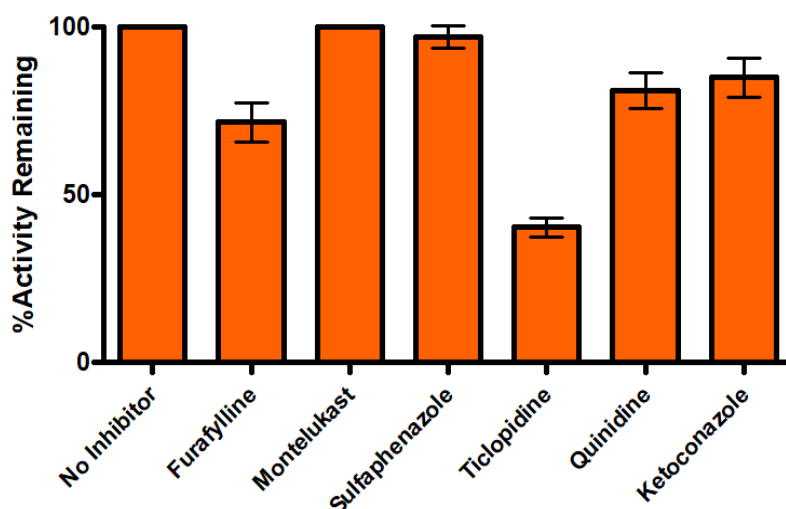


Figure 3.8: Chemical inhibition results showing the percentage enzyme activity remaining after the addition of specific inhibitors to the HLM incubation

Instead of calculating clearance, fm,CYP was calculated by measuring the rate of metabolite formation due to differences in approach to measuring kinetics. The slope of metabolite formation was calculated to obtain a rate k from which fm,CYP was calculated using a slightly modified version of the equation proposed by Njuguna *et al* seen below:

$$\left[1 - \left(\frac{k \text{ in presence of inhibitor}}{k \text{ with no inhibitor present}} \right) \right] * 100$$

The fm,CYP for proguanil calculated using this approach are shown in Table 3.8 below.

Table 3.8: fm,CYP for proguanil of the six major CYP isoforms

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	30.3	0	2.51	60.7	19.3	14.3

From the data in Table 3.9, CYP2C19 appears to be the major isoform involved in the *in vitro* microsomal metabolism of proguanil, as expected, with moderate contributions from CYP1A2, CYP2D6, and CYP3A4. Cross reactivity corrections were then applied as in work done in Chapter 2 to give the corrected fm,CYP values, seen in Table 3.9.

Table 3.9: Corrected fm,CYP values for proguanil of the six major CYP isoforms

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	28.8	0.0	0.0	44.8	12.8	13.6

The data shows contributions from four of the six isoforms tested with CYP2C19 being shown to be the major isoform involved, as expected. The contribution of CYP2C19 and CYP3A has been previously reported, with CYP1A2 involvement being previously suggested.^{23,24} However, the involvement of CYP2D6 has not been previously reported. To confirm the overall metabolic profile, as well as the involvement of CYP1A2 and CYP2D6 in proguanil *in vitro* metabolism, studies were conducted using rCYP enzymes.

3.6 Recombinant enzyme studies

To confirm the involvement of the above-mentioned enzymes in the conversion of proguanil to cycloguanil, proguanil was incubated with rCYP enzymes generated from baculovirus transfected cell strains. The conditions used mirrored those used during the determination of proguanil fm,CYP with only the protein source differing. These conditions are summarised in Table 3.10 below.

Table 3.10: Incubation conditions for the determination of proguanil metabolism by rCYPs

Parameter	Conditions tested
Enzyme Concentration (pmol/mL)	50
Time (min)	0; 15; 30; 45; 60
Standard Curve Range (nM)	1-1500
K_M (μM)	96.6
Substrate Concentration (μM)	10

Shown in Figure 3.9 below are the results of the rCYP incubations. Although fm,CYP was not determined for CYP3A5, due to the difficulty in establishing HLM kinetic experiments as a result of a lack of a truly specific substrate and inhibitor, the ability of CYP3A5 to convert proguanil to cycloguanil was tested due to the involvement of CYP3A4 in proguanil metabolism. This was done since CYP3A4 and CYP3A5 often overlap in the compounds they can metabolise.

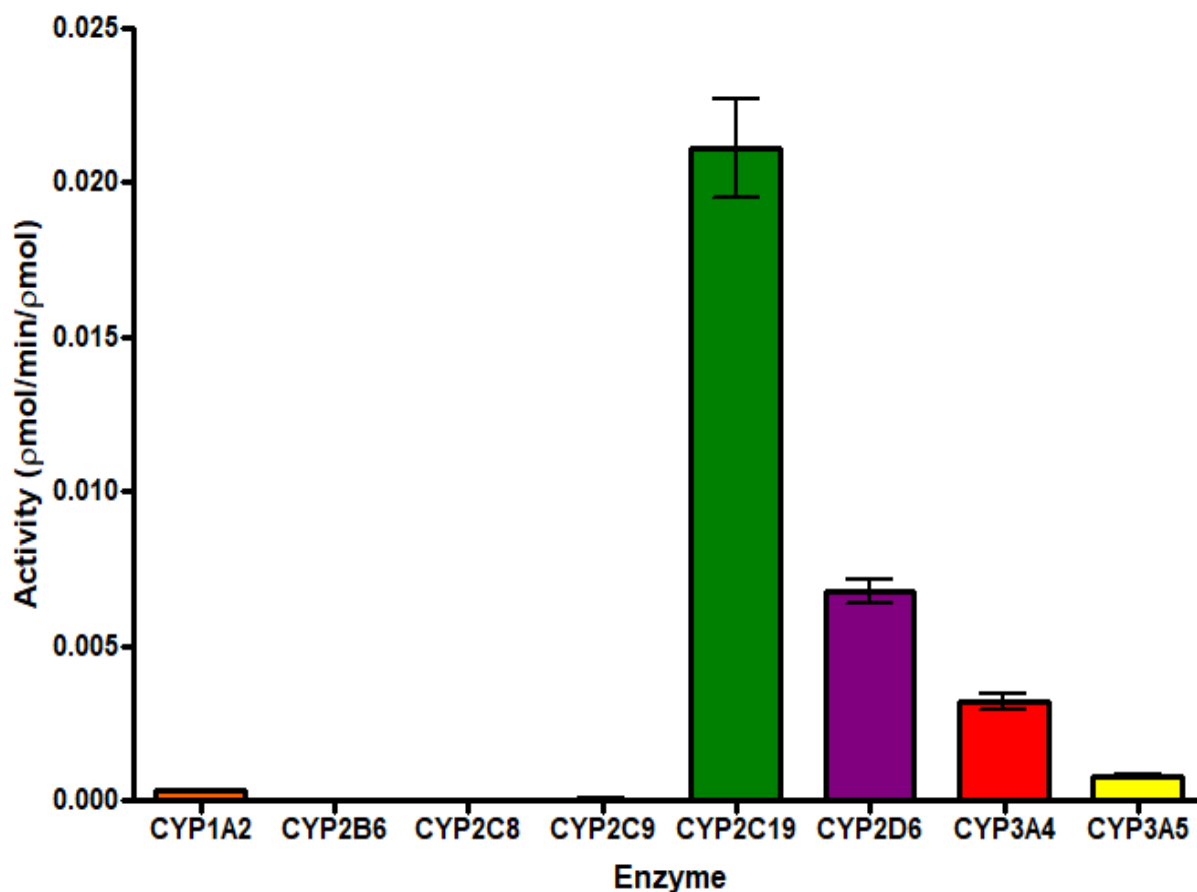


Figure 3.9: Results from the rCYP incubations showing the enzyme activity of tested CYP enzymes in converting proguanil to cycloguanil

The data in the above figure confirms the metabolic pattern determined during the fm,CYP studies discussed in the preceding section. With this pattern confirmed, full kinetics studies using rCYPs were undertaken to characterise the reactions between proguanil and the enzymes responsible for its *in vitro* metabolism. The conditions used mirrored those used during the determination of proguanil enzyme kinetics in HLM with only the protein source differing. These conditions are summarised in Table 3.11 below.

Table 3.11: Incubation parameters for the determination of proguanil enzyme kinetics

Parameter	Conditions tested
Enzyme Concentration (pmol/mL)	50
Time (min)	0; 15; 30; 45; 60
Substrate Concentration Range (μ M)	5-300
Standard Curve Range (nM)	1-1500

In Figures 3.10-3.14 are substrate saturation plots for the determination of proguanil CYP1A2, CYP2C19, CYP2D6, CYP3A4 and CYP3A5 kinetics.

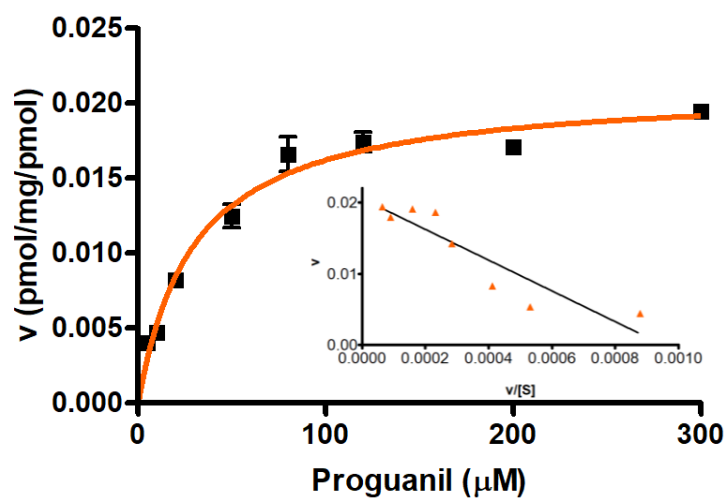


Figure 3.10: Substrate saturation curve for the determination of proguanil CYP1A2 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics

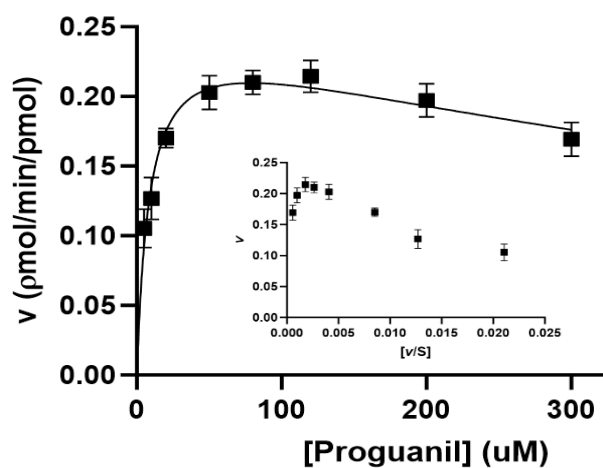


Figure 3.11: Substrate saturation curve for the determination of proguanil CYP2D6 kinetics. Inset: Eadie-Hofstee plot showing substrate inhibition kinetics

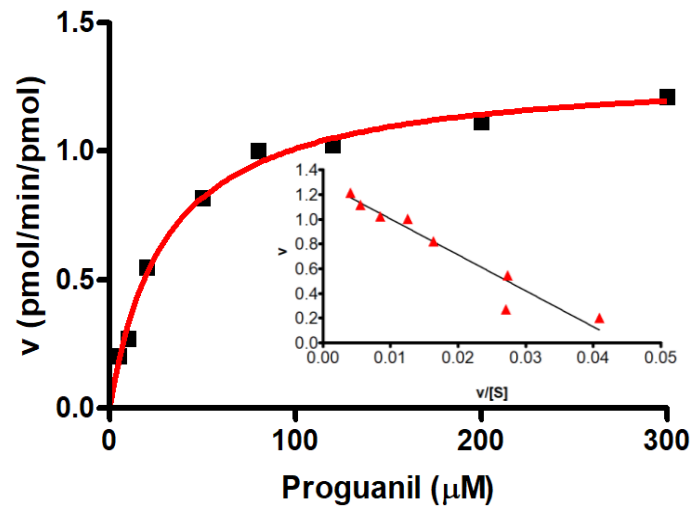


Figure 3.14: Substrate saturation curve for the determination of proguanil CYP2C19 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics

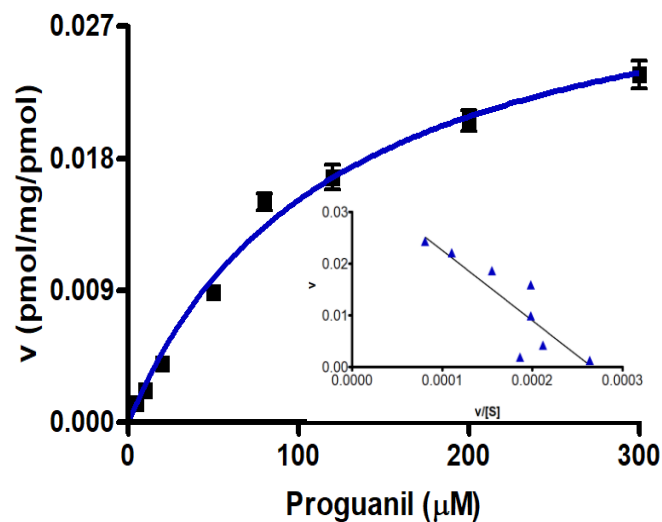


Figure 3.13: Substrate saturation curve for the determination of proguanil CYP3A4 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics

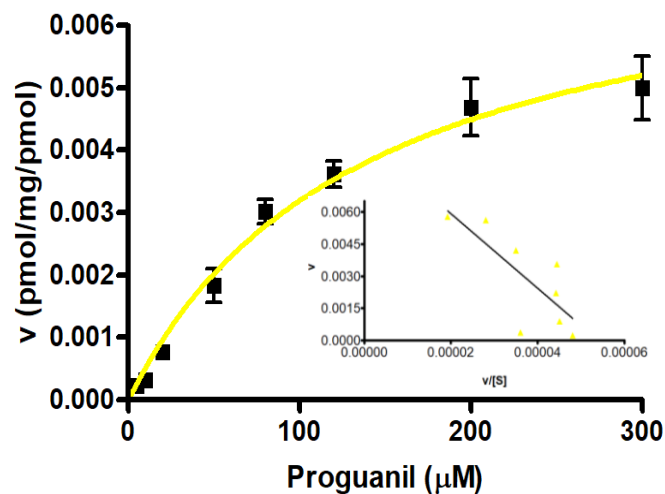


Figure 3.12: Substrate saturation curve for the determination of proguanil CYP3A5 kinetics. Inset: Eadie-Hofstee plot confirming simple Michaelis-Menten kinetics

In Table 3.12 is summarized the enzyme kinetics parameters determined for the conversion of proguanil to cycloguanil by the enzymes previously determined to be involved in its *in vitro* metabolism.

Table 3.12: Enzyme kinetic parameters for the conversion of proguanil to cycloguanil via specific rCYP isoforms. Data gathered from two independent incubations

Enzyme	K_M (μM) [95%CI]	V_{max} (pmol/mg/pmol) [95%CI]
CYP1A2	29.8 [25.4-34.1]	0.0210 [0.0202-0.0219]
CYP2C19	30.2 [22.8-37.5]	1.32 [1.23-1.40]
CYP2D6	3.87 [0.750-6.99]	0.210 [0.186-0.235]
CYP3A4	108 [65.8-151]	0.0340 [0.0283-0.0397]
CYP3A5	137 [116-159]	0.00758 [0.00703-0.00814]

The conversion of proguanil to cycloguanil by CYP1A2 and CYP2C19 was shown to be a moderate K_M reactions, whereas the conversions by CYP3A4 and CYP3A5 were high K_M reactions. The conversion of proguanil to cycloguanil by CYP2D6 was shown to be a low K_M reaction and to follow substrate inhibition kinetics. This was expected since it has been shown that proguanil inhibits CYP2D6 *in vitro*.³³

The enzymatic reactions had similar proportions for V_{max} as was seen during the initial enzyme reaction incubations. It was shown again that CYP2C19 was the main enzyme involved in proguanil metabolism while the involvement of CYP1A2 and CYP2D6 in this *in vitro* metabolism was confirmed.

The *in vitro* metabolic profile for proguanil was subsequently used to inform and assist in the development of PBPK models to predict proguanil exposure.

3.7 Proguanil *in vitro* *in vivo* extrapolation

The measured *in vitro* metabolism data was then scaled up to represent the unbound *in vivo* metabolic clearance values. In the case of the data generated in HLM, this was achieved by multiplying the measured HLM CL_{int} value by both the MPPGL used in Simcyp, as well as the average liver weight, in grams, per kg body weight. After this, the calculated CL_{int} was divided by the fraction of proguanil unbound in HLM.³³ In the case of the data generated in rCYPs, this was achieved by multiplying the measured rCYP CL_{int} value for each CYP isoform involved in the conversion of proguanil to cycloguanil by the relevant enzyme abundance value observed in Simcyp, then all values by average liver weight, in grams, per kg body weight, and then

lastly by the relevant ISEF value for each enzyme also found in Simcyp. This data was then compared to the relevant unbound *in vivo* clearance ($CL_{u,int}$) data, obtained by dividing the reported clearance data by the unbound fraction of proguanil in human plasma,^{21,33} as a means to determine how well the measured *in vitro* data would be able to predict clearance, as well as the formation of cycloguanil, in Simcyp. This comparison is shown in Table 3.13 below.

It should be noted that all *in vitro* data measures only the conversion of proguanil to cycloguanil. As such, the *in vitro* data must be compared to the *in vivo* $CL_{Cycloguanil}$ data for the most accurate comparison.

Table 3.13: Extrapolated and observed intrinsic clearance of proguanil

<i>In vitro</i> Microsomal Clearance				
	K_M (μ M)	V_{max} (pmol/mg/min)	CL_{int} (μ L/min/mg)	CL_{int} (mL/min/kg)
HLM	96.6	16.2	0.168	0.491
Recombinant Enzyme Clearance				
	K_M (μ M)	V_{max} (pmol/mg/pmol)	CL_{int} (μ L/min/pmol)	CL_{int} (mL/min/kg)
CYP1A2	29.8	0.0210	0.000708	0.000150
CYP2C19	30.2	1.32	0.0437	0.000981
CYP2D6	3.87	0.210	0.0542	0.00195
CYP3A4	108	0.0340	0.000315	0.000275
CYP3A5	137	0.00758	0.0000553	0.0000363
<i>In vivo</i> Clearance*				
			CL_{int} (mL/min/kg)	$CL_{u,int}$ (mL/min/kg)
<i>In vivo</i> CL_r			5.68	16.7
<i>In vivo</i> $CL_{Cycloguanil}$			3.40	10.0
<i>In vivo</i> CL_{CPB}			1.40	4.13
<i>In vivo</i> CL_{Add}			7.83	23.0
<i>In vivo</i> $CL_{Oral,Total}$			18.3	53.9

**In vivo* clearance data obtained from Jeppesen *et al.* 1997.²¹

As the above table illustrates, there is a significant under estimation of *in vivo* $CL_{Cycloguanil}$ when the *in vitro* data is extrapolated to *in vivo* conditions. It is thus unlikely that the measured *in vitro* data could accurately predict *in vivo* outcomes.

3.8 Proguanil PBPK Modelling

PBPK models were developed using the population-based PBPK software Simcyp (Simcyp Ltd, a Certara company, Sheffield, UK; version 21). The development strategy for this model followed established best practice.³⁴ This process entailed model development and optimization, followed by model verification and lastly, the application of the model to scenarios of interest.

3.8.1 Development of Proguanil model

Proguanil drug input parameters are listed in Tables 3.14-3.17 below.

Table 3.14: Input parameter values used to simulate the pharmacokinetics of proguanil

Input Parameter	Description	Value	Unit	Reference
MW	Molecular weight	253.73	g/mol	Charman <i>et al.</i> 2020 ³³
Log $D_{7.4}$	Octanol-water partition	0.27		Charman <i>et al.</i> 2020 ³³
Log $P_{O:W}$		2.98		Calculated from Log $D_{7.4}$ in Simcyp
Compound Type	Acid, base or neutral	Diprotic base		Charman <i>et al.</i> 2020 ³³
pK_a	pKa in water	10.4; 6.64		Charman <i>et al.</i> 2020 ³³
B/P ratio	Blood to plasma concentration ratio	3.3		Charman <i>et al.</i> 2020 ³³
$f_{u(inc)}$	Fraction unbound in vitro			
	Human Plasma	0.34		
	Albumax	0.8		Charman <i>et al.</i> 2020 ³³
	HLM	0.33		
f_a	Fraction available from dosage	0.836	%	Predicted by Simcyp
k_a	Absorption rate constant	0.572	1/h	Predicted by Simcyp
Q_{gut}	Nominal flow in gut model	7.75	L/h	Predicted by Simcyp
$P_{eff,man}$	Effective permeability in man	1.31	(10^{-4} cm/s)	Predicted based on Caco-2 Data
$f_{u(gut)}$	Unbound fraction in enterocytes	0.00668		Predicted by Simcyp
S_o	Intrinsic Solubility	2.0	mg/mL	Charman <i>et al.</i> 2020 ³³
V_{ss}	Volume of distribution at steady-state	15.7	L/kg	Edstein <i>et al.</i> 1988 ¹⁴
K_p Scalar	Scalar applied to all predicted tissue K_p values	0.46		Input into Simcyp to obtain V_{ss} of 15.7

Table 3.15: Permeability input parameters, in the form of Caco-2 data, for the simulation of proguanil pharmacokinetics.
Data sourced from Charman et al. 2020³³ & Katneni et al. 2018³⁵

Reference Compounds				
Unit P_{app} (10^{-6} cm/s)				
Compound	Description	A-B Range	B-A Range	Efflux Ratio Range
Lucifer Yellow	Minimal Permeability Marker	<2	Not assessed	-
Propranolol	High Permeability Marker	58	52	0.9-1.0
Rhodamine 123	P-gp efflux Marker	0.7-1.6	10-28	6-39
Atenolol		0.39		
Cimetidine		1.7		
Metoprolol		50		
Verapamil		41	45	1.1
Compounds in bold were used to predict a scalar from which $P_{eff,man}$ was predicted using proguanil Caco-2 data below				
Unit P_{app} (10^{-6} cm/s)				
Compound	Description	A-B	B-A Range	Efflux Ratio
Proguanil	-	3.5	7.9	2.3
Given proguanil efflux ratio >2, Mean of the A-B and B-A was used i.e 5.7 with passive and active transport				

Values in bold used in model

Table 3.16: Interaction input parameters, in the form of inhibition constants, for the simulation of proguanil pharmacokinetics

Interaction Properties				
Interaction	Description	Value	Unit	Reference
CYP2D6 (Weak Inhibitor)	K_i	2.2	μM	Charman et al. 2020 ³³

Table 3.17: Elimination input parameters for proguanil for the simulation of proguanil pharmacokinetics

Input Parameter	Description	Value	Unit	Reference
CL_r	Renal Clearance	26.8		
$CL_{met,Cycloguanil}$	Clearance to cycloguanil	16.0		
$CL_{met,CPB}$	Clearance to CPB	6.62	L/h	Jeppesen <i>et al.</i> 1997 ²¹
CL_{Add}	Additional Clearance	36.9		
$CL_{Oral,Total}$	Total Oral Clearance	86.4		
V_{max}	<i>In vitro</i> maximum velocity measured in HLM	16.2	pmol/mg/min	
K_M	<i>In vitro</i> Michaelis-Menten constant measured in HLM	96.6	μM	Measured
HLM CL_{int}	Intrinsic clearance measured in HLM	0.168	(μL/min/mg)	
CYP1A2 rCYP	Recombinant CYP Activity	0.000708	(μL/min/pmol)	Measured
CYP2C19 rCYP		0.0437		
CYP2D6 rCYP		0.0542		
CYP3A4 rCYP		0.000315		
CYP3A5 rCYP		0.0000553		
CYP1A2 CL_{int}	Intrinsic clearance determined for enzyme by RTT	0.0650	(μL/min/pmol)	Simcyp
CYP2C19 CL_{int}		1.12		
CYP2D6 CL_{int}		0.155		
CYP3A4 CL_{int}		0.00736		
CYP3A5 CL_{int}		0.00355		

Values in bold used in model

A single-dose PK study published by Jeppesen *et al* was used as the basis for the development of this model.²¹ PK simulations during the development phase were performed using the Simcyp Healthy Volunteer population. The trial design was replicated as closely as possible, by using the same age range and number and gender of participants for example, with these virtual trials being simulated ten times in each run. The fasted state Advanced Dissolution Absorption and Metabolism (ADAM) and full PBPK model modes were employed.

An initial simulation was attempted using the above-mentioned input data to determine whether the *in vitro* rCYP data would be able to predict proguanil clearance as well as cycloguanil formation. Since cycloguanil exposure could not be measured when using HLM clearance, this was not attempted. Figure 3.15 below shows simulations done using the measured *in vitro* metabolism data. The green line represents the simulated concentration-time profile while the orange dots represent the data obtained from Jeppesen *et al.*²¹ overlayed over the simulated profiles.

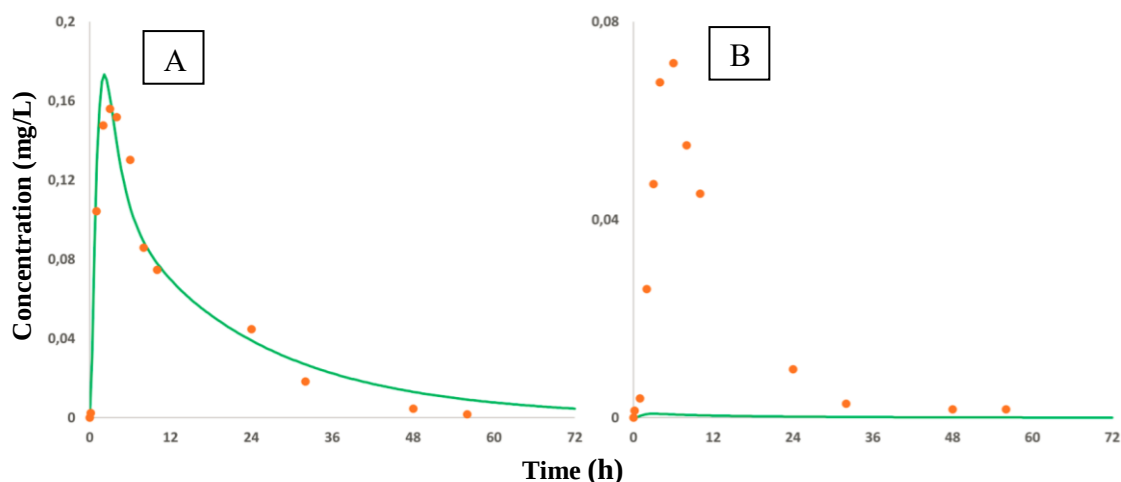


Figure 3.15: Simulated (green line) and observed (orange data points) mean plasma concentration-time profiles of proguanil (A) and cycloguanil (B) after a single oral dose of 200 mg proguanil with trial parameters matching that of Jeppesen *et al.*, (10 trials of 6 male subjects, 22-30 yrs). Model run with elimination being determined by rCYP clearance.

As can be seen, the measured *in vitro* rCYP clearance could reasonably accurately predict proguanil clearance but significantly underpredicted cycloguanil exposure, likely due to an underprediction in *in vitro* $CL_{met,Cycloguanil}$. To overcome this, a retrograde modelling approach was employed. The Reverse Translation Tool (RTT) in Simcyp was used to calculate proguanil clearance. As part of this approach, 28.8% of proguanil $CL_{met,Cycloguanil}$ was assigned to CYP1A2, 44.8% assigned to CYP2C19, 12.8% assigned to CYP2D6 and 13.6% assigned to CYP3A. These assignments were based on the fm,CYP values obtained from the chemical inhibition studies described above. Since CYP3A5 activity was shown to be approximately 30% of CYP3A4 activity, the 13.6% assignment of hepatic clearance to CYP3A was assigned as 9.5% CYP3A4 and 4.1% CYP3A5. In addition, the $CL_{met,CPB}$ for the conversion of proguanil to CPB was summed with the reported additional clearance with this new value, 43.5 L/h, being assigned in the RTT as additional clearance. Figure 3.16 below shows simulations done when the retrograde model was employed to predict mefloquine clearance parameters using the RTT. Table 3.18 shows the PK parameters obtained from the developed model as well as the observed data from Jeppesen *et al.*²¹

Table 3.18: Simulated and observed PK parameters based on the developed model and data from Jeppesen *et al.*

PK Parameter	Model	Literature
C_{max} (mg/L) (5 th and 95 th)	0.160 (0.150-0.170)	0.156
AUC (mg/L·h) (5 th and 95 th)	2.10 (1.90-2.27)	2.45

Figure 3.16 below illustrates that the developed model could describe the concentration-time profile of proguanil under the stated conditions reasonably well. The simulated PK parameter mean values were within two-fold of the observed values, $C_{\max}$ (mean 1.03-fold, range 0.934 to 1.10-fold) and AUC (mean 0.860-fold, range 0.734 to 0.930-fold).

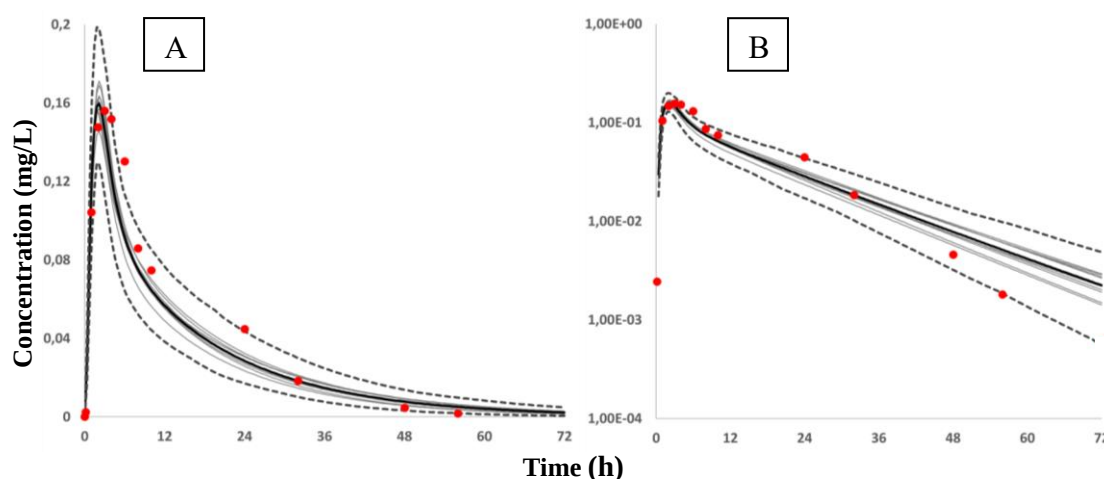


Figure 3.16: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of proguanil after a single oral dose of 200 mg proguanil (A), (10 trials of 6 male subjects, 22-30 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Jeppesen et al. 1997. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

3.8.2 Development of Cycloguanil model

As alluded to above, the proguanil model was further expanded to include cycloguanil as a metabolite. Cycloguanil drug input parameters are listed in Tables 3.19-3.22 below. In contrast to the proguanil model wherein the ADAM model mode was used, the first order absorption model was used for the cycloguanil model given its status as a metabolite.

Table 3.19: Input parameter values used to simulate the pharmacokinetics of cycloguanil

Input Parameter	Description	Value	Unit	Reference
MW	Molecular weight	251.72	g/mol	Charman <i>et al.</i> 2020 ³³
Log $D_{7.4}$	Octanol-water partition	-1.10		Charman <i>et al.</i> 2020 ³³
Log $P_{O:W}$		1.854		Calculated from LogD in Simcyp
Compound Type	Acid, base or neutral	Base		Charman <i>et al.</i> 2020 ³³
pK_a	pKa in water	11.4		Charman <i>et al.</i> 2020 ³³
B/P ratio	Blood to plasma concentration ratio	0.710		Charman <i>et al.</i> 2020 ³³
$f_{u(inc)}$	Fraction unbound in <i>vitro</i>			
	Human Plasma	0.75		
	Albumax	0.88		Charman <i>et al.</i> 2020 ³³
	HLM	0.72		
f_a	Fraction available from dosage	1	%	Assumed
Q_{gut}	Nominal flow in gut model	1.77	L/h	Predicted by Simcyp
$P_{eff,man}$	Effective permeability in man	0.216	(10^{-4} cm/s)	Predicted based on Caco-2 Data
$f_{u(gut)}$	Unbound fraction in enterocytes	1		Predicted by Simcyp
S_o	Intrinsic Solubility	2.0	mg/mL	Charman <i>et al.</i> 2020 ³³
V_{ss}	Volume of distribution at steady-state	1.47	L/kg	Parameter optimization to fit to clinical data
K_p Scalar	Scalar applied to all predicted tissue K_p values	2		Parameter optimization to fit to clinical data

Table 3.20: Permeability input parameters, in the form of Caco-2 data, for the simulation of cycloguanil pharmacokinetics. Data sourced from Charman *et al.* 2020³³ & Katneni *et al.* 2018³⁵

Reference Compounds				
Unit P _{app} (10 ⁻⁶ cm/s)				
Compound	Description	A-B Range	B-A Range	Efflux Ratio Range
Lucifer Yellow	Minimal Permeability Marker	<2	Not assessed	-
Propranolol	High Permeability Marker	58	52	0.9-1.0
Rhodamine 123	P-gp efflux Marker	0.7-1.6	10-28	6-39
Atenolol		0.39		
Cimetidine		1.7		
Metoprolol		50		
Verapamil		41	45	1.1
Compounds in bold were used to predict a scalar from which Peff,man was predicted using cycloguanil Caco-2 data below				
Unit P _{app} (10 ⁻⁶ cm/s)				
Compound	Description	A-B	B-A Range	Efflux Ratio
Cycloguanil		1.2	0.77	0.7

Values in bold used in model

Table 3.21: Elimination input parameters for proguanil for the simulation of cycloguanil pharmacokinetics

Input Parameter	Description	Value	Unit	Reference
HLM CL_{int}	Intrinsic clearance measured in HLM	0.1	(μ L/min/mg)	Charman <i>et al.</i> 2020 ³³ (Value reported as <7, this parameter was optimized to fit clinical data)
CL_r	Renal Clearance	35.0	L/h	Funck-Brentano <i>et al.</i> 1997 ²⁰

Values in bold used in model

Table 3.22: Interaction input parameters, in the form of inhibition constants, for the simulation of cycloguanil pharmacokinetics

Interaction Properties				
Interaction	Description	Value	Unit	Reference
CYP2D6 (Weak Inhibitor)	K_i	3.7	μ M	Charman <i>et al.</i> 2020 ³³

The same studies used for the development of the proguanil model were used for the development of the cycloguanil model. Ultimately, cycloguanil exposure was highly dependent on proguanil elimination, as a result of it being modelled as a metabolite of proguanil. Given this, the final cycloguanil model was dependent on which elimination method was selected for the proguanil model. In Figure 3.17 below is shown the concentration-time profile of cycloguanil after the RTT was used to predicted proguanil elimination. Table 3.23 shows the PK parameters obtained from the developed cycloguanil model as well as the observed data from Jeppesen *et al.*²¹

Table 3.23: Simulated and observed PK parameters based on the developed cycloguanil model and data from Jeppesen et al.

PK Parameter	Model	Literature
$C_{\max}$ (mg/L) (5 th and 95 th)	0.0718 (0.0536-0.0937)	0.0716
AUC (mg/L·h) (5 th and 95 th)	1.10 (0.848-1.38)	1.32

Figure 3.17 below illustrates that the developed model could describe the concentration-time profile of cycloguanil under the stated conditions reasonably well. The simulated PK parameter mean values were within two-fold of the observed values, $C_{\max}$ (mean 1.00-fold, range 0.715 to 1.37-fold) and AUC (mean 0.829-fold, range 0.596 to 1.05-fold).

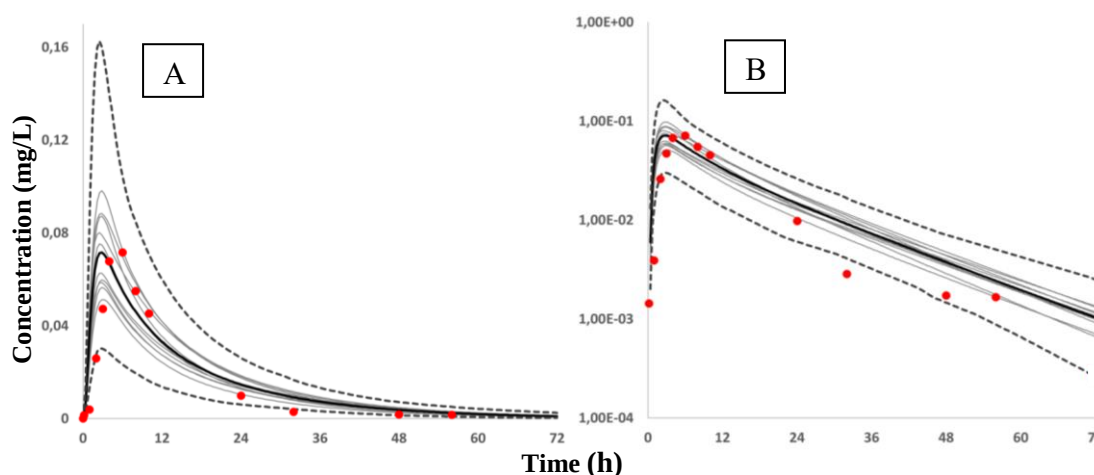


Figure 3.17: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of cycloguanil after a single oral dose of 200 mg proguanil (A), (10 trials of 6 male subjects, 22-30 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Jeppesen et al. 1997. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

3.8.3 Model Verification: Proguanil

The model developed above was then verified by simulating proguanil concentration-time profiles using different dosage regimens. These simulated parameters were then compared to known literature data where their dosage parameters were used while maintaining the same compound input parameters. Other study parameters, such as proportion of females involved in the study and the age range of the participants, were also changed to represent the study more accurately. Table 3.24 below lists the different studies, and their study details, used to verify the developed model. When a study was conducted in Thai individuals, the Simcyp Chinese Healthy Volunteer population was used to better represent those individuals. In all other cases, the Simcyp Healthy Volunteer population was used.

Table 3.24: Summary of literature sources used in proguanil/cycloguanil model verification

Pharmacokinetic studies used in model verification		
Study Details	Study type, dosage and description (Duration)	Reference
Thai HV 21-29 yrs 9 M	SD, 200 mg (2 days)	Wattanagoon <i>et al.</i> 1987 ⁶
Caucasian HV 20-31 yrs 12 M	SD, 200 mg (2 days)	Funck-Brentano <i>et al.</i> 1997 ²⁰
Caucasian HV 20-26 yrs 10 M 8 F	SD, 100 mg (1 day)	Van Luin <i>et al.</i> 2010 ³⁶
Caucasian HV 20-30 yrs 7 M	SD, 200 mg (3 days)	Helsby <i>et al.</i> 1990 ¹²
Caucasian HV 24-32 yrs 6 M	MD, 200 mg once daily for 7 days (8 days)	Edstein <i>et al.</i> 1988 ¹⁴
Caucasian HV 24-33 yrs 6 M	MD, 200 mg once daily for 7 days (8 days)	Edstein <i>et al.</i> 1990 ¹⁵
Caucasian HV 24-33 yrs 5 M 1 F	MD, 200 mg once daily for 14 days (16 days)	Helsby <i>et al.</i> 1993 ¹³
Caucasian HV 22-30 yrs 6 M	Interaction. Fluvoxamine was administered as a 100 mg dose once daily for 7 days. On the seventh day, 200 mg proguanil was administered along with fluvoxamine (9 days)	Jeppesen <i>et al.</i> 1997 ²¹
Caucasian HV 20-31 yrs 12 M	Interaction. Omeprazole was administered as a 40 mg dose once daily for 7 days. On the seventh day, 200 mg proguanil was administered along with omeprazole (9 days)	Funck-Brentano <i>et al.</i> 1997 ²⁰
Caucasian HIV patients 35-60 yrs 18 M 2 F	Interaction. HIV patients who received chronic efavirenz treatment were given a single 100 mg proguanil dose (1 day)	Van Luin <i>et al.</i> 2010 ³⁶

HV=Healthy Volunteers; HIV=Human immunodeficiency virus

Where reported PK data did not provide corresponding summary data, observed data points in reported figures were retrieved using WebPlotDigitizer v4.6.³⁷ These were then superimposed onto simulated profiles for visual predictive checks. PK data was retrieved by doing non-compartmental analysis (NCA) of reported figures using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA) with a method based on curve stripping. In the case of the studies conducted by Edstein *et al.* 1988,¹⁴ and Edstein *et al.* 1990,¹⁵ where data was only reported for a single study day in the form of a figure, data was retrieved as described here and

comparison between observed and simulated data was done for only the corresponding simulated day.

In Figures 3.18-3.20 below are representative concentration-time profiles for model verification. These represent single dose, multiple dose, and DDI studies. Complete data sets for all the other studies used for model verification can be found in the appendices at the end of the document.

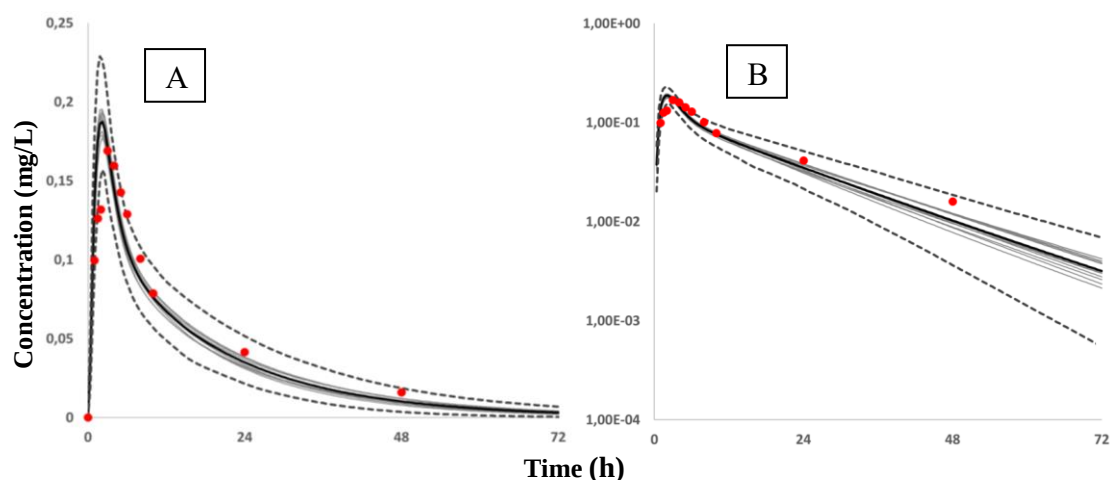


Figure 3.18: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of proguanil after a single oral dose of 200 mg proguanil (A), (10 trials of 9 male subjects, 21-29 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Wattanagoon et al., 1987. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

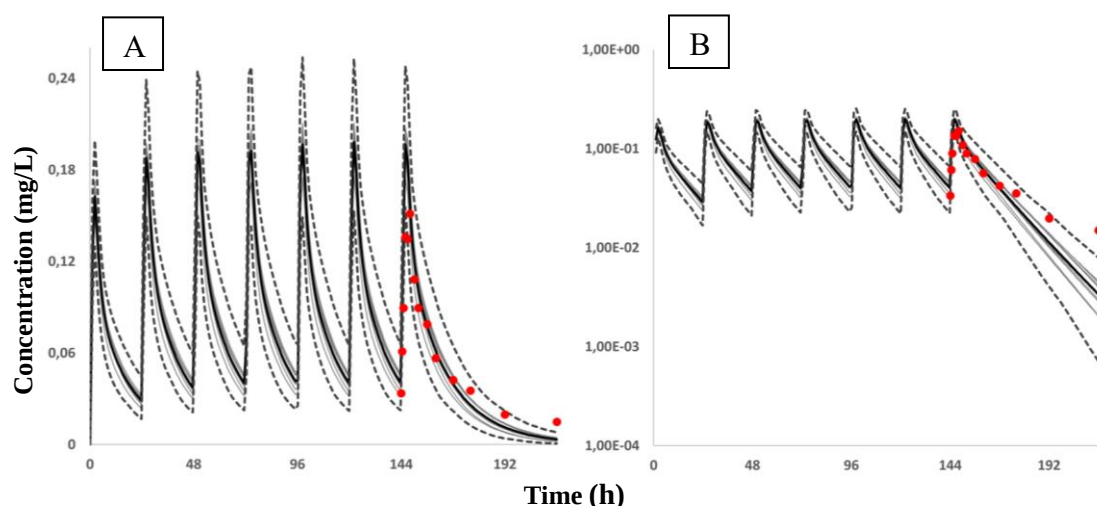


Figure 3.19: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of proguanil after multiple oral doses of 200 mg proguanil (A), (10 trials of 6 male subjects, 24-33 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Edstein et al., 1990. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

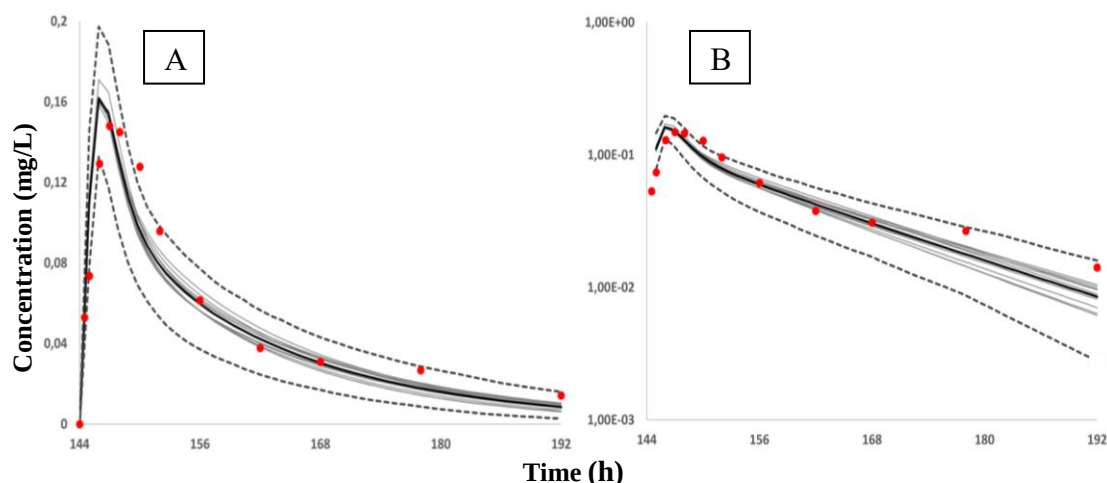


Figure 3.20: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of proguanil after single oral dose of 200 mg preceded by pre-treatment with 40 mg of omeprazole, taken once a day for 6 consecutive days before proguanil administration. Proguanil was then administered on the 7th day along with the final omeprazole dose (A), (10 trials of 12 male subjects, 20-31 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Funck-Brentano *et al.*, 1997. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

It should be noted that the study conducted by Helsby *et al.* 1993,¹³ was excluded due to the poor quality of the reported figures wherefrom data would need to be extracted. A study conducted by Soyinka *et al.* 2010,³⁸ was also excluded since the reported clearance was significantly higher than those reported in other studies used.

These figures illustrate that the developed model could describe the concentration-time profile of proguanil under a variety of conditions reasonably well. The observed and predicted AUC ratios were 1.47 vs 1.15 for the interaction with fluvoxamine, 1.29 vs 1.01 for the interaction with omeprazole, and 0.705 vs 0.974 for the interaction with efavirenz. The observed and predicted C_{max} ratios were 1.27 vs 1.05 for the interaction with fluvoxamine, 1.06 vs 1.00 for the interaction with omeprazole, and 0.693 vs 0.971 for the interaction with efavirenz. The simulated PK parameter mean values were also within two-fold of the observed values for all studies used, with these PK parameters shown in Table 3.25 below. Thus, the proguanil model was considered verified.

Of note is that, despite still being within two-fold of the observed data, the model underpredicted the exposure profile of the study conducted by Helsby *et al.* 1990.¹² An attempt by researchers at Simcyp to develop and verify a model for proguanil, accessed through the global health repository within the Simcyp members area, also underpredicted proguanil exposure for this study; potentially indicating that this study is a minor outlier in comparison to other studies.³⁹ In addition, Simcyp researchers excluded the aforementioned studies conducted by Helsby *et al.* 1993 and Soyinka *et al.* 2010 for the same reasons stated previously.^{13,38,39}

Table 3.25: Comparison between reported and simulated PK parameters for the studies used for proguanil model verification

Study (Type)	PK Parameter	Model	Literature	Mean Fold Diff. (Range)
Wattanagoon <i>et al.</i> 1987 ⁶ (SD)	C _{max} (mg/L) (5 th and 95 th)	0.188 (0.178-0.195)	0.170	1.10 (1.04-1.15)
	AUC (mg/L·h) (5 th and 95 th)	2.54 (2.36-2.72)	2.98	0.853 (0.786-0.916)
Funck-Brentano <i>et al.</i> 1997 ²⁰ (SD)	C _{max} (mg/L) (5 th and 95 th)	0.162 (0.157-0.168)	0.139	1.16 (1.12-1.22)
	AUC (mg/L·h) (5 th and 95 th)	2.05 (1.92-2.16)	1.80	1.14 (1.04-1.21)
Van Luin <i>et al.</i> 2010 ³⁶ (SD)	C _{max} (mg/L) (5 th and 95 th)	0.0777 (0.0737-0.0830)	0.0821*	0.947 (0.898-1.02)
	AUC (mg/L·h) (5 th and 95 th)	0.846 (0.815-0.874)	0.826*	1.02 (0.985-1.06)
Helsby <i>et al.</i> 1990 ¹² (SD)	C _{max} (mg/L) (5 th and 95 th)	0.183 (0.161-0.209)	0.278*	0.657 (0.580-0.751)
	AUC (mg/L·h) (5 th and 95 th)	2.10 (1.85-2.37)	3.88*	0.541 (0.467-0.626)
Edstein <i>et al.</i> 1988 ¹⁴ (MD)	C _{max} (mg/L) (5 th and 95 th)	0.198 (0.155-0.251)	0.123**	1.61 (1.43-1.65)
	AUC (mg/L·h) (5 th and 95 th)	5.56 (3.25-8.61)	4.16**	1.34 (1.08-1.50)
Edstein <i>et al.</i> 1990 ¹⁵ (MD)	C _{max} (mg/L) (5 th and 95 th)	0.197 (0.155-0.248)	0.151**	1.30 (1.16-1.34)
	AUC (mg/L·h) (5 th and 95 th)	5.79 (3.35-9.04)	5.50**	1.05 (0.845-1.19)
Jeppesen <i>et al.</i> 1997 ²¹ (Interaction)	C _{max} (mg/L) (5 th and 95 th)	0.169 (0.161-0.178)	0.198*	0.851 (0.800-0.922)
	AUC (mg/L·h) (5 th and 95 th)	2.42 (2.19-2.53)	3.60*	0.673 (0.595-0.704)
Funck-Brentano <i>et al.</i> 1997 ²⁰ (Interaction)	C _{max} (mg/L) (5 th and 95 th)	0.162 (0.159-0.167)	0.148*	1.09 (1.07-1.16)
	AUC (mg/L·h) (5 th and 95 th)	2.09 (1.94-2.24)	2.32*	0.902 (0.838-0.977)
Van Luin <i>et al.</i> 2010 ³⁶ (Interaction)	C _{max} (mg/L) (5 th and 95 th)	0.0755 (0.0721-0.0791)	0.0569*	1.33 (1.26-1.40)
	AUC (mg/L·h) (5 th and 95 th)	0.824 (0.769-0.864)	0.583*	1.42 (1.31-1.50)

* Values not reported, data obtained through non-compartmental analysis of reported figures

**Data only reported for one study day, modelling data reported for corresponding simulated day

3.8.4 Model Verification: Cycloguanil

The studies used for proguanil model verification, shown in Table 3.24 above, were also used for cycloguanil model verification. In Figures 3.21-3.23 are the corresponding representative concentration-time profiles for cycloguanil model verification as was shown for proguanil model verification. These represent single dose, multiple dose, and DDI studies. Complete data sets for all the other studies used for model verification can be found in the appendices at the end of the document. It should be noted that no data on cycloguanil was reported in the study

conducted by Van Luin *et al.* 2010.³⁶ As a result, a comparison between the simulated and reported data could not be done.

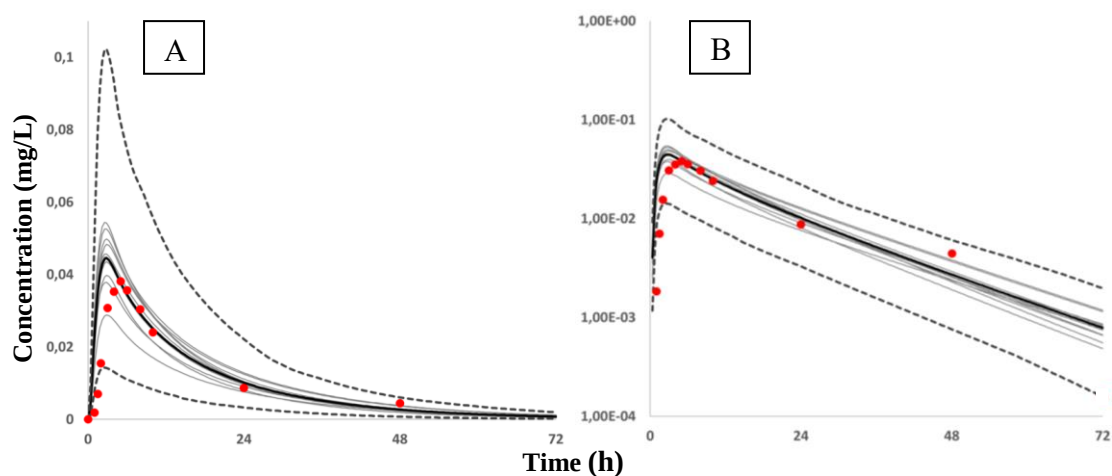


Figure 3.21: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of cycloguanil after a single oral dose of 200 mg proguanil (A), (10 trials of 9 male subjects, 21-29 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Wattanagoon *et al.*, 1987. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

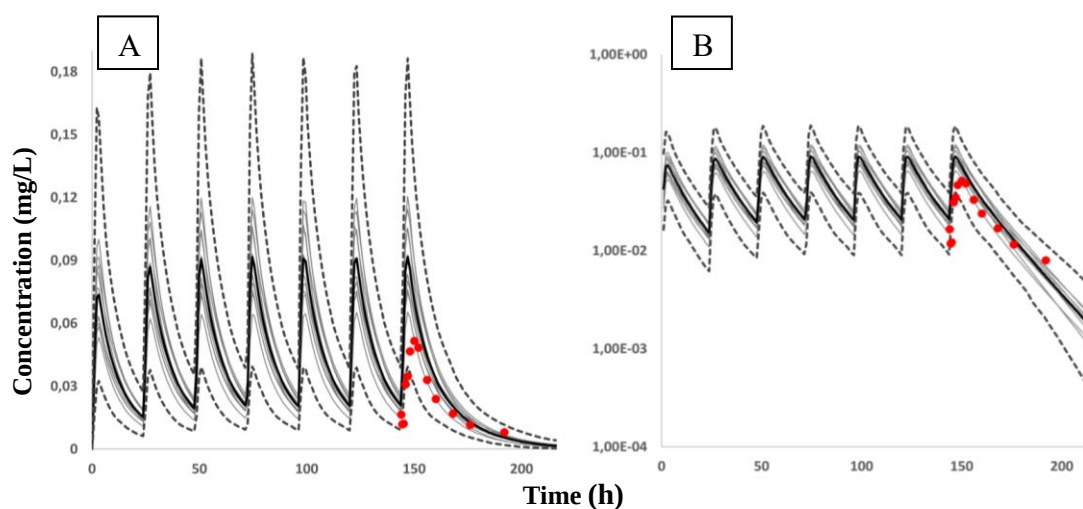


Figure 3.22: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of cycloguanil after a multiple oral doses of 200 mg proguanil (A), (10 trials of 6 male subjects, 24-33 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Edstein *et al.*, 1990. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

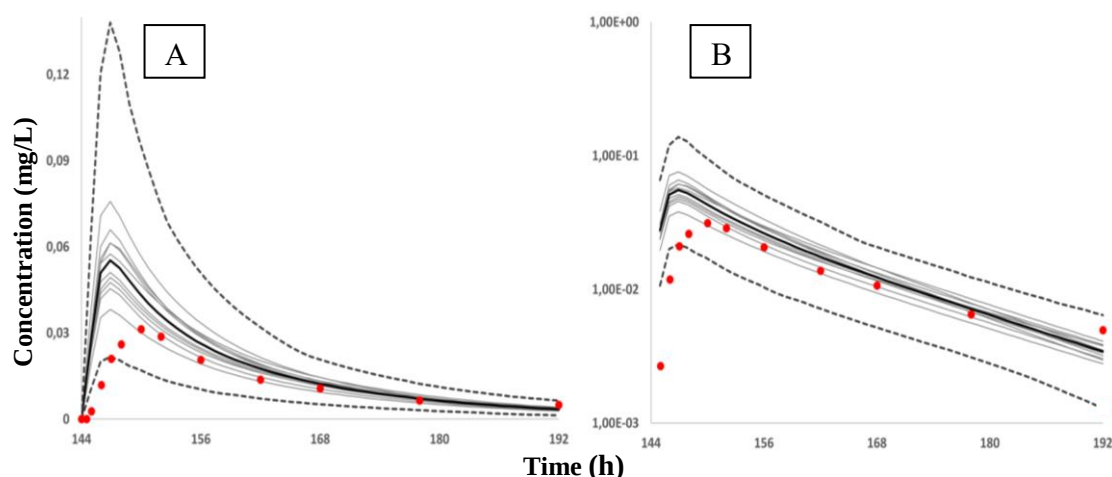


Figure 3.23: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of cycloguanil after single oral dose of 200 mg preceded by pre-treatment with 40 mg of omeprazole, taken once a day for 6 consecutive days before proguanil administration. Proguanil was then administered on the 7th day along with the final omeprazole dose (A), (10 trials of 12 male subjects, 20-31 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Funck-Brentano *et al.*, 1997. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

These figures illustrate that the developed cycloguanil model could describe the overall shape of cycloguanil PK under a variety of conditions reasonably well. However, the PK parameters, seen in Table 3.26 below, show that the model could not accurately predict the magnitude of cycloguanil exposure for every study. Specifically, the model could only predict the magnitude of exposure with decent accuracy for the single dose studies for which data was reported, albeit with Helsby *et al.* 1990 still being an outlier because of the underprediction in proguanil exposure. The model had some difficulty in predicting the exposure of the multi-dose studies, slightly overpredicting cycloguanil $C_{\max}$ and AUC. Despite this notable overprediction, these studies were still predicted within 2-fold of the observed data.

In addition, the model also slightly overpredicted cycloguanil PK parameters for the interaction studies, more so for the study conducted by Jeppesen *et al.*,²¹ than that conducted by Funck-Brentano *et al.*,²⁰ where the model still predicted the PK parameters within the 2-fold range. This is illustrated in the PK parameter ratios for these studies where the observed and predicted AUC ratios were 0.174 vs 0.223 for the interaction with fluvoxamine, and 0.482 vs 0.815 for the interaction with omeprazole. The observed and predicted $C_{\max}$ ratios were 0.065 vs 0.166 for the interaction with fluvoxamine, and 0.411 vs 0.798 for the interaction with omeprazole.

However, it should be noted that the model still predicted most of the verification studies with reasonable accuracy; with the only exceptions being the predicted $C_{\max}$ for the interaction with fluvoxamine and the predicted AUC for the Helsby *et al.* 1990 study being outside of the 2-fold range.^{12,21} Moreover, cycloguanil exposure, in the form of AUC, was well predicted for

the fluvoxamine interaction study and the difficulties faced with the Helsby *et al.* 1990 study likely resulting because of the underprediction of proguanil exposure for this investigation.¹² As a result of the above, the cycloguanil portion of the model was considered verified. However, the propensity of the model to slightly over-predict cycloguanil exposure for multi-dose and DDI studies should always be kept in consideration when the model is used for prediction in those types of investigations.

Table 3.26: Comparison between reported and simulated PK parameters for the studies used for cycloguanil model verification

Study (Type)	PK Parameter	Model	Literature *	Mean Fold Diff. (Range)
Wattanagoon <i>et al.</i> 1987 ⁶ (SD)	C _{max} (mg/L) (5 th and 95 th)	0.0446 (0.0329-0.0536)	0.0381	1.17 (0.756-1.43)
	AUC (mg/L·h) (5 th and 95 th)	0.723 (0.553-0.870)	0.803	0.901 (0.641-1.11)
Funck-Brentano <i>et al.</i> 1997 ²⁰ (SD)	C _{max} (mg/L) (5 th and 95 th)	0.0694 (0.0534-0.0880)	0.0762	0.910 (0.647-1.19)
	AUC (mg/L·h) (5 th and 95 th)	1.03 (0.815-1.21)	1.26	0.822 (0.596-0.961)
Van Luin <i>et al.</i> 2010 ³⁶ (SD)	C _{max} (mg/L) (5 th and 95 th)	0.0342 (0.0291-0.0392)	NR	ND
	AUC (mg/L·h) (5 th and 95 th)	0.466 (0.413-0.516)	NR	ND
Helsby <i>et al.</i> 1990 ¹² (SD)	C _{max} (mg/L) (5 th and 95 th)	0.0805 (0.0625-0.103)	0.124	0.652 (0.498-0.883)
	AUC (mg/L·h) (5 th and 95 th)	1.05 (0.823-1.34)	2.30	0.459 (0.353-0.586)
Edstein <i>et al.</i> 1988 ¹⁴ (MD)	C _{max} (mg/L) (5 th and 95 th)	0.0916 (0.0392-0.186)	0.0497	1.84 (1.31-2.30)
	AUC (mg/L·h) (5 th and 95 th)	2.88 (1.24-5.36)	1.86	1.55 (1.10-1.95)
Edstein <i>et al.</i> 1990 ¹⁵ (MD)	C _{max} (mg/L) (5 th and 95 th)	0.0918 (0.0393-0.186)	0.0516	1.78 (1.27-2.22)
	AUC (mg/L·h) (5 th and 95 th)	3.00 (1.28-5.63)	2.20	1.36 (0.969-1.72)
Jeppesen <i>et al.</i> 1997 ²¹ (Interaction)	C _{max} (mg/L) (5 th and 95 th)	0.0119 (0.00816-0.0170)	0.00463	2.58 (1.73-4.19)
	AUC (mg/L·h) (5 th and 95 th)	0.245 (0.174-0.330)	0.231	1.06 (0.736-1.60)
Funck-Brentano <i>et al.</i> 1997 ²⁰ (Interaction)	C _{max} (mg/L) (5 th and 95 th)	0.0553 (0.0415-0.0714)	0.00313	1.77 (1.22-2.42)
	AUC (mg/L·h) (5 th and 95 th)	0.842 (0.654-1.01)	0.0606	1.39 (1.01-1.75)
Van Luin <i>et al.</i> 2010 ³⁶ (Interaction)	C _{max} (mg/L) (5 th and 95 th)	0.0398 (0.0371-0.0423)	NR	ND
	AUC (mg/L·h) (5 th and 95 th)	0.532 (0.499-0.555)	NR	ND

NR=Data was not reported in literature; ND=Data could not be determined due to literature data not being reported

*All literature cycloguanil data was determined through non-compartmental analysis of reported figures

3.8.5 Model Application: Proguanil/Cycloguanil

Upon verification, the proguanil model was applied to various scenarios of interest. In all model application investigations, proguanil was administered in the model at a dosage of 200 mg. All model application investigations were done as 10 trials of 5 male and 5 female subjects with an age range of 20-65 years.

3.8.5.1 Model Application: Ethnic sensitivity

The verified model was run with the modified black South African tuberculosis population file (V16R1_SouthAfricanTB_Population); which can be found, along with the supporting information used to create it, in the global health repository within the Simcyp members area.^{39,40} Changes were made to this population file so as to reflect a healthy black South African population, within the bounds of the available information, so as to compare proguanil and cycloguanil disposition within this population group to the healthy volunteers' population group, made up of data derived from Caucasians. Specifically, changes were made to parameters relating to the previously mentioned blood proteins as well as body weight.

In Figure 3.24 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 3.27 below.

Table 3.27: Comparison between verified proguanil/cycloguanil model and model run with the modified black South African population file

	PK parameters	Verified Model	Black SA Pop.	C _{max} R/AUCR
Proguanil	C _{max} (mg/L)	0.187	0.180	0.963
	(5 th and 95 th)	(0.169-0.200)	(0.149-0.197)	
	AUC (mg/L·h)	2.15	2.06	0.958
	(5 th and 95 th)	(1.89-2.41)	(1.67-2.32)	
Cycloguanil	C _{max} (mg/L)	0.0786	0.139	1.77
	(5 th and 95 th)	(0.0610-0.0934)	(0.0963-0.176)	
	AUC (mg/L·h)	1.02 (0.887-1.14)	1.78	1.75
	(5 th and 95 th)		(1.34-2.10)	

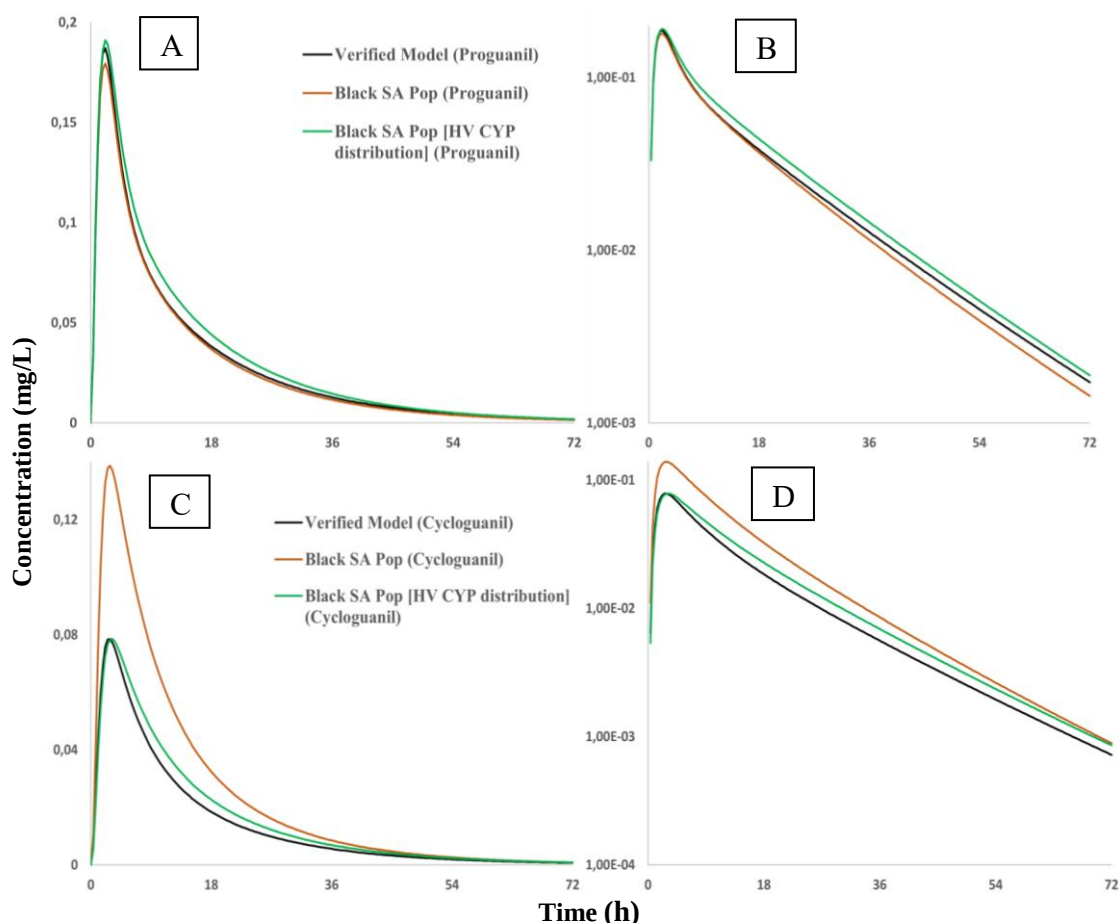


Figure 3.24: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil, for the prediction of the effect of ethnicity on proguanil/cycloguanil disposition

Minimal differences were seen in the predicted proguanil/cycloguanil exposure profiles between the two simulated populations. However, a notable increase in cycloguanil exposure was noticed during simulations run with the modified black South African population. Considering this, the representative black South African population file was edited so as to mirror the CYP enzyme genotype frequencies and abundances of the Healthy Volunteer population file. As seen in the above figure as well, this simulated profile was similar to the Healthy Volunteer population simulation. This illustrated that the difference between the two Health Volunteer population file and the black South African population file is an increased ability of the individuals represented in the black South African population file to metabolize proguanil and convert it to cycloguanil as a result in difference in CYP genotype frequencies and abundances. Subsequent simulations showed that the differences observed were because of changes in CYP abundances, with CYP genotype frequencies playing only a minor role.

The effect of CYP2C19 polymorphisms was investigated since this is known to be the main enzyme involved in proguanil clearance and cycloguanil formation. Moreover, there is

evidence of these polymorphisms having a clinical effect in the African population; with literature available to compare the model prediction to.²¹

To test the effect of CYP2C19 polymorphisms on proguanil metabolism and to observe whether the model could accurately predict proguanil and cycloguanil exposure for these polymorphisms, changes were made to the enzyme phenotype frequencies in the Simcyp simulator to create populations with phenotype frequencies seen in Table 3.28 below. The original proguanil/cycloguanil model, which was run with the default frequencies, was then run with these newly created populations.

Table 3.28: Enzyme frequencies in populations used to test effect of CYP2C19 genotype on proguanil/cycloguanil exposure

	Enzyme frequency in population				
	EM	PM	IM1	IM2	UM
Verified Model	0.424	0.023	0.246	0.26	0.047
1 PM CYP2C19	0	1	0	0	0

EM=Extensive metabolizers; PM=Poor metabolizers; IM1=Intermediate metabolizers; IM2=Intermediate metabolizers; UM=Ultra-rapid metabolizers

In Figure 3.25 below are the representative concentration-time profiles for this model application. In Table 3.29 below is summarized the PK parameters for these model runs.

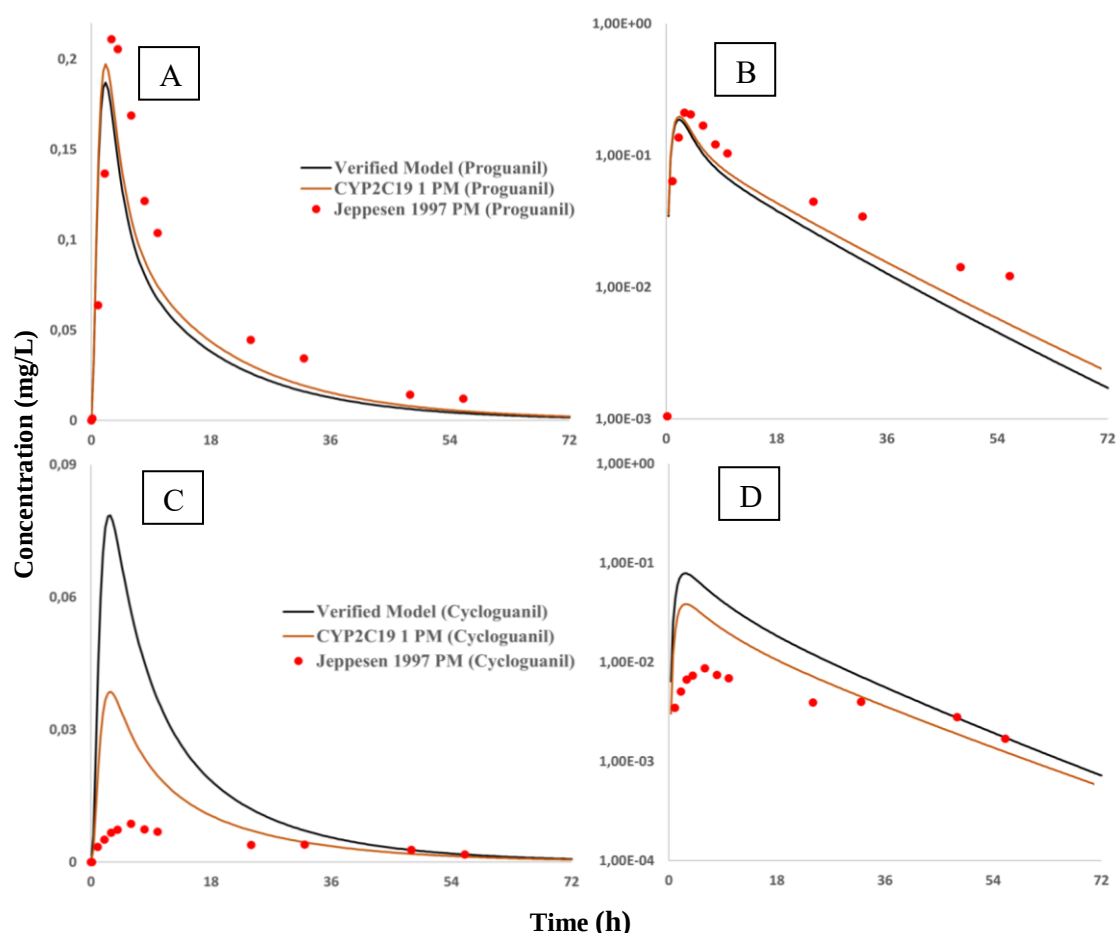


Figure 3.25: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil, for the prediction of the effect of proguanil administration to CYP2C19 poor metabolizers. Observed data, red dots, were extracted from Jeppesen et al. 1997

Table 3.29: Comparison between verified proguanil/cycloguanil model, the model run with populations with specific CYP2C19 polymorphisms and literature data extracted from Jeppesen et al. 1997.²¹

		Verified Model	1.0 PM CYP2C19	Literature*
Proguanil	C_{max} (mg/L)	0.187	0.197	0.211
	(5 th and 95 th)	(0.169-0.200)	(0.181-0.210)	
	AUC (mg/L·h)	2.15	2.41	
	(5 th and 95 th)	(1.89-2.41)	(2.20-2.62)	3.25
Cycloguanil	C_{max} (mg/L)	0.0786	0.0387	0.00868
	(5 th and 95 th)	(0.0610-0.0934)	(0.0290-0.0488)	
	AUC (mg/L·h)	1.02	0.559	
	(5 th and 95 th)	(0.887-1.14)	(0.434-0.668)	0.244

*Literature data determined through non-compartmental analysis of reported figures

While the model was able to predict proguanil exposure in a population of CYP2C19 PM with reasonable accuracy, it was not able to predict cycloguanil exposure with cycloguanil exposure being overpredicted. This is illustrated in Table 3.30 below. Precisely why this remains unclear but could be as a result of the other CYP enzymes involved in the conversion of proguanil to cycloguanil.

Table 3.30. Simulated vs observed interaction ratios comparing proguanil and cycloguanil PK parameters in CYP2C19 PM and wild type individuals. Observed data from Jeppesen et al 1997

		C _{max} R	AUCR
Observed	Proguanil	1.35	1.32
	Cycloguanil	0.121	0.185
Simulated	Proguanil	1.05	1.12
	Cycloguanil	0.492	0.548

Another attempt, with data obtained from a different literature study, was made to ascertain the verified model's ability to predict proguanil/cycloguanil exposure in CYP2C19 PM. The enzyme frequencies seen in Table 3.29 above were used with literature data obtained from Helsby, *et al.* 1990.¹² In Figure 3.26 below are the representative concentration-time profiles for this model application. In Table 3.31 below is summarized the PK parameters for these model runs.

Table 3.31: Comparison between verified proguanil/cycloguanil model, the model run with populations with specific CYP2C19 polymorphisms and literature data extracted from Helsby *et al.* 1990

		Verified Model	1.0 PM CYP2C19	Literature*
Proguanil	C _{max} (mg/L) (5 th and 95 th)	0.187 (0.169-0.200)	0.197 (0.181-0.210)	0.255
	AUC(mg/L·h) (5 th and 95 th)	2.15 (1.89-2.41)	2.41 (2.20-2.62)	6.61
Cycloguanil	C _{max} (mg/L) (5 th and 95 th)	0.0786 (0.0610-0.0934)	0.0387 (0.0290-0.0488)	0.0348
	AUC (mg/L·h) (5 th and 95 th)	1.02 (0.887-1.14)	0.559 (0.434-0.668)	1.84

*Literature data determined through non-compartmental analysis of reported figures

Table 3.32. Simulated vs observed interaction ratios comparing proguanil and cycloguanil PK parameters in CYP2C19 PM and wild type individuals. Observed data from Helsby *et al.* 1990

		C _{max} R	AUCR
Observed	Proguanil	0.917	1.70
	Cycloguanil	0.281	0.800
Simulated	Proguanil	1.05	1.12
	Cycloguanil	0.492	0.548

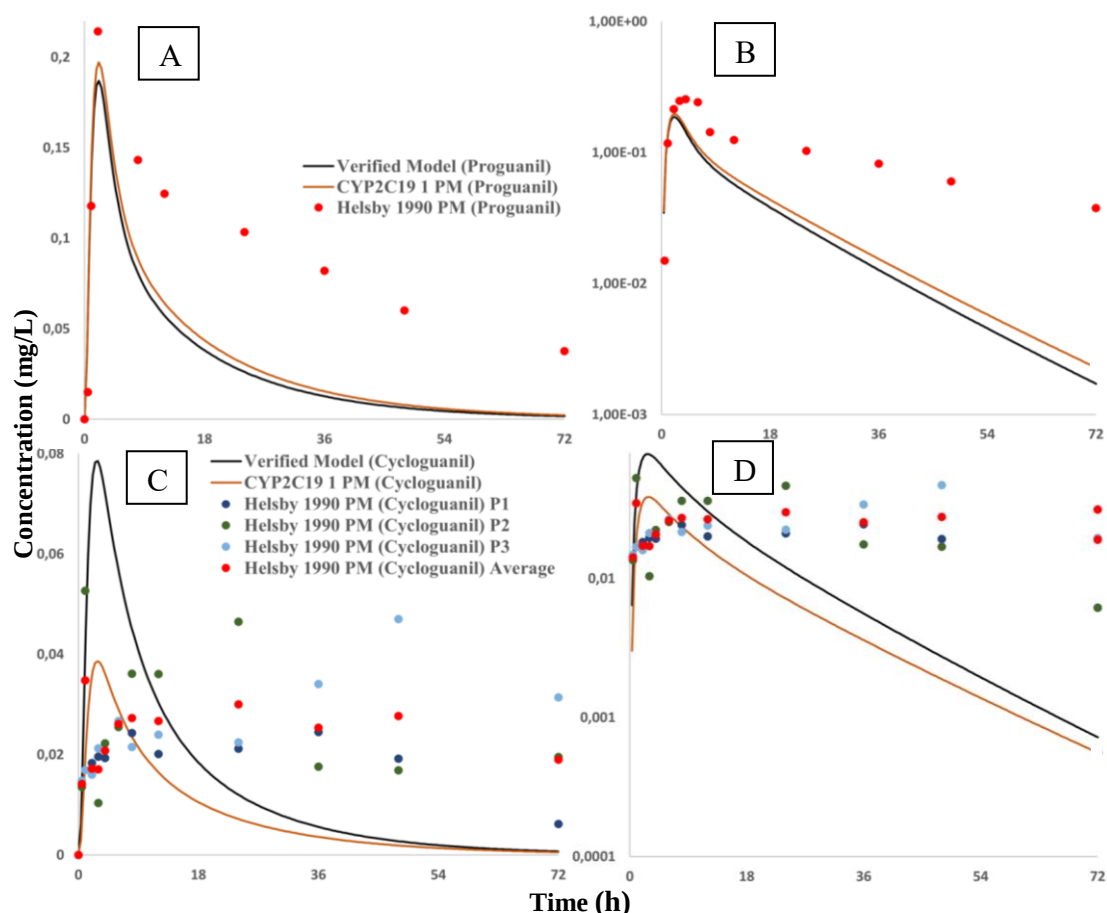


Figure 3.26: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil, for the prediction of the effect of proguanil administration to CYP2C19 poor metabolizers. Observed data, different coloured dots, were extracted from Helsby *et al.* 1990

The verified model was able to predict the $C_{\max}$ for both proguanil and cycloguanil reasonably. However, the elimination profiles of both drugs were not accurately predicted, with both proguanil and cycloguanil elimination being overpredicted. This is illustrated in Table 3.32. This is in contrast to the Jeppesen *et al.* study where the model could predict proguanil and cycloguanil clearance reasonably well, but could not accurately predict $C_{\max}$.²¹ In both cases, AUC could not be accurately predicted, albeit with one study being over, and the other study being underpredicted.

The effect of CYP1A2 polymorphisms on proguanil/cycloguanil exposure was also investigated. As previously mentioned, there is evidence of these polymorphisms having a clinical effect in the African population; a group wherein proguanil is administered due to the high prevalence of malaria on the continent.^{41,42} Moreover, the confirmation of the involvement of CYP1A2 in the *in vitro* metabolism of proguanil in the work previously described provides added motivation for this investigation.

To test the effect of CYP1A2 polymorphisms on proguanil/cycloguanil exposure, changes were made to the enzyme phenotype frequencies in the Simcyp simulator to create populations with phenotype frequencies seen in Table 3.33 below. The original proguanil/cycloguanil model, which was run with the default frequencies, was then run with these newly created populations.

Table 3.33: Enzyme frequencies in populations used to test effect of CYP1A2 genotype on proguanil/cycloguanil exposure

	Enzyme frequency in population				
	EM	PM	IM1	IM2	UM
Verified Model	1	0	0	0	0
0.5 PM CYP1A2	0.5	0.5	0	0	0
1 PM CYP1A2	0	1	0	0	0

EM=Extensive metabolizers; PM=Poor metabolizers; IM1=Intermediate metabolizers; IM2=Intermediate metabolizers; UM=Ultra-rapid metabolizers

In Figure 3.27 below are the representative concentration-time profiles for this model application. In Table 3.34 below is summarized the PK parameters for these model runs.

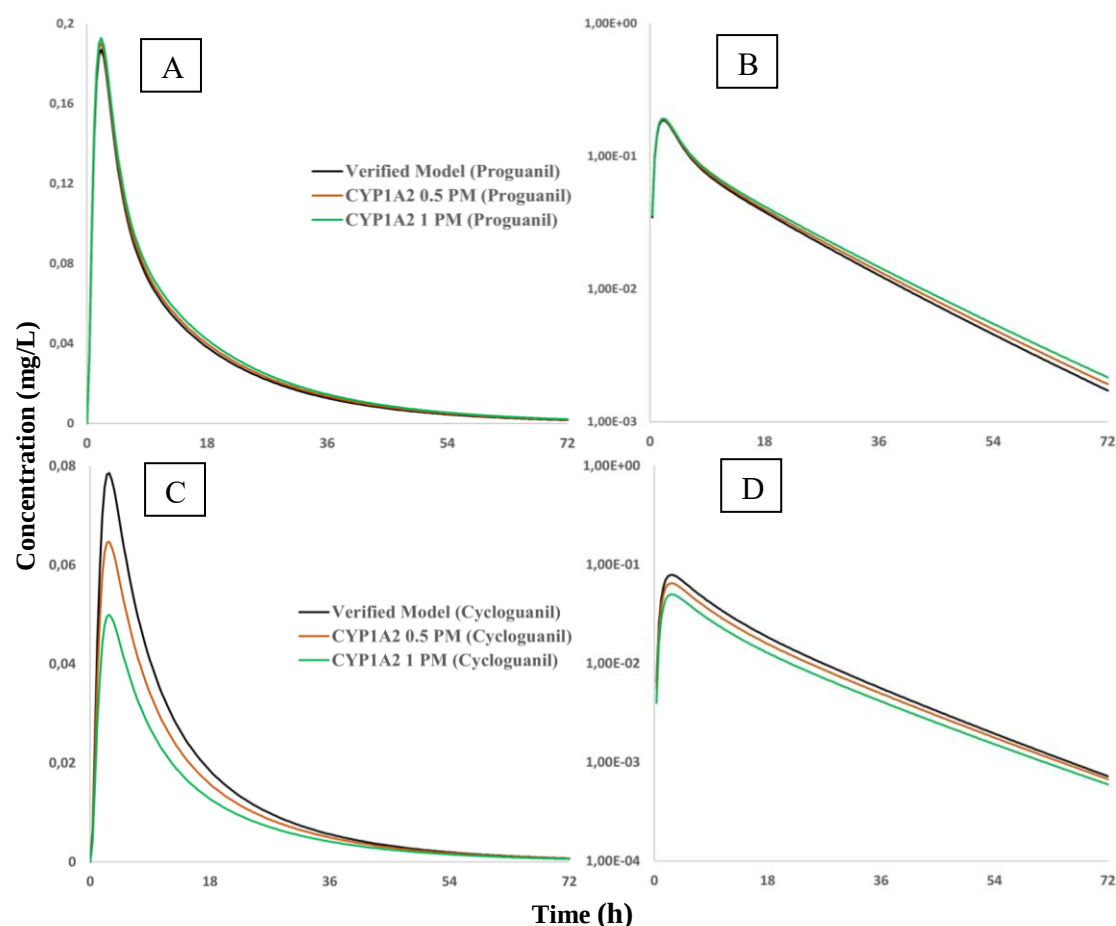


Figure 3.27: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil, for the investigation of the effect of CYP1A2 polymorphisms on proguanil and cycloguanil exposure.

Table 3.34: Comparison between verified proguanil/cycloguanil model and model run with populations with specific CYP1A2 polymorphisms

		Verified Model	0.5 PM CYP1A2	$C_{max}R/AUCR$	1.0 PM CYP1A2	$C_{max}R/AUCR$
Proguanil	C_{max} (mg/L)	0.187	0.190	1.02	0.193	1.03
	(5 th and 95 th)	(0.169-0.200)	(0.173-0.205)		(0.179-0.208)	
	AUC (mg/L·h)	2.15	2.24	1.04	2.33	1.08
	(5 th and 95 th)	(1.89-2.41)	(1.98-2.47)		(2.10-2.54)	
Cycloguanil	C_{max} (mg/L)	0.0786	0.0647	0.823	0.0499	0.635
	(5 th and 95 th)	(0.0610-0.0934)	(0.0514-0.0813)		(0.0346-0.0652)	
	AUC (mg/L·h)	1.02	0.865	0.848	0.690	0.676
	(5 th and 95 th)	(0.887-1.14)	(0.747-0.988)		(0.518-0.798)	

The simulated individuals with reduced CYP1A2 activity had a minor but notable increase in cycloguanil AUC and, by extension, cycloguanil exposure.

The effect of polymorphisms in the other enzymes involved in the conversion of proguanil to cycloguanil, namely CYP2D6 and CYP3A5, was also explored like how those in CYP2C19 and CYP1A2 were. This investigation is summarized in Figure 3.28.

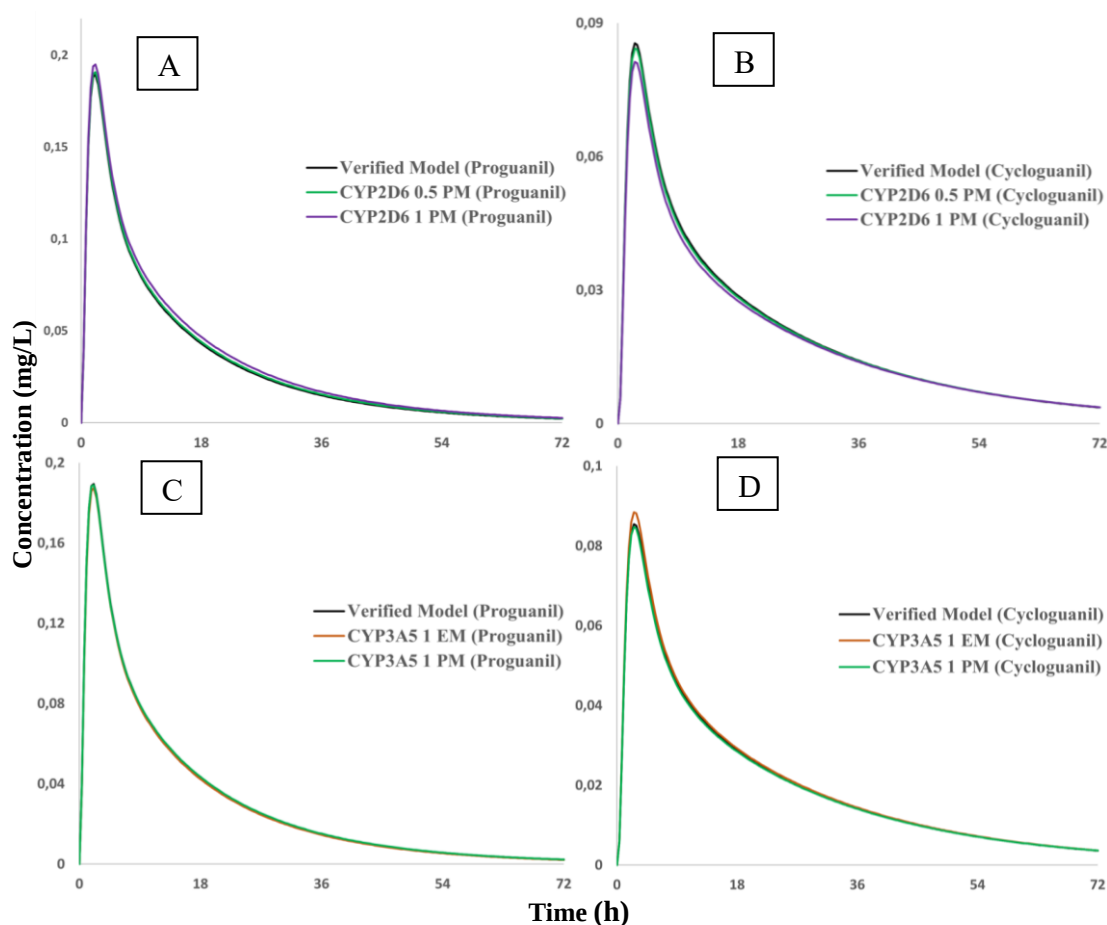


Figure 3.28: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A) and (C), and cycloguanil (B) and (D) (10 trials of 5 male and 5 female subjects, 20-65 yrs) for the investigation of the effect of CYP2D6 and CYP3A5 polymorphisms on proguanil and cycloguanil exposure.

As is illustrated in the above figures, little to no effect was observed. This likely underscores the importance of CYP2C19 to proguanil metabolism as well as the effect of the number of enzymes involved proguanil metabolism.

3.8.5.2 Model Application: DDI

The interaction between anti-HIV medications and antimalarials is of special importance in Africa, given the prevalence of malaria and HIV co-infection. As a result, an attempt has been made here to predict the consequences of this interaction with the use of the verified model, despite this interaction being investigated clinically by Van Luin *et al.*³⁶ since they did not report cycloguanil exposure data. Specifically, the effect of chronic efavirenz usage, as in the case of HIV-infected individuals, on proguanil/cycloguanil exposure was predicted. In this case, efavirenz was administered as a 600 mg dose once daily throughout the duration of the

simulation, with 200 mg proguanil administered on the 8th simulated day to mimic conditions in chronic efavirenz use.

In Table 3.35 below is summarized the PK parameters for these model runs. In Figure 3.29 below are the representative concentration-time profiles for this model application.

Table 3.35: Comparison of the effect of efavirenz, administered as a chronic medication, on proguanil/cycloguanil exposure

	Efavirenz Status	C _{max} (mg/L) (5 th and 95 th)	C _{max} R	AUC (mg/L·h) (5 th and 95 th)	AUCR
Proguanil	No Interaction	0.183 (0.168-0.193)	-	2.10 (1.90-2.29)	-
	Efavirenz	0.181 (0.166-0.190)	0.989	2.03 (1.84-2.21)	0.967
Cycloguanil	No Interaction	0.0776 (0.0602-0.0918)	-	1.00 (0.858-1.12)	-
	Efavirenz	0.0894 (0.0715-0.105)	1.15	1.14 (1.01-1.26)	1.14

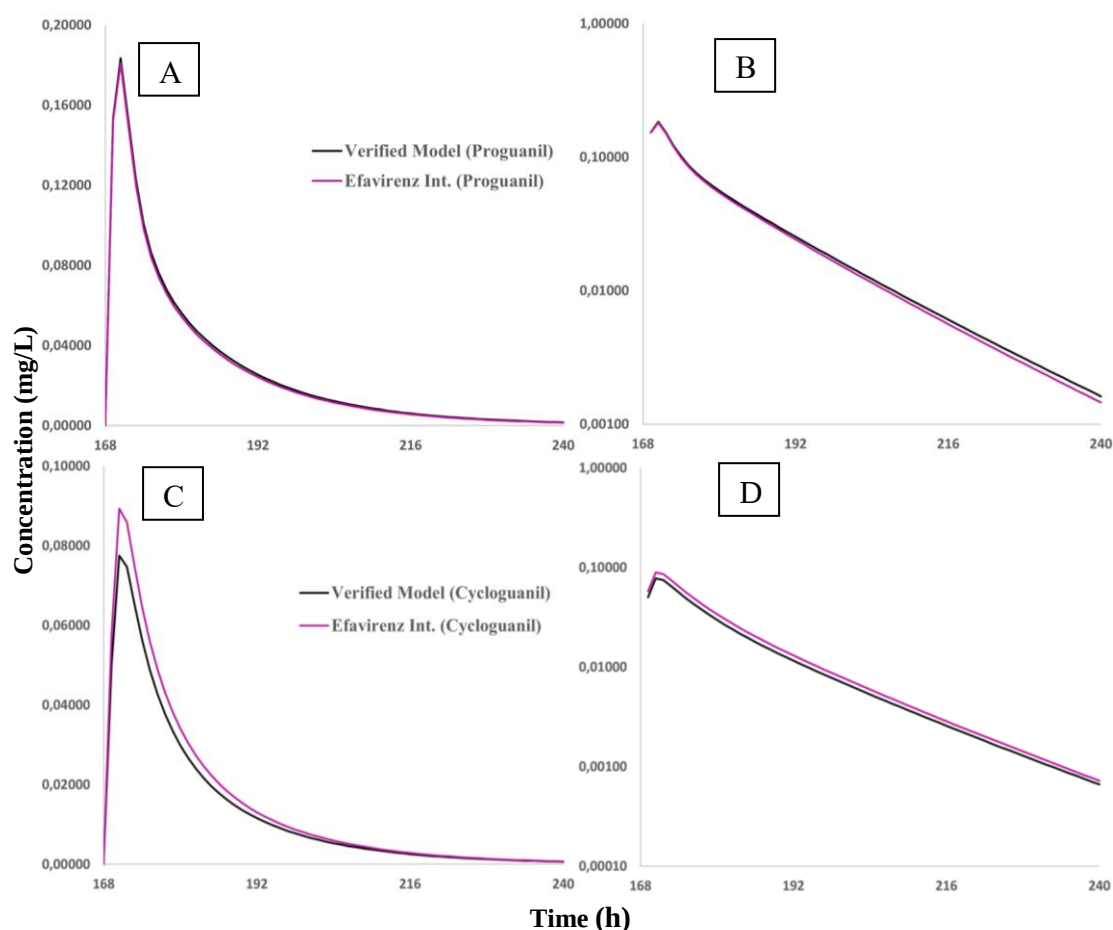


Figure 3.29: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil, for the investigation of the effect of efavirenz, administered as a chronic medication, on proguanil and cycloguanil exposure.

A minimal effect was observed between the two simulated runs. Overall, a decrease in proguanil exposure was accompanied by an increase in cycloguanil exposure, likely because of the induction effect efavirenz has on CYP2C19.

With the involvement of CYP2D6 in the *in vitro* metabolism of proguanil being uncovered here, potential DDIs between proguanil and an inhibitor of CYP2D6, namely quinidine, was explored using the verified model. The potential interaction between a CYP2D6 inhibitor as well as a CYP2C19 inhibitor, namely omeprazole, was also investigated to determine whether any increase in exposure was additive. This was done by running the verified model with quinidine as an inhibitor on its own, omeprazole as an inhibitor on its own, and then quinidine and omeprazole as inhibitors together. Given the relatively minor effect single doses of inhibitors were previously observed to have, only multiple dose effects were investigated.

In the case of these investigations, the relevant inhibitor/s were administered once daily for 7 consecutive days prior to proguanil administration and were then dosed simultaneously with

proguanil on the 8th simulated day, with inhibitor administration continuing for 3 days post proguanil administration. As with all model application investigations, proguanil was administered at a dose of 200 mg. The inhibitors quinidine and omeprazole were administered at dosages of 200 mg once daily and 20 mg twice daily respectively. In Table 3.36 below is summarized the PK parameters for these model runs. In Figure 3.30 below are the representative concentration-time profiles for this model application.

Table 3.36: Comparison of the effect of co-administration of multiple doses of CYP2D6 and CYP2C19 inhibitors with proguanil on proguanil/cycloguanil exposure

Inhibitor status		C _{max} (mg/L) (5 th and 95 th)	C _{max} R	AUC (mg/L·h) (5 th and 95 th)	AUCR
Proguanil	No Inhibitor	0.182 (0.168-0.193)	-	2.06 (1.91-2.24)	-
	Quinidine	0.185 (0.170-0.198)	1.02	2.17 (1.99-2.46)	1.05
	Omeprazole	0.186 (0.170-0.201)	1.02	2.18 (1.97-2.39)	1.06
	Both Inhibitors	0.187 (0.173-0.198)	1.03	2.18 (2.03-2.38)	1.06
Cycloguanil	No Inhibitor	0.0780 (0.0600-0.0921)	-	1.03 (0.851-1.15)	-
	Quinidine	0.0723 (0.0535-0.0877)	0.927	0.962 (0.766-1.08)	0.934
	Omeprazole	0.0615 (0.0452-0.0760)	0.788	0.815 (0.641-0.985)	0.791
	Both Inhibitors	0.0559 (0.0382-0.0710)	0.717	0.750 (0.569-0.882)	0.728

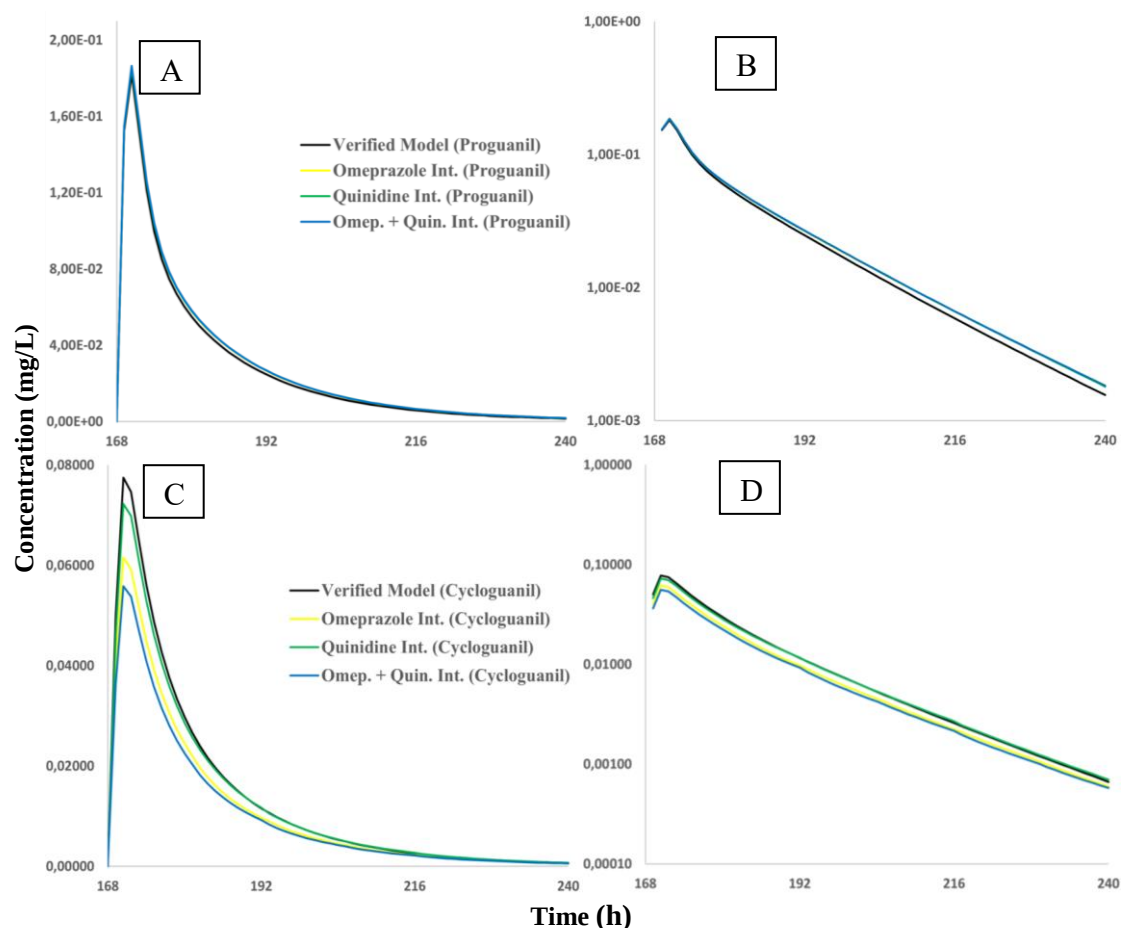


Figure 3.30: Simulated mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 5 male and 5 female subjects, 20-65 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil, for the investigation of the effect of CYP2C19 and CYP2D6 inhibitors, administered together or alone over multiple doses, on proguanil and cycloguanil PK.

A minor difference in the proguanil and cycloguanil PK profile was observed when quinidine was co-administered with proguanil in the simulated runs. The effect with co-administration of both omeprazole and quinidine was additive but, given the miniscule effect quinidine has on proguanil and cycloguanil exposure, this effect was minor.

3.9 Discussion

3.9.1 CYP1A2, CYP2D6 and CYP3A contribute to Proguanil metabolism

The proguanil fm,CYP investigation identified the involvement of a new CYP, namely CYP2D6, to the *in vitro* metabolism of proguanil which has not been previously reported. This investigation also confirmed the involvement of CYP1A2 which was previously identified by Coller *et al.*,²³ but could not be confirmed due to their lack of access to recombinantly expressed CYP1A2 enzymes.²³ It should be noted that the involvement of an additional CYP in the *in vitro* metabolism of proguanil was suggested by Coller *et al.*²³ as well. Their investigation confirmed the previously known involvement of CYP2C19 and CYP3A4, while uncovering the potential involvement of CYP1A2. Subsequent incubations where all three of these

enzymes were inhibited at once did not yield 100% inhibition of cycloguanil formation indicating the potential involvement of another CYP enzyme. The work here suggests that this enzyme is CYP2D6.

The fm,CYP investigation also identified CYP3A5 as a contributor to proguanil *in vitro* metabolism; which could have been inferred given the overlap of substrates between this enzyme and CYP3A4.

3.9.2 *In vitro* microsomal and rCYP metabolism underpredicts cycloguanil formation

Using the aforementioned *in vitro* rCYP metabolism data, a PBPK model was developed for both proguanil and cycloguanil as a metabolite formed because of proguanil elimination. The model was unable to correctly predict cycloguanil clinical PK when using only *in vitro* rCYP metabolism data to predict proguanil elimination. As a result a retrograde PBPK modelling approach, using the Simcyp RTT, was used to predict drug elimination. The resulting data showed a 91.8-fold, 25.6-fold, 2.86-fold, 23.4-fold and 64.2-fold difference between the clearance measured using rCYPs and clearance calculated using the RTT for CYP1A2, CYP2C19, CYP2D6, CYP3A4 and CYP3A5, respectively.

As such, this difference excluded the possibility of using a pure bottom-up approach to proguanil modelling. Thus, with the use of the RTT, a middle-out approach was used to develop the model which was later verified with the use of a variety of literature sources.

3.9.2.1 PBPK model of proguanil/cycloguanil shows difference in cycloguanil exposure between a black African population and a Caucasian population

Upon completion of the verification process, the proguanil/cycloguanil model was then applied to scenarios of interest. A 1.74-fold difference in cycloguanil exposure was observed between the verified model and the model run with a representative black South African population; indicating that a key difference in the Simcyp Healthy Volunteers population file and the modified black South African population file is potentially an increase in clearance because of increased CYP expression or abundances in the latter file. This was confirmed by editing the representative black South African population file to mirror the CYP enzyme distribution of the Healthy Volunteer population file and simulating proguanil/cycloguanil PK with this altered population file. This simulation showed that it was indeed an increase in CYP mediated metabolism of proguanil that resulted in the simulated increase in cycloguanil exposure, with subsequent modelling showing that differences in CYP abundances had the most prominent effect. However, since the publication wherefrom the black South African population file was

adapted does not make mention of changes in CYP abundance to the model, it is difficult to explain the source of the difference in abundance between the two population files.⁴⁰ Given that the publication wherefrom the black South African population file was adapted made use of an older version of Simcyp and modified the Healthy Volunteer population file from that older version to create the black South African population file, it is possible that the differences in abundances are updates made by Simcyp between the two versions. This will need to be explored further.

However, it has been shown that a Nigerian population has an increased ability to clear proguanil as compared to the Caucasian populations used in model verification here; which further buttresses this finding.³⁸ This has significant clinical implications given the prevalence of malaria on the African continent. The work done here, as well as that observed clinically, suggests that a different dose of proguanil would be needed for Africans as compared to Caucasians when proguanil is used to treat malaria. Of course, this would need to be studied and compared clinically.

3.9.2.2 CYP polymorphisms have an impact on cycloguanil but not proguanil exposure

Model application also showed that proguanil exposure was minimally affected by the various conditions tested. Specifically, polymorphisms conveying reduced activity to the variety of enzymes uncovered and confirmed to be involved in proguanil metabolism here, were shown to have little to no impact on proguanil PK. Also, despite its effect of cycloguanil, when the model was applied to a representative black South African population, only minor changes were seen in overall proguanil exposure. The only minor deviation from this trend was seen when CYP2C19 was involved in these interactions.

Specifically, as previously stated, the model could predict a notable decrease in cycloguanil exposure when proguanil was administered to CYP2C19 poor metabolizers. A 0.546-fold difference between the verified model and the model run with a population of CYP2C19 poor metabolizers was predicted. This is significantly higher than the 0.185 ratio observed in the study done by Jeppesen *et al.*,²¹ and is notably lower than the 0.8-fold difference observed in the study done by Helsby *et al.* 1990.¹² This indicates that there exists significant inter-individual variability in proguanil/cycloguanil PK not only in wild type individuals, but also in individuals that carry CYP2C19 poor metabolizer genes. This could be because of the way poor metabolizers are classified. As an example, a study conducted by Bolaji *et al.* used a measurement of proguanil/cycloguanil ratio in urine as an indicator of poor metabolizer

status.⁴³ Using an antimode value for this proguanil/cycloguanil ratio of 10, established in a study done by Ward *et al.*,⁷ they were able to group the participants in their study into extensive metabolizers, those with a proguanil/cycloguanil ratio below 10, and poor metabolizers, those with a proguanil/cycloguanil ratio above 10. However, the range of proguanil/cycloguanil ratios of the poor metabolizer participants was from 10.2 to 39.6. Such a large variability in what constitutes a poor metabolizer would be extremely difficult for any model to correctly predict.

Moreover, the number of CYPs involved in the metabolism of proguanil provides difficulties as well since CYP2C19 metabolizer status is not the sole driver of cycloguanil formation. The relative activity of other CYPs involved in the metabolism of proguanil, something that is subject to high interindividual variability, as well as the metabolizer status of these other enzymes would need to be accounted for to accurately predict cycloguanil exposure in CYP2C19 PM. This data was not available in the tested studies.

Additionally, it has previously been shown that both proguanil and cycloguanil are substrates of the organic cation transporter (OCT); an active cellular transporter.⁴⁴ It has also been shown that OCT1 deficiency, as a result of genetic polymorphisms in the OCT1 gene that reduce the ability of OCT1 to transport substrates, affects hepatocellular concentrations as well as the PK of cycloguanil in a clinical study.⁴⁵ This provides an added layer of variability to understanding plasma levels of cycloguanil as a result of proguanil administration since, for example, a CYP2C19 extensive metabolizer could have cycloguanil plasma concentrations similar to that of a poor metabolizer if that individual has a genetic polymorphism that results in OCT1 deficiency.

Interestingly, a small but notable difference in cycloguanil exposure was noted when proguanil was administered to CYP1A2 poor metabolizers in the model. A 0.674-fold fold difference between the verified model and the model run with a population of CYP1A2 poor metabolizers was predicted, which is quite like that predicted during the aforementioned investigation done for CYP2C19. Given that the model could predict this shift in CYP2C19 poor metabolizers, albeit not always the magnitude of the shift, our model suggests the potential for a similar shift to occur in CYP1A2 poor metabolizers; which would need to be confirmed clinically.

However, other than these examples, cycloguanil exposure remained relatively unchanged during model application with no significant trends observed. Although, this should be taken in the context of the model overpredicting cycloguanil exposure when these types of interaction

studies have been done; with this overprediction seen to be roughly 1.75-fold during model verification. In addition, why the model predicted a notable difference in cycloguanil exposure in CYP1A2 poor metabolizers but not in poor metabolizers of CYP2D6 and CYP3A5 is likely because of a higher proportion of proguanil conversion to cycloguanil being attributed to CYP1A2 than the other two enzymes. As discussed previously, when proguanil clearance was modelled using the RTT, 28.8% of said clearance was attributed to CYP1A2, while only 12.8% and 4.1% was attributed to CYP2D6 and CYP3A5, respectively. Researchers have previously uncovered the involvement of CYP1A2 in the metabolism of proguanil, especially of its conversion to cycloguanil, but have downplayed its importance for a variety of reasons in favour of highlighting the importance of CYP2C19.^{19,21,23} While CYP2C19 is certainly the most important enzyme involved in proguanil metabolism, the model, buttressed by the *in vitro* investigation done here, shows that CYP1A2 is of importance as well.

3.9.2.3 DDIs do not significantly impact proguanil/cycloguanil exposure

Neither efavirenz as an inducer or quinidine as an inhibitor, of CYP2C19 and CYP2D6 respectively, had any significant effect on proguanil exposure. Co-administration of proguanil with the CYP2C19 inhibitor omeprazole in the verified model did lead to a noticeable increase in proguanil exposure. However, this change was minor and would not necessarily be of clinical significance for proguanil; as has been shown in the case of CYP2C19 poor metabolizers in literature. Moreover, even though a minor increase in proguanil exposure was noted when quinidine and omeprazole were co-administered, this increase was not of great significance either, further underlining the lack of effect that co-administration of quinidine has on proguanil PK.

Complicating the matter of how important each CYP enzyme is in the metabolism of proguanil is the number of enzymes involved in the conversion of proguanil to cycloguanil. The *fm*, CYP investigation, literature, as well as model outputs clearly show the importance of CYP2C19 to proguanil metabolism. However, the involvement of the four other CYP enzymes makes it difficult to elucidate the importance of changes in their genotype or the effect potential inducers or inhibitors may have on proguanil or cycloguanil exposure. For example, CYP2D6 may play a significant role in the clearance of proguanil or its conversion to cycloguanil in an individual which is wild type for this enzyme or is not simultaneously taking a medication that inhibits it. However, in the case of someone who is a poor metabolizer of this enzyme, such as carriers of the CYP2D6*4 gene, or who is taking an inhibitor, any of the other four CYP enzyme involved

in the metabolism of proguanil could metabolize the drug instead, thereby negating this potential effect.

On the other hand, this does point to a potential strategy in future drug development. Given the potential negative effects DDIs have on drug regulatory approval, designing a drug that is metabolized in relatively equal amounts by several enzymes could circumvent this issue. This is of course incredibly difficult to do, especially in early drug development where efficacy and tolerability are paramount. But, if DDIs do become an issue in regulatory approval for a potential new drug, shifting its metabolism away from any one enzyme could provide a way to avoid the said DDIs.

3.9.2.4 Model limitations: Proguanil/Cycloguanil

The proguanil portion of the model was able to accurately predict proguanil exposure over a variety of different tested conditions. These included single doses of 100 or 200 mg, as well as multiple doses and interactions with a variety of inhibitors of proguanil metabolism and a potential inducer in efavirenz. It was also able to predict the increase in proguanil exposure because of administration to individuals who are CYP2C19 poor metabolizers. The proguanil model did show a resistance to the effect of inhibitors or inducers of CYP enzymes, or when simulations were done with individuals who had CYP polymorphisms different from the wild-type. Proguanil exposure has been shown to be stable in literature, but this resistance in the model was shown to lead to a slight underprediction in proguanil exposure when administered to individuals who are CYP2C19 poor metabolizers. Despite this, proguanil exposure PK was well predicted in several simulations. Thus, the proguanil portion of the model was considered verified.

In contrast, the cycloguanil portion of the model had some difficulty in predicting cycloguanil exposure outside of single dose proguanil administration. Generally, cycloguanil exposure was overpredicted when multiple doses of proguanil were administered or proguanil was administered with a potential inhibitor of proguanil metabolism. The model also had difficulty in predicting the fate of cycloguanil in individuals who are CYP2C19 poor metabolizers for the same reason, though it could predict one of the two studies used better than the other; indicating inter-individual variability between the studied populations.

A potential reason for these relatively slight but notable overpredictions of cycloguanil exposure, as well as the resistance to change observed in the proguanil model, could be as a result of not fully taking into account the conversion of proguanil to CPB. Given the previously

mentioned lack of access to a chemical standard for this compound, determining the *in vitro* metabolic characteristics of its formation because of proguanil metabolism proved difficult. Attempts at measuring its formation during the *in vitro* metabolism investigation undertaken here were made with the use of metabolite identification software that was able to tentatively identify it in some of the incubations done. However, it was formed in very small quantities and not under any of the established *in vitro* conditions used to study proguanil *in vitro* metabolism. Only being able to measure this metabolite in small quantities during HLM incubations, instead of having larger quantities of the compound in the form of a chemical standard, precluded attempts to optimize the developed LC-MS/MS methods to better quantify it. Furthermore, a lack of literature data on its physicochemical and PK properties would also have hindered attempts to accurately predicting its formation using a PBPK model.

However, despite the cycloguanil portion of the model having some difficulty in accurately predicting the magnitude of cycloguanil exposure under these conditions, the model predictions were still reasonably accurate, predicting most studies within 2-fold, and was able to predict the general trend observed clinically. Namely, the model could predict that a reduction in cycloguanil exposure would take place when proguanil was administered with an inhibitor of CYP2C19 or to CYP2C19 poor metabolizers. This both underlines the importance of CYP2C19 in the conversion of proguanil to cycloguanil and that the model could predict this importance; with a general reduction in the CYP2C19 clearance of proguanil leading to a reduction in the formation of cycloguanil. As a result, the cycloguanil portion of the model still has value in predicting potential trends in cycloguanil formation when proguanil is administered with potential inhibitors or inducers of proguanil metabolism or to individuals that carry a specific genotype outside of the wild type for a particular CYP enzyme involved in proguanil metabolism. This carries clinical significance given proguanil's status as a prodrug, although the actual magnitude of exposure will potentially be slightly overpredicted.

3.10 Conclusions: Chapter 3

In this chapter, data from *in vitro* metabolism experiments were used to inform PBPK modelling of proguanil and cycloguanil. Proguanil and cycloguanil PK is reasonably well described *in vivo*, while *in vitro* metabolism data found in literature has previously identified several CYP enzymes involved in proguanil metabolism. Despite this wealth of information, gaps in the data on proguanil did exist. While the involvement of CYP1A2 in proguanil metabolism was previously uncovered, it was not confirmed, with the same study that uncovered the potential involvement of CYP1A2 also suggesting that another CYP enzyme

played a role too. The work done here not only confirmed the involvement of CYP1A2 but also uncovered the other enzyme suggested to be involved in proguanil metabolism; namely CYP2D6. The CYP phenotyping studies done here also uncovered the involvement of CYP3A5, but this could be inferred from the known involvement of CYP3A4 since these enzymes overlap in the substrates they are able to metabolize.

Overall, cycloguanil formation in HLM was found to be a high K_M reaction, within the range of previously published data. The *in vitro* metabolism data was then used in a PBPK model of proguanil, and cycloguanil as its metabolite, built in Simcyp. The proguanil and cycloguanil models were then verified using clinical data. However, the cycloguanil portion of the model had a propensity to slightly overpredict cycloguanil exposure when multiple doses of proguanil were administered or when proguanil was administered with an inhibitor of its metabolism. Generally, the model overpredicted cycloguanil exposure in multi dose and DDI studies by roughly 1.75-fold, while it was found during model application that this overprediction was roughly 2-fold in studies in CYP2C19 poor metabolizers for some PK parameters. Despite the model having some difficulty in predicting the magnitude of cycloguanil exposure, its predictions were still reasonably accurate and it was able to predict the general trends in observed during these studies; namely, if a reduction in cycloguanil exposure was observed in literature, the model was able to predict that a reduction would take place. As a result of this, despite the slight overpredictions, the cycloguanil portion of the model was considered verified and has value in predicting the trends, if not necessarily the magnitude of cycloguanil exposure.

Upon verification, the model was used to investigate several scenarios of interest. These included ethnic sensitivity testing by means of a representative black South African population as well as populations with different phenotypes of the CYP enzymes confirmed here to be involved the conversion of proguanil to cycloguanil. The potential effect of co-administration of inhibitors or inducers of these same CYP enzymes with proguanil was also investigated. Despite the wide range of tested conditions, it was found that they had a minimal to negligible effect on proguanil PK. This mirrors the general trend seen in literature.

This trend was also seen in cycloguanil exposure, with only two exceptions. Both the administration of proguanil to CYP2C19 poor metabolizers and a representative black South African population showed notable differences from the administration to Healthy Volunteers in the model. However, the differences the model predicted were unlikely to be of clinical significance. This finding is complicated by the models overprediction of cycloguanil

exposure. This is seen most clearly in the case of proguanil administration to CYP2C19 poor metabolizers. The difference the model predicted was not of minor magnitude, but it is known from literature that these poor metabolizers reach sub-therapeutic levels of cycloguanil; which is most certainly of clinical significance.

This overprediction could be because of the conversion of proguanil to CPB not being fully taken into account. Looking forward, efforts could be made to acquire or synthesize a chemical standard for this compound after which its physicochemical properties and metabolic profile could be determined to better incorporate it into the model; thereby improving cycloguanil prediction. Moreover, the effect of active transporters, namely OCT, was not included in the model. Since this could be a potential source of clinically observed variability seen in CYP2C19 poor metabolizers' ability to convert proguanil to cycloguanil, incorporating this knowledge into the model will help predictions of cycloguanil exposure when the OCT1 genotype of a studied population is known.

Overall, the number of enzymes confirmed to be involved in the metabolism of proguanil likely means that changes in their genotype or co-administration of a drug that inhibits or induces them will have little effect. This is except for CYP2C19, which was shown in literature and reaffirmed here to be the most important enzyme involved in proguanil metabolism.

3.11 References

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Chapter 4 Ritonavir

4.1 Chapter 4 Overview

In this chapter, the *in vitro* microsomal metabolism of ritonavir is evaluated. A ritonavir PBPK model is then used to investigate gaps in knowledge of ritonavir DDIs with ritonavir as the perpetrator drug.

4.2 General Introduction: Ritonavir

Ritonavir is a symmetry-based HIV protease inhibitor, discovered and developed by Abbot Laboratories.¹ It was approved for the treatment of HIV by the FDA in 1996. However, the 600 mg twice daily dosage necessary for the antiviral activity of ritonavir to take full effect was not fully palatable, with many patients incapable of tolerating this dose for an extended period of time due to the gastrointestinal side effects it induced.² As a result, the primary indication of ritonavir currently is as a pharmacologic enhancer of other anti-retroviral drugs due to its ability to inhibit CYP3A4, a key enzyme involved in the first pass metabolism of HIV protease inhibitors.³ For the treatment of HIV, it is mainly used in conjunction with lopinavir, with the lopinavir/ritonavir (LPV/r) combination, first approved by the FDA in 2000, showing improved immune outcomes and substantial viral suppression in patients.⁴

In addition to its use in HIV treatment, ritonavir, in combination with ombitasvir, paritaprevir and dasabuvir, was approved by the FDA for the treatment of hepatitis C virus genotype 1 in 2014.⁵ Owing to its mechanism of action, along with other protease inhibitors, it is also under investigation for its use in treating certain forms of cancer.⁶ A combination of nirmatrelvir and ritonavir as an enhancer, under the trade name paxlovid, has gained FDA approval for the treatment of COVID-19.⁷ Furthermore, evidence suggests that LPV/r can be used to treat COVID-19 as well, but large scale clinical trials are required to attain more robust results.⁸

LPV/r is administered as a tablet, a soft gel capsule, or as an oral solution, typically in a regimen of 400 mg lopinavir/100 mg ritonavir orally twice daily.⁴ Ritonavir is well absorbed with its oral bioavailability ranging from 60-80%.⁹ Its exposure as measured by area under the curve (AUC) is roughly 15% higher when administered with food, while a decrease of 7% is observed for the oral solution.⁹ Ritonavir is highly protein bound, greater than 98%, to both α^1 -acid glycoprotein and albumin.⁹

Ritonavir is rapidly and extensively metabolized both *in vitro* and *in vivo*.^{9–11} It is metabolized to a range of metabolites *in vitro*, with 3 major metabolites formed in HLMs; namely a decarbamoylation product (R1), a C-hydroxylation product (R2), and a N-dealkylation product (R3).^{10–12} R2 was the most abundantly formed metabolite in HLMs, accounting for more than 50% of metabolite formation.¹⁰ The chemical structures of ritonavir and these three metabolites are illustrated in Figure 4.1 below.

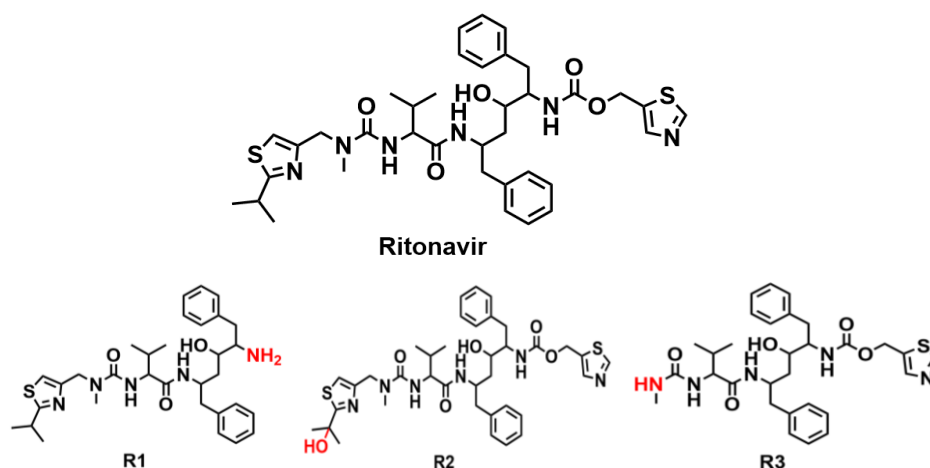


Figure 4.1: Chemical structures of ritonavir and its metabolites

Overall, ritonavir metabolism is chiefly mediated by CYP3A, with CYP2D6 also playing a lesser, but yet prominent, role.^{10,11} With reference to the aforementioned metabolites, R1 and R3 are likely formed solely by CYP3A, whereas the formation of R2 is likely carried out by both CYP3A and CYP2D6.¹⁰ In the study conducted by Kumar *et al.*, discrepancies arose when comparing data gathered in rCYPs and HLMs, chiefly an overestimation of K_M in the latter. They theorized that this overestimation could be as a result of minor contributions from other CYP isoforms which they were not able to investigate.¹⁰ Additionally, despite the ketoconazole and quinidine inhibitor concentrations being well above K_i values for CYP3A and CYP2D6 at the time, metabolite formation could not be fully suppressed. This was likely due to the high affinity of ritonavir for CYP3A, but could also point to the involvement of other CYP isoforms. Koudriakova *et al.*, were able to measure the enzyme kinetic parameters of ritonavir in microsomes containing expressed human CYP2D6, CYP3A4, and CYP3A5.¹¹

Below is a summary of the process followed to develop the method for ritonavir kinetic determinations.

4.3 Chromatographic development and analysis

Ritonavir was infused on an AB Sciex 4500Q LC-MS/MS in electrospray positive mode. The spectra of the protonated molecular ions as well as their fragments are illustrated in Figure 4.2 below.

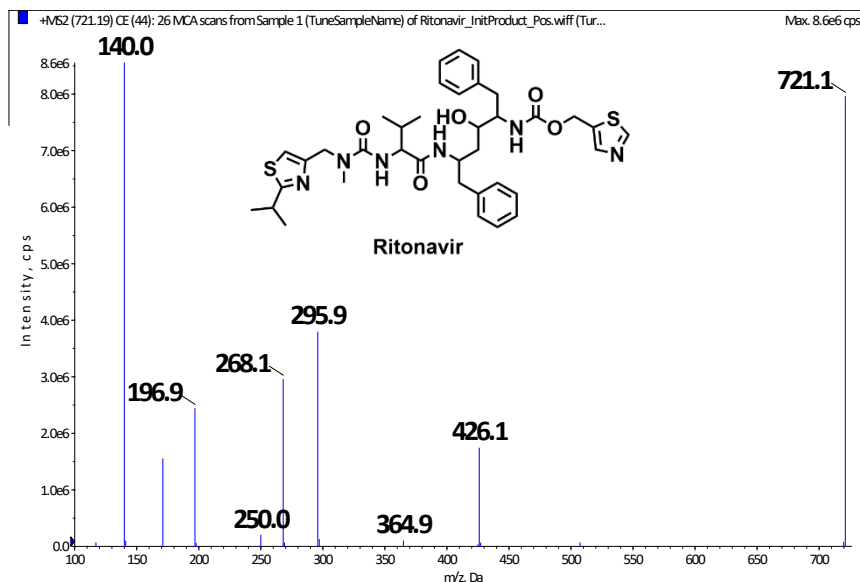


Figure 4.2: Initial product ion mass spectrum of ritonavir in positive mode showing the [M+H]⁺ ion at m/z 721 and the product ion at 197

The product ions shown here were selected during chromatographic development as they showed the best sensitivity and reproducible chromatography and have also been reported for ritonavir LC-MS/MS analysis^{10,12,13}. The final analytical method is summarized in Table 4.1 with the chromatography illustrated in Figure 4.3.

Table 4.1: Summary of the analytical conditions for the developed method

Ritonavir	
Analytical Conditions	
Q1 [M+H]	721
Q3 [M+H]	197
Internal Standard	Carbamazepine
Column (Dimensions)	Gemini NX-C18 (2.0 x 50 mm; 5 µm)
Injection volume (µL)	5
Gradient Program %B (min)	10(0) → 95(2.5) → 95(3.5) → 10(3.6) → 10(5)
Flow rate (µL/min) ^a	1000

^aFlow split 1:2 into MS

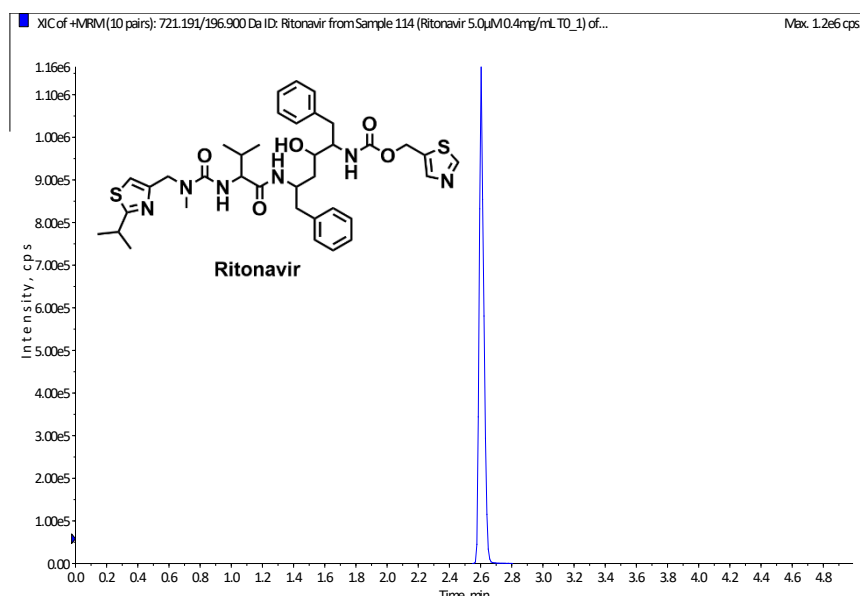


Figure 4.3: Representative chromatogram for ritonavir

4.3.1 Flow injection analyses

Upon obtaining optimal chromatographic conditions, an on-column flow injection analysis (FIA) was carried out to optimise the MS source parameters, such as curtain gas, ion spray voltage, and source temperature, as well as compound parameters to ensure optimal response and sensitivity from the MS for this reaction. The resulting optimized MS/MS parameters are summarized in Table 4.2 below.

Table 4.2: Summary of the optimized MS conditions for mefloquine and carboxymefloquine

	Ritonavir
Q1 (m/z)	719
Q3 (m/z)	197
Source Temp. °C	550
Curtain Gas	40
Ion Source Gas 1	30
Ion Source Gas 2	30
Ion Spray Voltage	4500
CE (eV)	59
EP	10
DP	41

This method was partially validated to ensure reproducibility. The results of this partial validation are seen in the appendix.

4.3.2 Sample extraction

Protein precipitation was chosen as the extraction method for the methods developed here; as described in Chapter 2.

4.3.3 Development of biological method

Unlike mefloquine and proguanil, ritonavir is rapidly metabolized to a number of metabolites in microsomes. This would have complicated V_{max} and K_M determination since each metabolite would have required monitoring and quantitation. Substrate depletion was therefore the preferred method for kinetics determination.

Reaction linearity was performed at two substrate concentrations, in duplicate. The range of conditions evaluated is summarised in Table 4.3 below.

Table 4.3: Incubation conditions used to test linearity of ritonavir kinetics

Substrate (Conc. μM)	Microsomal Protein (mg/ml)	Duration (min)
Ritonavir (0.5,5)	0.2; 0.4; 0.8; 1.2	60

The results of this linearity determination is summarized in Figure 4.4 below.

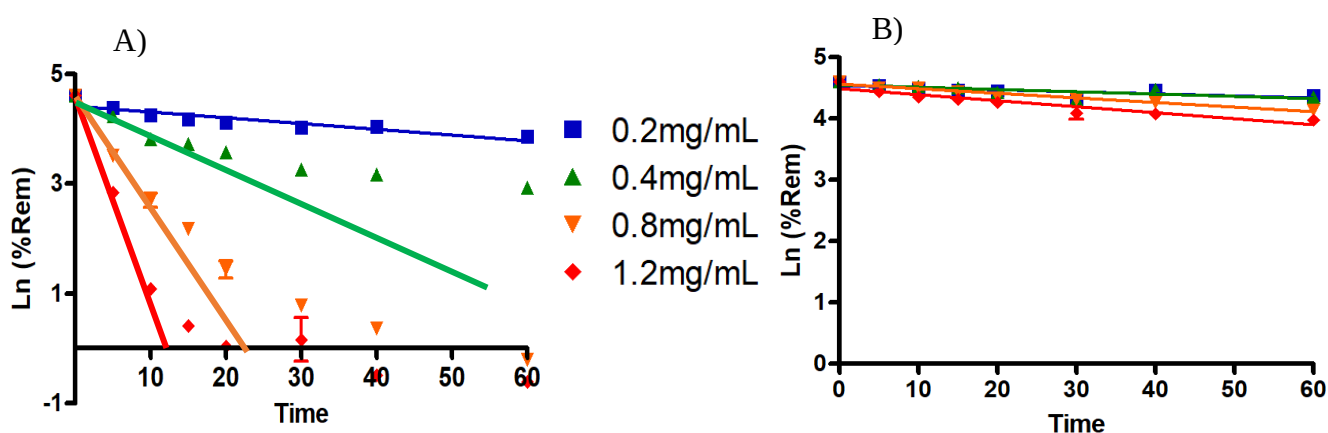


Figure 4.4: Summary of linearity determination data. A) 0.5 μM Substrate concentration. B) 5 μM Substrate concentration

The measured microsomal metabolism of ritonavir was rapid at 0.5 μM with moderate metabolism seen at 5 μM . This data is summarised in Table 4.4. As illustrated in Figure 4.4, for all protein concentrations tested, substrate depletion at the 5 μM substrate concentration followed first-order kinetics for the duration of the incubation. In contrast to this, substrate depletion at the 0.5 μM substrate concentration only followed first-order kinetics for the duration of the incubation at the 0.2 mg/mL protein concentration.

Table 4.4: Summary of percentage remaining data after ritonavir linearity determination

	%Remaining after 60min	
Protein Concentration	0,5 μM	5 μM
0,2mg/mL	47	79
0,4mg/mL	18	77
0,8mg/mL	<5	62
1,2mg/mL	<5	52

Further incubations were performed to elucidate which incubation conditions would better describe ritonavir kinetics using the substrate depletion approach. The final conditions used to measure ritonavir kinetics is summarised in Table 4.5 below.

Table 4.5: Final incubation parameters for the determination of ritonavir kinetics

Parameter	Conditions tested
Protein Concentration (mg/mL)	0.02
Time (min)	0; 5; 10; 20; 30
Substrate Concentration Range (nM)	12.5-5000

The results of the log transformed linearity determination is summarized in Figure 4.5 below.

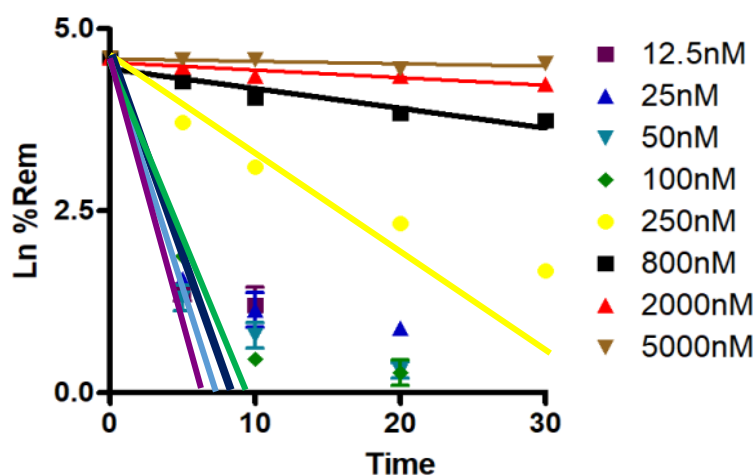


Figure 4.5: Summary of linearity determination data for ritonavir.

The data in Figure 4.5 follows the general trend of the incubations done up to this point with first-order kinetics followed for the duration of the incubation only at the higher substrate concentrations. Subsequent to this, ritonavir enzyme kinetics were calculated based on the available data using the work reported by Nath and Atkins,¹⁴ who mathematically validated Obach and Reed-Hagen's substrate depletion approach.¹⁵ After transformation of substrate depletion data, data points that were not in the linear portion of the log transformation graph, and thus were not part of a first-order reaction, were excluded. The remaining data points were then used to calculate the k_{dep} , the slope of the substrate depletion vs time plot, at each substrate concentration. These k_{dep} values were subsequently plotted against substrate concentration to yield the sigmoidal curve seen in Figure 4.6 below.

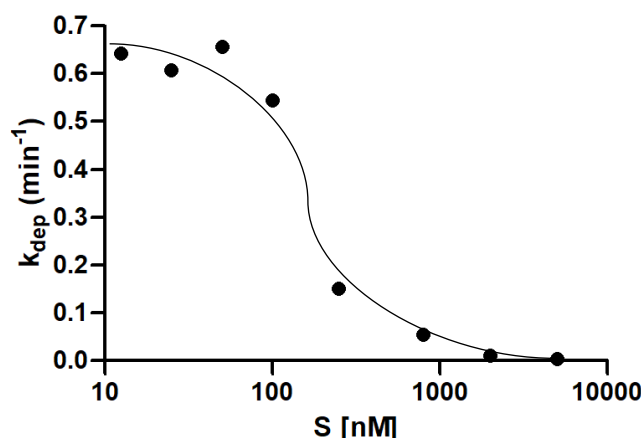


Figure 4.6: Plot of depletion rate constant versus time for ritonavir microsomal metabolism

A nonlinear regression (curve fit) analysis was done using the one site binding (hyperbola) function on GraphPad Prism 4.0 software to analyse the data set. The equation used to do this analysis was the one below, proposed by Obach and Reed-Hagen:

$$k_{dep} = k_{dep([S]=0)} \cdot \left(1 - \frac{[S]}{[S] + K_m}\right) \quad (11)$$

where K_M is the Michaelis constant, k_{dep} is the apparent first-order rate constant of substrate depletion, $[S]$ is the substrate concentration and $k_{dep([S]=0)}$ is the theoretical k_{dep} at infinitesimally low substrate concentrations. In our case, the k_{dep} measured at the lowest substrate concentration was used to represent $k_{dep([S]=0)}$. An apparent K_M for this reaction was calculated using this analysis while V_{max} was calculated using an equation derived from first principles by Nath and Atkins:

$$k_{dep([S] \rightarrow 0)} = \frac{V_{max}}{K_m} \quad (12)$$

The measured kinetic parameters using these methods are summarized in Table 4.6 below.

Table 4.6: Summary of measured enzyme kinetics for ritonavir. Data gathered from two independent incubations

Kinetic Parameters	Mean	95% CI
K_M (nM)	135	0.0-280
V_{max} (nM·min ⁻¹)	87.1	ND

CI = Confidence Interval, 95% CI for $k_{dep([S] \rightarrow 0)}$ not determined

4.3.4 Measurement of fm,CYP

The measurement of ritonavir fm,CYP was undertaken as in previous Chapters. The conditions used to measure ritonavir fm,CYP are shown in Table 4.7 below.

Table 4.7: Incubation conditions used to measure ritonavir fm,CYP

Parameter	Conditions tested
Protein Concentration (mg/mL)	0.02
Time (min)	0; 5; 10; 20; 30
K_M (nM)	135
Substrate Concentration (nM)	25

The HLM in buffer pre-mix was pre-incubated with NADPH along with the mechanistic inhibitors for 20 minutes before the incubation was started by the addition of ritonavir. The competitive inhibitors were added to the incubation plate at the same time as the compound and the incubation was carried as described above. In Figure 4.7 below are the results of the chemical inhibitor incubations for ritonavir.

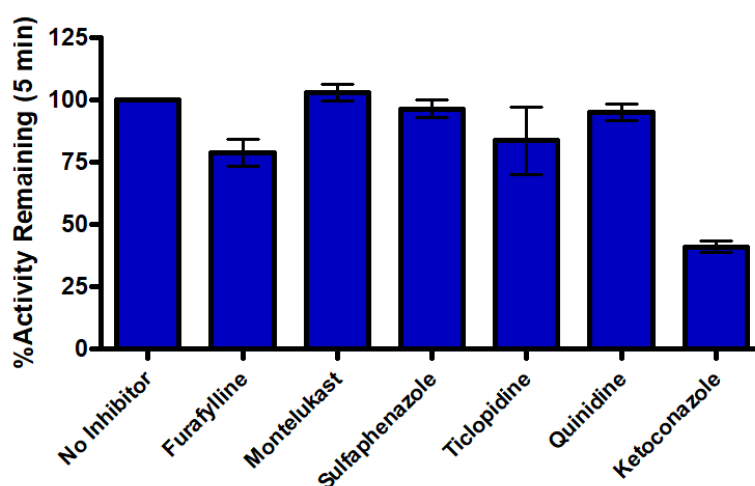


Figure 4.7: Chemical inhibition results showing the percentage enzyme activity remaining after the addition of specific inhibitors to the HLM incubation for ritonavir

This data was then used to calculate fm,CYP using the method described by Njuguna *et al.*¹⁶ Using this method, fm,CYP was calculated by measuring the rate of substrate depletion where the slope of normalised log transformed substrate depletion was measured to obtain rate k_{dep} . The intrinsic clearance (CL_{int}) was then calculated from k_{dep} using the following equation:

$$CL_{int} = \frac{0.693}{in\ vitro\ t_{\frac{1}{2}}} * \frac{mL\ incubation}{mg\ microsomes}, \text{ where } in\ vitro\ t_{\frac{1}{2}} = \frac{-0.693}{k_{dep}} \quad (13)$$

The fm,CYP was then calculated from the calculated CL_{int} using the following equation:

$$fm, CYP = \left[1 - \left(\frac{CL_{int} \text{ in presence of inhibitor}}{CL_{int} \text{ with no inhibitor present}} \right) \right] * 100 \quad (14)$$

In Table 4.8 below is summarised the fm,CYP data calculated for ritonavir.

Table 4.8: Calculated ritonavir fm,CYP data

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	0	17.7	4.68	0	31.3	96.9

From the data in Table 4.8, CYP3A is shown to be the major isoform involved in the *in vitro* microsomal metabolism of ritonavir with a significant contribution from CYP2D6, as expected. Cross reactivity corrections were then applied as in work described in Chapter 2 to give the corrected fm,CYP values, seen in Table 4.9, below.

Table 4.9: Corrected ritonavir fm,CYP data

	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	Ticlopidine	Quinidine	Ketoconazole
fm,CYP	0	0	1.4	0	26.1	72.5

The data collected shows that CYP3A is the major isoform involved in the *in vitro* microsomal metabolism of ritonavir, with a more moderate but significant contribution from CYP2D6; which is what has been found in literature.^{10,11} There is also a minor contribution from CYP2C9, which has also been suggested in literature.¹⁰

4.4 Ritonavir PBPK modelling

During the course of this work, a ritonavir PBPK model was developed and published in literature and was made available in the global health repository within the Simcyp members area.^{17,18} Given that no new discoveries were made with reference to ritonavir fm,CYP during the *in vitro* metabolism investigation done here, attention instead turned to making use of this published model to investigate and explain some gaps in knowledge in ritonavir DDIs with the use of the mefloquine and proguanil/cycloguanil model developed and verified here since both drugs are metabolized by CYP3A; which is a major source of DDIs typically seen with ritonavir administration.

4.4.1 Ritonavir Model Optimization

Simcyp has an available lopinavir substrate file (v18_lit_Lopinavir_Wagner_2017), published by Wagner *et al.* with supporting information, accessed through the global health repository within the Simcyp members area, which can be used in DDI studies.^{18,19} Interestingly, neither

this file nor the published ritonavir file take into account the induction of CYP1A2 which is known to occur with LPV/r co-administration.²⁰

This induction appears to arise as a result of ritonavir since an investigation into the interaction between lopinavir and several CYP isoforms suggested that lopinavir had no effect on CYP1A2.²¹ Moreover, another study, which forms part of the ritonavir product label, indicates that co-administration of ritonavir with theophylline, a known CYP1A2 substrate, resulted in a 43% reduction in theophylline AUC and a 32% reduction in its $C_{\max}$.^{22,23}

Given that the work done here uncovered and confirmed the involvement of CYP1A2 in the metabolism of mefloquine, the induction effect of CYP1A2 by ritonavir could potentially explain the lack of inhibitory interaction between it and mefloquine despite its status as a strong CYP3A inhibitor; as suggested by Rattanapunya *et al.*²⁴ To investigate this, an attempt was made to recapture the induction effect of ritonavir on theophylline with the use of the published ritonavir inhibitor file as well as the theophylline substrate file available on Simcyp.

This was performed by running the available theophylline file with the published ritonavir file in a DDI study; first with its default parameters and then after manually optimizing the CYP1A2 $\text{Ind}_{\max}$ parameter to successfully simulate a 43% reduction in theophylline AUC. This manual optimization was carried out by inputting different CYP1A2 $\text{Ind}_{\max}$ values until the 43% reduction in theophylline AUC was successfully simulated. This DDI investigation was conducted based on the study parameters as performed by Hsu *et al.*²² During this study, ritonavir was administered to 11 participants at a dose of 500 mg twice daily for 10 days along with theophylline at a dosage of 3 mg/kg three times a day for 15 days. In Figure 4.8 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.10 below.

Table 4.10: Comparison of the effect of ritonavir co-administration with theophylline, administered with its default interaction parameters and with an optimized CYP1A2 $\text{Ind}_{\max}$ parameter, and the effect this has on theophylline exposure

Inhibitor status	$C_{\max}$ (mg/L) (5 th and 95 th)	$C_{\max}R$	AUC (mg/L·h)(5 th and 95 th)	AUCR
No Inhibitor	12.5 (10.2-15.5)	-	78.1 (61.0-103)	-
Ritonavir (Default)	12.4 (11.0-14.6)	0.992	77.6 (65.8-92.0)	0.994
Ritonavir (CYP1A2 Ind.)	8.25 (6.49-11.1)	0.660	44.5 (32.9-66.6)	0.560

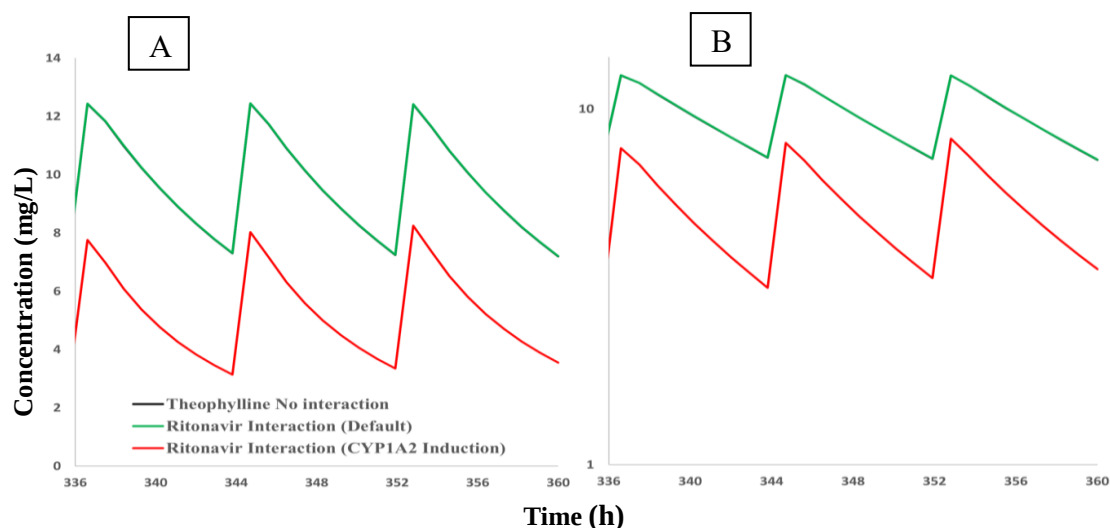


Figure 4.8: Simulated mean plasma concentration-time profiles of multiple 3 mg/kg oral doses of theophylline taken three times a day for 15 days (A), (10 trials of 6 male and 5 female subjects, 20-50yrs) along with 500 mg ritonavir taken twice daily for 10 consecutive days, and the data plotted with the y-axis on a log scale (B), for the investigation of the induction effect of ritonavir on CYP1A2 to recapture the clinically observed effect this has on theophylline exposure. The default (green line) and no interaction (black line) overlap and thus only the green line is visible.

The figure and accompanying PK parameters show that the default ritonavir file, wherein no account is taken of CYP1A2 induction, has a negligible effect on theophylline exposure. Parameter optimization in Simcyp allowed the estimation of CYP1A2 $\text{Ind}_{\text{max}} = 82 \mu\text{M}$, which yielded a reduction of theophylline AUC of 43% and a 34% reduction in its C_{max} ; which closely mirrors the outcomes observed by Hsu *et al.* Combined with the lack of effect the default ritonavir file has on theophylline exposure, this shows that the difference between the theophylline exposure without an inhibitor and with the ritonavir inhibitor file with optimized CYP1A2 induction parameters is as a result of CYP1A2 induction. The interaction parameters for the ritonavir inhibitor file with an optimized CYP1A2 Ind_{Max} parameter are shown in Table 4.11 below.

Table 4.11: Interaction parameters of the published ritonavir inhibitor file with an optimized CYP1A2 Ind_{max} parameter

Interactions								
Competitive inhibition			Mechanism-Based Inhibition			Induction/Suppression		
Enzyme	K_i	$f_{u_{mic}}$	K_{app}	k_{inact}	$f_{u_{mic}}$	Ind _{max}	IndC ₅₀	$f_{u_{inc}}$
CYP1A2	n/a	n/a	n/a	n/a	n/a	82	1	1
CYP2C9	n/a	n/a	n/a	n/a	n/a	3.33	0.07	1
CYP2D6	0.04	1	n/a	n/a	n/a	n/a	n/a	n/a
CYP3A4	0.00194	1	0.18	19.8	1	68.5	1	1
CYP3A5	0.00194	1	0.18	19.8	1	68.5	1	1

K_i = concentration of inhibitor that supports half maximal inhibition (μM); $f_{u_{mic}}$ = fraction of unbound drug in *in vitro* microsomal incubation; K_{app} = concentration of mechanism-based inhibitor associated with half maximal inactivation rate (μM); k_{inact} = inactivation rate of the enzyme ($1/\text{h}$); Ind_{max} = maximal fold induction/suppression over enzyme (1=no induction/suppression; >1=induction; <1 (but >0)=suppression; IndC₅₀ = test compound concentration that supports half maximal induction/suppression (μM); $f_{u_{inc}}$ = fraction of unbound drug in the *in vitro* incubation; n/a parameter not used in inhibitor file

As a means of verifying the optimized CYP1A2 induction effect of ritonavir as well as to establish whether the simulations performed with this updated file remain accurate when lopinavir and ritonavir are administered together in Simcyp, an attempt was made to recapture the induction effect of LPV/r co-administration with a different CYP1A2 substrate; namely caffeine. A study conducted by Yeh *et al.* observed a 43% increase in CYP1A2 activity upon administration of LPV/r using caffeine as a marker for this enzyme and an attempt was made to accurately predict this effect.²⁰

This was done by running the caffeine substrate file available in Simcyp with the published ritonavir inhibitor file along with the published lopinavir inhibitor file in a DDI study; first with the ritonavir inhibitor file's default parameters and then with the file with the optimized CYP1A2 Ind_{max}. An assessment of the difference in caffeine AUC between the different model runs would be used to assess whether the induction effect of LPV/r on CYP1A2 matched the observed effect seen in literature. This DDI investigation was conducted based on the study parameters as performed by Yeh *et al.*²⁰ During this study, LPV/r was administered to 6 male and 8 female participants, with an age range of 24-30 years, at a dose of 400 mg lopinavir and 100 mg ritonavir twice daily for 10 days prior to a single 2 mg/kg dose of caffeine on the 11th day. In Figure 4.9 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.12 below.

Table 4.12: Comparison of the effect of LPV/r co-administration with caffeine, with ritonavir administered with its default interaction parameters and with an optimized CYP1A2 Ind_{max} parameter, and the effect this has on caffeine exposure

Inhibitor status	C_{max} (mg/L) (5 th and 95 th)	C_{maxR}	AUC (mg/L·h) (5 th and 95 th)	AUCR
No Inhibitor	3.32 (3.11-3.50)	-	26.6 (21.6-31.2)	-
LPV/r (Default)	3.32 (3.11-3.51)	1.00	26.6 (21.6-31.2)	1.00
LPV/r (CYP1A2 Ind.)	2.65 (2.44-2.85)	0.798	13.3 (10.4-15.8)	0.500

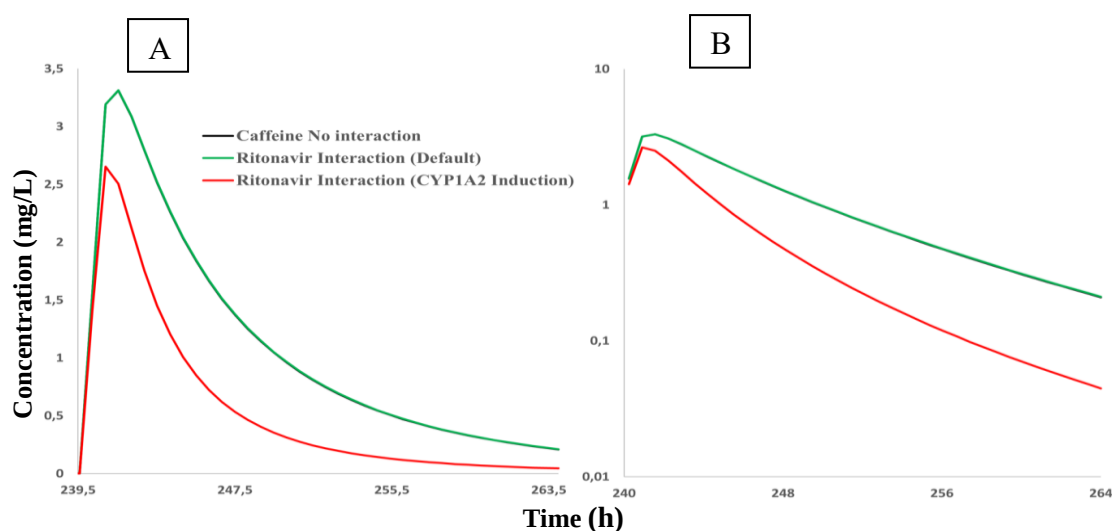


Figure 4.9: Simulated mean plasma concentration-time profiles of a single 2 mg/kg dose of caffeine (A), (10 trials of 6 male and 5 female subjects, 20-50yrs) preceded by pre-treatment with 400 mg lopinavir in combination with 100 mg ritonavir taken twice daily for 10 consecutive days prior to the caffeine dose, and the data plotted with the y-axis on a log scale (B), for the investigation of the induction effect of ritonavir on CYP1A2 to recapture the clinically observed effect this has on caffeine exposure. The default (green line) and no interaction (black line) overlap and thus only the green line is visible.

Co-administration of LPV/r in Simcyp with the default ritonavir file showed almost no ability to inhibit or induce caffeine metabolism, and by extension clearance. However, when this same interaction was simulated with the ritonavir file updated with an optimized CYP1A2 Ind_{max} , a 50% reduction was noted in caffeine AUC. This shows that the reduction in AUC was as a result of the induction effect on CYP1A2 of the updated ritonavir file. Moreover, the 50% reduction in AUC is similar to the 43% increase in CYP1A2 activity upon co-administration of LPV/r noted by Yeh *et al.*, given that this reduction in AUC in the model is as a result of CYP1A2 induction. This provided confidence that the lopinavir and updated ritonavir files could accurately predict clinical DDIs seen with LPV/r administration.

4.4.2 Ritonavir Model Application

Ritonavir is a known mixed inducer and inhibitor of CYP3A, with this knowledge being incorporated into the model published by Arora *et al.*¹⁷ A possible explanation for the lack of interaction between ritonavir and mefloquine then, as proposed by Khaliq *et al.*,²⁵ could be that the induction of CYP3A by ritonavir could be minimizing its effect as an inhibitor of the same

enzyme. To investigate this, the published ritonavir inhibitor file was run with its default parameters, as published by Arora *et al.*,¹⁷ then as either an inhibitor or inducer of CYP3A along with the developed and verified mefloquine model. The latter simulations were achieved by altering the ritonavir inhibitor file so as to remove its effect as an inhibitor or inducer for the relevant model run. The interaction parameters for the published ritonavir inhibitor file are seen in Table 4.13 below.

Table 4.13: Interaction parameters of the published ritonavir inhibitor file

Interactions								
Competitive inhibition			Mechanism-Based Inhibition			Induction/Suppression		
Enzyme	K_i	$f_{u_{mic}}$	K_{app}	k_{inact}	$f_{u_{mic}}$	Ind_{max}	$IndC_{50}$	$f_{u_{inc}}$
CYP2C9	n/a	n/a	n/a	n/a	n/a	3.33	0.07	1
CYP2D6	0.04	1	n/a	n/a	n/a	n/a	n/a	n/a
CYP3A4	0.00194	1	0.18	19.8	1	68.5	1	1
CYP3A5	0.00194	1	0.18	19.8	1	68.5	1	1

K_i = concentration of inhibitor that supports half maximal inhibition (μM); $f_{u_{mic}}$ = fraction of unbound drug in *in vitro* microsomal incubation; K_{app} = concentration of mechanism-based inhibitor associated with half maximal inactivation rate (μM); k_{inact} = inactivation rate of the enzyme (1/h); Ind_{max} = maximal fold induction/suppression over enzyme (1=no induction/suppression; >1=induction; <1 (but >0)=suppression; $IndC_{50}$ = test compound concentration that supports half maximal induction/suppression (μM); $f_{u_{inc}}$ = fraction of unbound drug in the *in vitro* incubation; n/a parameter not used in inhibitor file

In these model application investigations, ritonavir was administered as a 100 mg dose twice daily throughout the duration of the simulation to mimic conditions seen in chronic HIV treatment, with 1000 mg mefloquine administered on the 8th simulated day. All model application investigations were performed as 10 trials of 5 male and 5 female subjects with an age range of 20-65 years. In Figure 4.10 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.14 below.

Table 4.14: Comparison of the effect of ritonavir, administered as an inducer, inhibitor or mixed inducer/inhibitor of CYP3A, on mefloquine exposure

Inhibitor status	C_{max} (mg/L) (5 th and 95 th)	C_{maxR}	AUC (mg/L·h) (5 th and 95 th)	AUCR
No Inhibitor	1.34 (1.22-1.45)	-	516 (411-614)	-
Ritonavir (Default)	1.35 (1.23-1.46)	1.01	827 (650-993)	1.60
Ritonavir (CYP3A Inhibitor)	1.32 (1.20-1.46)	0.985	811 (698-1000)	1.57
Ritonavir (CYP3A Inducer)	1.31 (1.19-1.44)	0.978	467 (362-584)	0.905

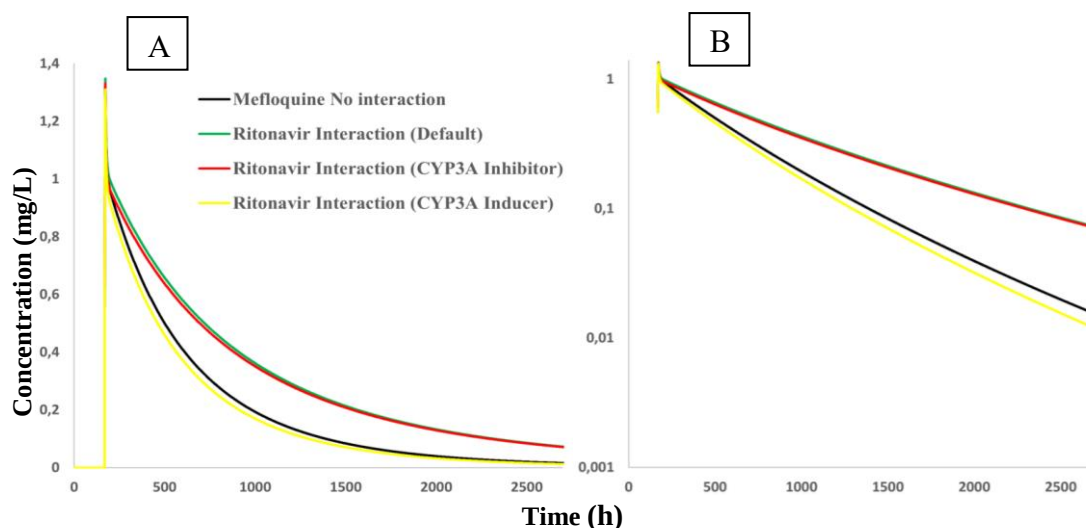


Figure 4.10: Simulated mean plasma concentration-time profiles of a single oral dose of 1000 mg of mefloquine (A), (10 trials of 5 male and 5 female subjects, 20-65 yrs) with ritonavir administered at a dose of 100 mg twice a day, throughout the study, and the data plotted with the y-axis on a log scale (B), for the investigation of the mixed induction and inhibition effect of ritonavir on mefloquine PK.

The figure and accompanying PK parameters show that ritonavir has a limited ability to induce the metabolism of mefloquine based on the published ritonavir inhibitor file. This indicates that the lack of interaction between mefloquine and ritonavir is not as a result of its status as a mixed inhibitor and inducer of CYP3A.

4.4.3 Application of updated ritonavir model

4.4.3.1 Ritonavir interaction with mefloquine

To test whether the induction of CYP1A2 by ritonavir was the reason for its minimal interaction with mefloquine, despite its status as a strong CYP3A inhibitor, the updated ritonavir file and the published lopinavir file were used to recapture the observed reduction in mefloquine exposure noted by Rattanapunya *et al.* upon co-administration with LPV/r.

This was conducted by replicating the DDI study parameters as performed by Rattanapunya *et al.* with the ritonavir file along with the published lopinavir file; first with the ritonavir file's default parameters and then with the file with the optimized CYP1A2 Ind_{max}. During the study performed by Rattanapunya *et al.*, mefloquine was administered at dosages of 750 mg and 500 mg on the first and second study day, respectively, to 8 male and 6 female subjects with an age range of 20-40 years old. During the DDI portion of the study, mefloquine administration was preceded by LPV/r administration at a dosage of 400 mg lopinavir and 100 mg ritonavir twice daily for 14 days. Mefloquine, in combination with artesunate, was then administered on the 15th and 16th days at the dosages described above, with LPV/r administration continuing for 3 days upon mefloquine administration for a total of 34 LPV/r doses.

In Figure 4.11 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.15 below. The fold error is illustrated in Figure 4.12.

Table 4.15: Comparison of the effect of LPV/r administration with mefloquine, with ritonavir administered with its default interaction parameters and with an optimized CYP1A2 Ind_{Max} parameter, and the effect this has on mefloquine exposure

	Inhibitor status	C _{max} (mg/L) (5 th and 95 th)	C _{max} R	AUC (mg/L·h) (5 th and 95 th)	AUCR
Literature	No Inhibitor	2.90 (2.44-3.35)	-	1160 (924-1397)	-
	LPV/r	2.34 (2.05-2.63)	0.807	751 (471-1031)	0.647
Model	No Inhibitor	1.65 (1.18-2.27)	-	1010 (352-2280)	-
	LPV/r (Default)	1.68 (1.19-2.32)	1.02	1100 (399-2500)	1.09
	LPV/r (CYP1A2 Ind.)	1.63 (1.17-2.25)	0.988	961 (338-2260)	0.951

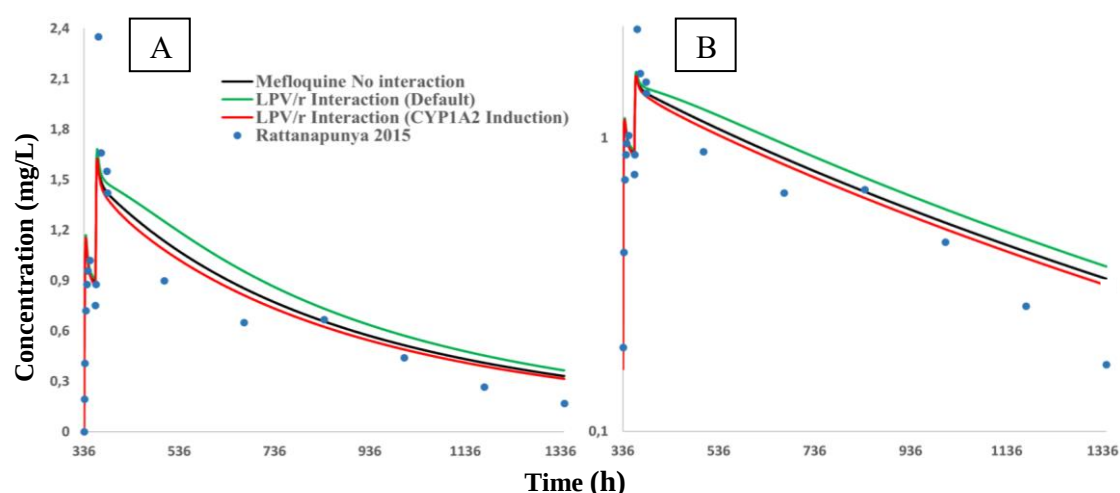


Figure 4.11: Simulated mean plasma concentration-time profiles of mefloquine after a 750 mg dose followed by a 500 mg dose on the next day. This was preceded by pre-treatment with 400 mg of lopinavir and 100 mg of ritonavir, taken twice daily for 14 consecutive days before mefloquine dose and 3 consecutive days after the first mefloquine dose (A), (10 trials of 8 male and 6 female subjects, 20-40 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Rattanapunya et al., 2015.

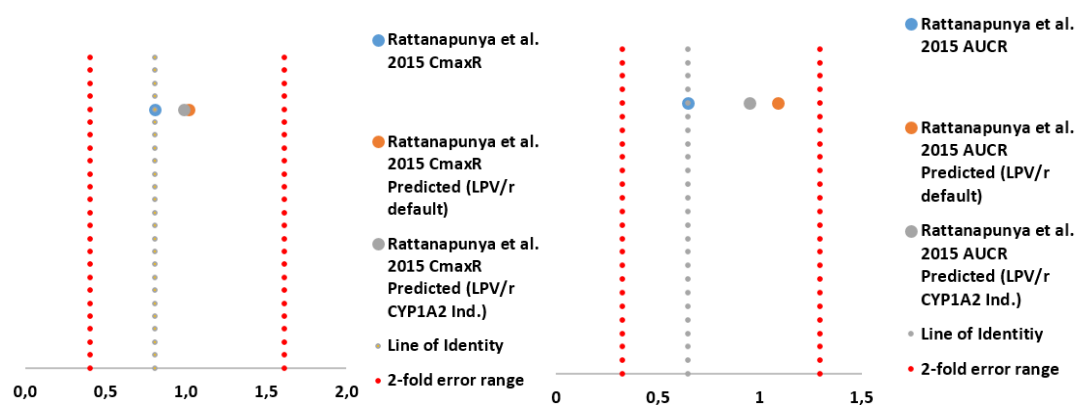


Figure 4.12: Visual representation of predicted vs observed C_{max}R and AUCR for study conducted by Rattanapunya et al. 2015

The above data makes clear that the default ritonavir file, where it only inhibits and induces CYP3A and induces CYP2C9, has little effect on mefloquine exposure. Furthermore, it slightly increased mefloquine exposure, as a result of CYP3A inhibition, instead of predicting the decrease noted by Rattanapunya *et al.* The updated ritonavir file on the other hand, wherein CYP1A2 induction is accounted for, correctly predicted the decrease in mefloquine; although the predicted decrease was not of the same magnitude as the observed. Of note, the model could not accurately predict mefloquine $C_{\max}$ for this study. This is likely due to the limitation of this model discussed in Chapter 2 where the model underpredicts $C_{\max}$ at dosages above 1000 mg; as is the case here.

In addition to this, the importance of the discovery of the involvement of CYP1A2 to mefloquine metabolism and its subsequent incorporation into the mefloquine model was also investigated with the use of the updated ritonavir file. This was achieved by comparing the model developed and verified here with the mefloquine model developed and verified by the Simcyp research team and made available in the Simcyp members' area since this model does not include CYP1A2 metabolism as one of its clearance pathways. The simulations were conducted as described above for the prediction of the study done by Rattanapunya *et al.* with the Simcyp mefloquine substrate file used instead of the mefloquine substrate file developed here.

In Figure 4.13 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.16 below.

Table 4.16: Comparison of the effect of LPV/r administration with mefloquine, with ritonavir administered with its default interaction parameters and with an optimized CYP1A2 $Ind_{\max}$ parameter, and the effect this has on mefloquine exposure using the mefloquine substrate file available on Simcyp

	Inhibitor status	$C_{\max}$ (mg/L) (5 th and 95 th)	$C_{\max}R$	AUC (mg/L·h) (5 th and 95 th)	AUCR
Literature	No Inhibitor	2.90 (2.44-3.35)	-	1160 (924-1397)	-
	LPV/r	2.34 (2.05-2.63)	0.807	751 (471-1031)	0.647
Simcyp Model	No Inhibitor	1.80 (0.961-3.14)	-	749 (196-1640)	-
	LPV/r (Default)	2.02 (1.06-3.55)	1.12	1010 (318-2100)	1.35
	LPV/r (CYP1A2 Ind.)	2.02 (1.06-3.55)	1.12	1010 (318-2100)	1.35

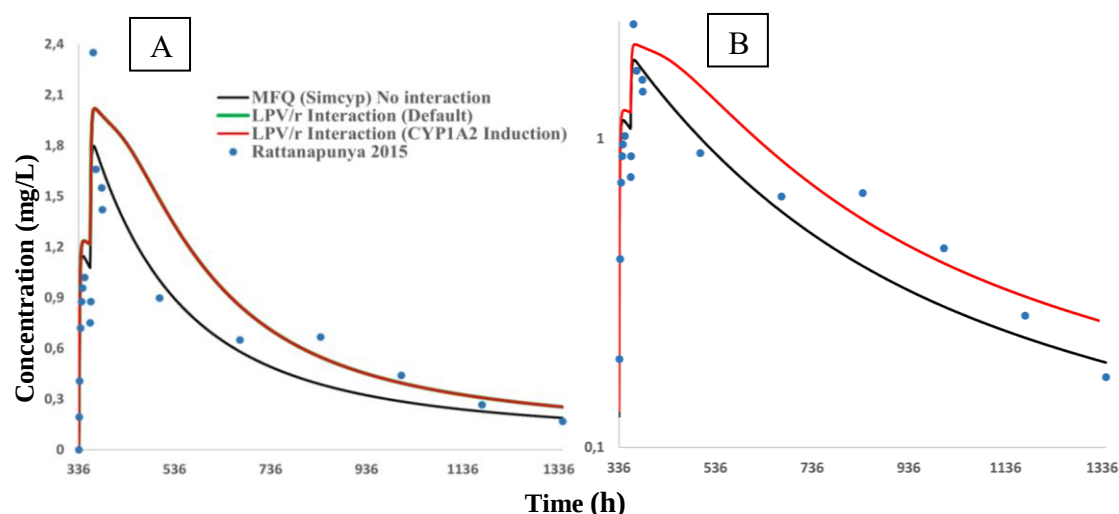


Figure 4.13 Simulated mean plasma concentration-time profiles of mefloquine (using the mefloquine substrate file available on Simcyp) after a 750 mg dose followed by a 500 mg dose on the next day. This was preceded by pre-treatment with 400 mg of lopinavir and 100 mg of ritonavir, taken twice daily for 14 consecutive days before mefloquine dose and 3 consecutive days after the first mefloquine dose (A), (10 trials of 8 male and 6 female subjects, 20-40 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Rattanapunya *et al.*, 2015. The default (green line) and interaction (red line) overlap and thus only the red line is visible.

The above figure and PK data illustrate that the addition of the induction effect on CYP1A2 of ritonavir had no effect on mefloquine exposure in these simulations. Both DDI simulations were identical, with both predicting a notable increase in exposure. This is of course the opposite of the reported data. These simulations show the importance of incorporating elimination by CYP1A2 metabolism into mefloquine PBPK models.

4.4.3.2 Ritonavir interaction with proguanil

Since the involvement of CYP1A2 in the metabolism of proguanil was confirmed in the work done here, along with the known and confirmed contribution of CYP3A, an attempt was made to recapture the decrease in proguanil exposure noted upon co-administration of proguanil and both LPV/r and atazanavir/ritonavir (ATV/r).

This was achieved by replicating the DDI study parameters as performed by Van Luin *et al.* with the ritonavir file along with the published lopinavir file and an atazanavir substrate file available on Simcyp; first with the ritonavir file's default parameters and then with the file with the optimized CYP1A2 Ind_{max}. During the study performed by Van Luin *et al.*, proguanil was administered as a single 100 mg dose to healthy volunteers as well as to HIV positive patients who were receiving chronic treatment with either LPV/r or ATV/r at dosages of 400mg lopinavir 100 mg ritonavir twice daily or 300 mg lopinavir 100 mg ritonavir once daily, respectively.²⁶ Eighteen healthy volunteers participated in the study along with 19 patients receiving LPV/r with a further 19 receiving ATV/r treatment.

In Figures 4.14 and 4.16 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.17 and the interaction ratios in Table 4.18, below. The fold error is illustrated in Figures 4.15 and 4.16. It should be noted that Van Luin *et al.* did not monitor or report cycloguanil plasma concentrations. As a result, only data for proguanil are available for comparison.

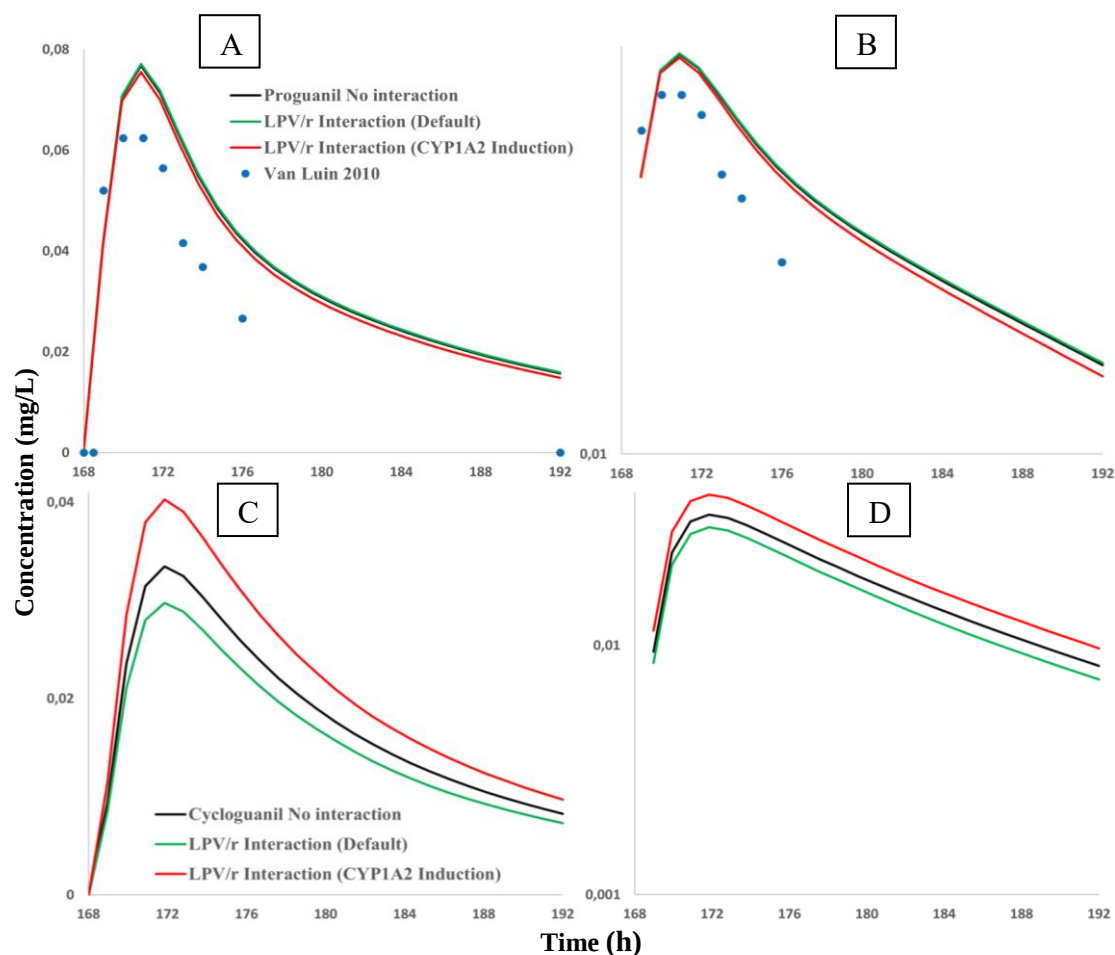


Figure 4.14 Simulated mean plasma concentration-time profiles of proguanil (A), cycloguanil (C) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil after single oral dose of 100 mg proguanil preceded by pre-treatment with 400 mg lopinavir and 100 mg ritonavir, taken twice daily for 7 consecutive days before proguanil administration. Proguanil was then administered on the 8th day along with the final LPV/r dose, (10 trials of 18 male and 2 female subjects, 35-60 yrs). Observed data, blue dots, were extracted from Van Luin. *et al.* 2010 with the patients involved in the study receiving chronic LPV/r treatment.

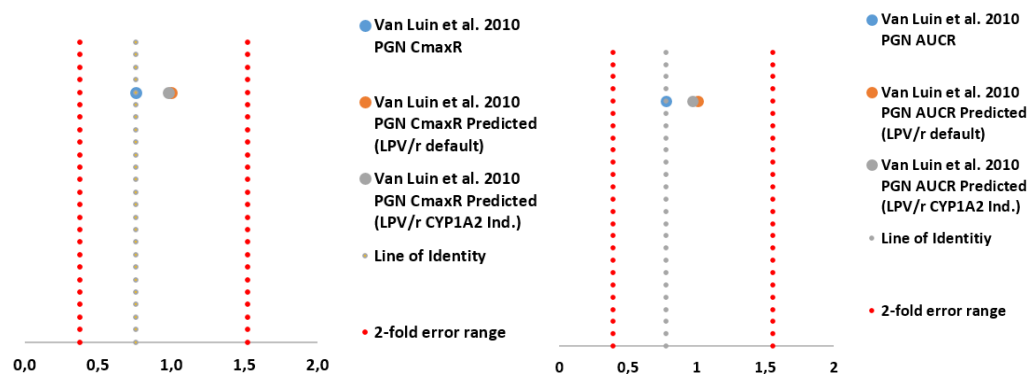


Figure 4.15: Visual representation of predicted vs observed C_{maxR} and AUCR for the interaction with LPVr in the study conducted by Van Luin et al. 2010

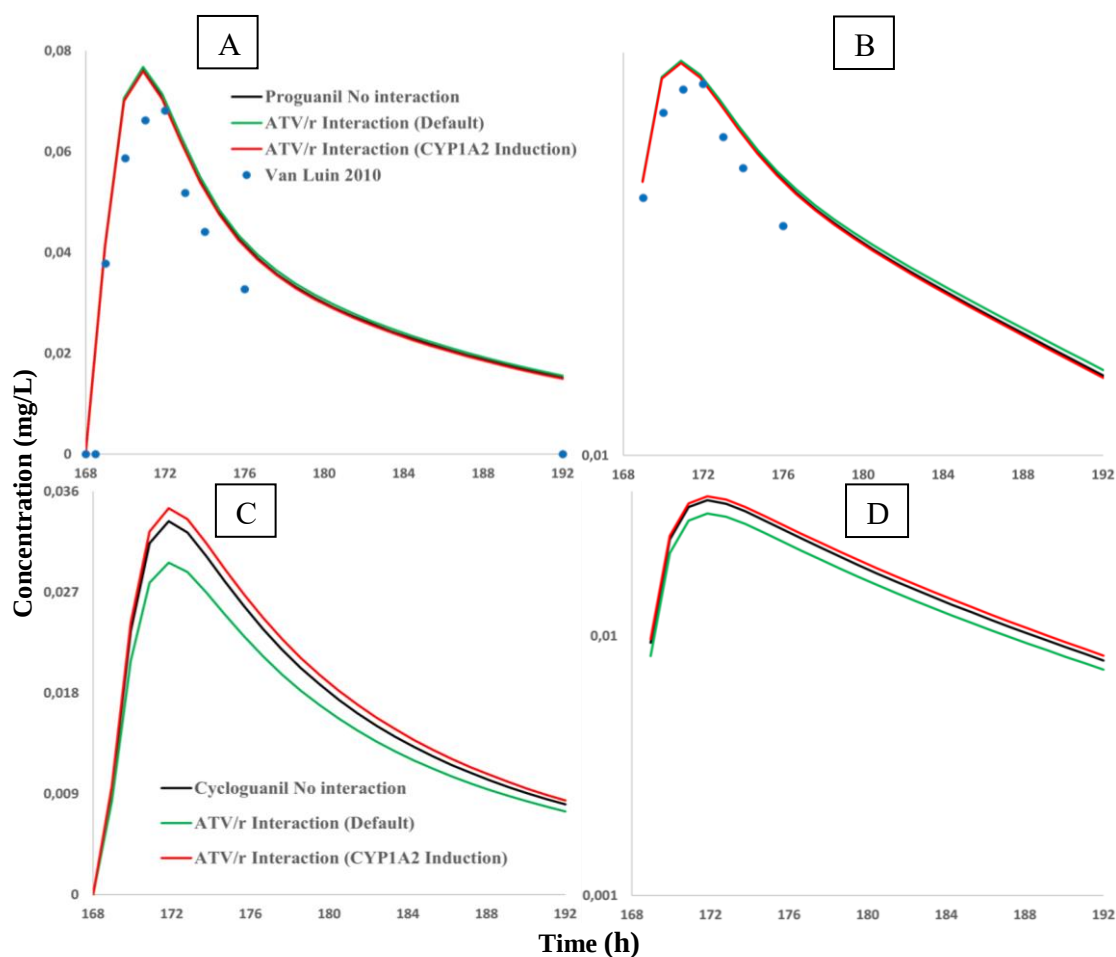


Figure 4.16: Simulated mean plasma concentration-time profiles of proguanil (A), cycloguanil (C) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil after single oral dose of 100 mg proguanil preceded by pre-treatment with 300 mg lopinavir and 100 mg ritonavir, taken once a day for 7 consecutive days before proguanil administration. Proguanil was then administered on the 8th day along with the final ATV/r dose, (10 trials of 18 male and 2 female subjects, 35-60 yrs). Observed data, blue dots, were extracted from Van Luin. et al. 2010 with the patients involved in the study receiving chronic ATV/r treatment.

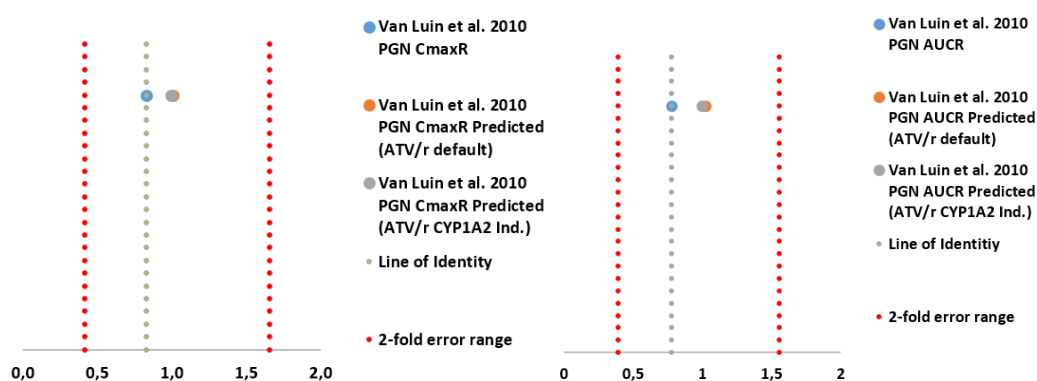


Figure 4.17: Visual representation of predicted vs observed C_{maxR} and AUCR for the interaction with ATV/r in the study conducted by Van Luin et al. 2010

Table 4.17: Comparison of the effect of LPV/r and ATV/r administration with proguanil, with ritonavir administered with its default interaction parameters and with an optimized CYP1A2 Ind_{Max} parameter, and the effect this has on proguanil and cycloguanil exposure

	Inhibitor status	C_{max} (mg/L) (5 th and 95 th)	AUC (mg/L·h) (5 th and 95 th)
Literature (Proguanil)	No Inhibitor*	0.0821 (NR)	0.826 (NR)
	LPV/r*	0.0624 (NR)	0.642 (NR)
Model (Proguanil)	No Inhibitor	0.0768 (0.0738-0.0796)	0.853 (0.811-0.905)
	LPV/r (Default)	0.0771 (0.0739-0.0806)	0.859 (0.805-0.900)
	LPV/r (CYP1A2 Ind)	0.0755 (0.0726-0.0779)	0.827 (0.790-0.872)
Model (Cycloguanil)	No Inhibitor	0.0335 (0.0288-0.0398)	0.437 (0.378-0.508)
	LPV/r (Default)	0.0297 (0.0253-0.0363)	0.388 (0.330-0.463)
	LPV/r (CYP1A2 Ind)	0.0403 (0.0369-0.0468)	0.522 (0.485-0.595)
Literature (Proguanil)	No Inhibitor*	0.0821 (NR)	0.826 (NR)
	ATV/r*	0.0681 (NR)	0.642 (NR)
Model (Proguanil)	No Inhibitor	0.0761 (0.0735-0.0792)	0.837 (0.797-0.880)
	ATV/r (Default)	0.0768 (0.0742-0.0799)	0.851 (0.810-0.896)
	ATV/r (CYP1A2 Ind)	0.0759 (0.0734-0.0789)	0.832 (0.791-0.876)
Model (Cycloguanil)	No Inhibitor	0.0333 (0.0287-0.0398)	0.433 (0.369-0.507)
	ATV/r (Default)	0.0296 (0.0252-0.0362)	0.389 (0.332-0.464)
	ATV/r (CYP1A2 Ind)	0.0345 (0.0291-0.0423)	0.450 (0.386-0.543)

NR=Data was not reported; *Data was determined through non-compartmental analysis of reported figures

Table 4.18. Interaction ratios for the comparison of the effect of LPV/r and ATV/r administration with proguanil, with ritonavir administered with its default interaction parameters and with an optimized CYP1A2 Ind_{Max} parameter, and the effect this has on proguanil and cycloguanil exposure

	Inhibitor status	C _{max} R	AUCR
Literature (Proguanil)	No Inhibitor*	-	-
	LPV/r*	0.760	0.777
Model (Proguanil)	No Inhibitor	-	-
	LPV/r (Default)	1.00	1.01
	LPV/r (CYP1A2 Ind)	0.983	0.970
Model (Cycloguanil)	No Inhibitor	-	-
	LPV/r (Default)	0.887	0.888
	LPV/r (CYP1A2 Ind)	1.20	1.19
Literature (Proguanil)	No Inhibitor*	-	-
	ATV/r*	0.829	0.777
Model (Proguanil)	No Inhibitor	-	-
	ATV/r (Default)	1.01	1.02
	ATV/r (CYP1A2 Ind)	0.997	0.994
Model (Cycloguanil)	No Inhibitor	-	-
	ATV/r (Default)	0.889	0.898
	ATV/r (CYP1A2 Ind)	1.04	1.04

Minimal differences were seen between all simulations. However, it should be noted that a minor decrease in proguanil exposure, and a corresponding increase in cycloguanil exposure, was seen when the induction effect of ritonavir on CYP1A2 was taken into account. This is the opposite of what was seen when simulations were conducted using the default ritonavir file, where instead a slight increase in proguanil exposure, and a corresponding decrease in cycloguanil exposure, took place; as a result of the induction of CYP1A2 not being taken into account.

4.4.3.3 Ritonavir interaction with ethinyl estradiol

An attempt was also made to test the updated ritonavir inhibitor file with a validated Simcyp substrate file. Ethinyl estradiol was chosen for this investigation since its elimination is partly mediated by CYP1A2, with this knowledge captured in the Simcyp file, and literature data for this interaction was available to measure the accuracy of the simulated interaction.

This investigation was carried out replicating the DDI study parameters as performed by Ouellet *et al.* with the ritonavir file's default parameters and then with the file with the optimized CYP1A2 Ind_{max}.²⁷ During the study performed by Ouellet *et al.*, ethinyl estradiol was administered as a single 50 µg oral dose to healthy volunteers. After a 15 day washout period,

all subjects then received twice daily doses of ritonavir for 16 days with 300 mg every 12 hours on day 15, 400 mg every 12 hours on day 16, and 500 mg every 12 hours thereafter. Ritonavir was administered via oral syringe as an 80 mg/mL solution. The second ethinyl estradiol dose was then administered on day 29, together with the morning dose of ritonavir. Twenty three women, with an age range of 19-45 years, completed the study. In Figure 4.18 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.19. The fold error is illustrated in Figure 4.19.

Table 4.19: Comparison of the effect of ritonavir co-administration with ethinyl estradiol, with ritonavir administered with its default interaction parameters and with an optimized CYP1A2 Ind_{Max} parameter, and the effect this has on ethinyl estradiol exposure

	Inhibitor status	C _{max} (µg/L) (5 th and 95 th)	C _{max} R	AUC (µg/L·h) (5 th and 95 th)	AUCR
Literature	No Inhibitor	0.0893 (NR)	-	1.67 (NR)	-
	Ritonavir	0.0610 (NR)	0.683	0.993 (NR)	0.595
Simcyp Model	No Inhibitor	0.166 (0.0490-0.442)	-	1.68 (0.532-3.51)	-
	Ritonavir (Default)	0.159 (0.0511-0.410)	0.958	1.86 (0.564-4.00)	1.11
	Ritonavir (CYP1A2 Ind.)	0.129 (0.0435-0.295)	0.777	1.07 (0.253-2.43)	0.637

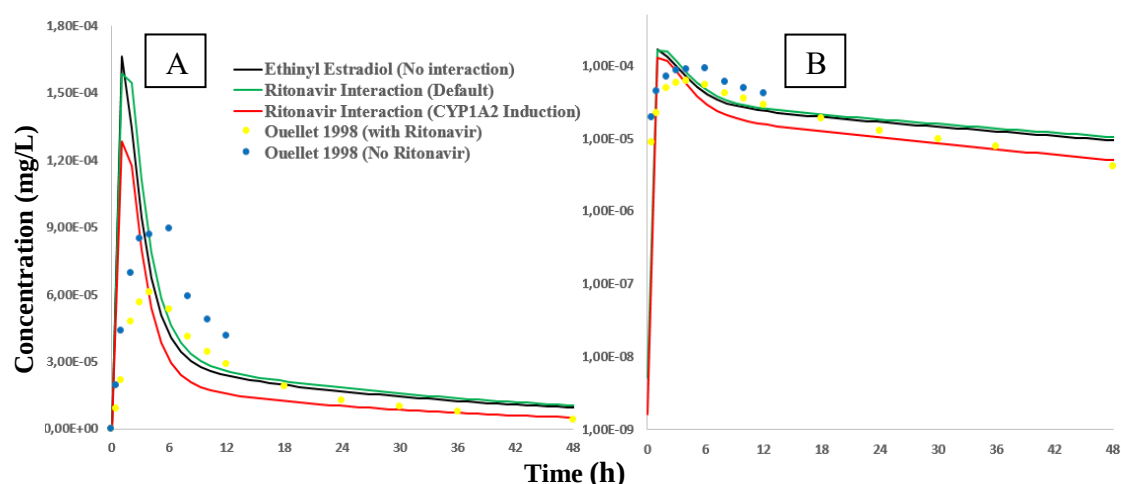


Figure 4.18: Simulated mean plasma concentration-time profiles of ethinyl estradiol (A), and the data plotted with the y-axis on a log scale (B) after single oral dose of 50 µg ethinyl estradiol on its own and preceded by pre-treatment with ritonavir with its dosage escalated to 500 mg twice daily for 16 days. Ethinyl estradiol was then administered on the 14th day, (10 trials of 23 female subjects, 19-45 yrs). Observed data, blue and yellow dots, were extracted from Ouellet. et al. 1998.

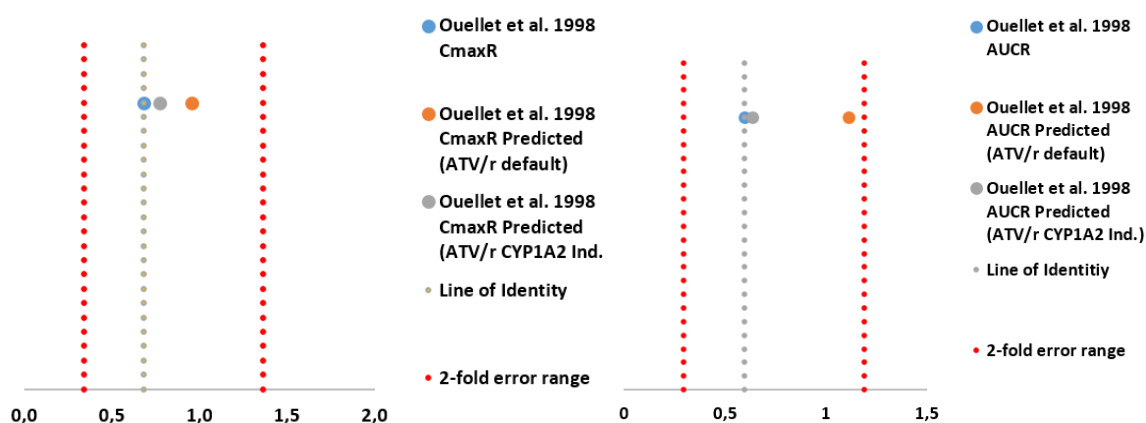


Figure 4.19: Visual representation of predicted vs observed C_{maxR} and AUCR for study conducted by Ouellet et al. 1998

The above data once again illustrates the importance of incorporating the induction effect of ritonavir on CYP1A2 when investigating ritonavir DDIs with CYP1A2 substrates. The default ritonavir file is unable to simulate the clinically observed reduction in ethinyl estradiol exposure. However, when CYP1A2 induction is accounted for, this reduction is predicted with reasonable accuracy (observed and predicted AUC ratio 0.595 vs 0.637, observed and predicted C_{max} ratio 0.680 vs 0.777).

Interestingly, ethinyl estradiol elimination is also mediated by CYP3A and CYP2C9, both enzymes that ritonavir interacts with as seen in Table 4.11 and Table 4.13. As a result, an attempt was made to ascertain which of these interactions drive the overall interaction between ritonavir and ethinyl estradiol.

This was investigated by editing the ritonavir inhibitor file so that it only carries out one of its many interactions at a time. Ritonavir's interaction as a CYP3A inhibitor, CYP3A inducer, CYP2C9 inducer, and CYP1A2 inducer were investigated. All other study parameters were as described above. In Figure 4.20 below are the representative concentration-time profiles for this model application with the corresponding PK parameters in Table 4.20.

Table 4.20: Comparison of the effect of ritonavir co-administration with ethinyl estradiol, with ritonavir administered with its default interaction parameters and as an inhibitor or inducer of a single CYP with which it interacts as a means of identifying which interaction drives its interaction with ethinyl estradiol

	Inhibitor status	C_{max} ($\mu\text{g/L}$) (5 th and 95 th)	C_{maxR}	AUC ($\mu\text{g/L}\cdot\text{h}$) (5 th and 95 th)	AUCR
Literature	No Inhibitor	0.0893 (NR)	-	1.67 (NR)	-
	Ritonavir	0.0610 (NR)	0.683	0.993 (NR)	0.595
Simcyp Model	No Inhibitor	0.166 (0.0490-0.442)	-	1.68 (0.532-3.51)	-
	Ritonavir (Default)	0.159 (0.0511-0.410)	0.958	1.86 (0.564-4.00)	1.11
	Ritonavir (CYP3A Inh.)	0.165 (0.0521-0.430)	0.994	2.14 (0.667-4.10)	1.27
	Ritonavir (CYP3A Ind.)	0.143 (0.0486-0.375)	0.861	1.36 (0.430-2.92)	0.810
	Ritonavir (CYP2C9 Ind.)	0.152 (0.0501-0.402)	0.916	1.64 (0.516-3.35)	0.976
	Ritonavir (CYP1A2 Ind.)	0.144 (0.0478-0.375)	0.867	1.37 (0.399-3.00)	0.815

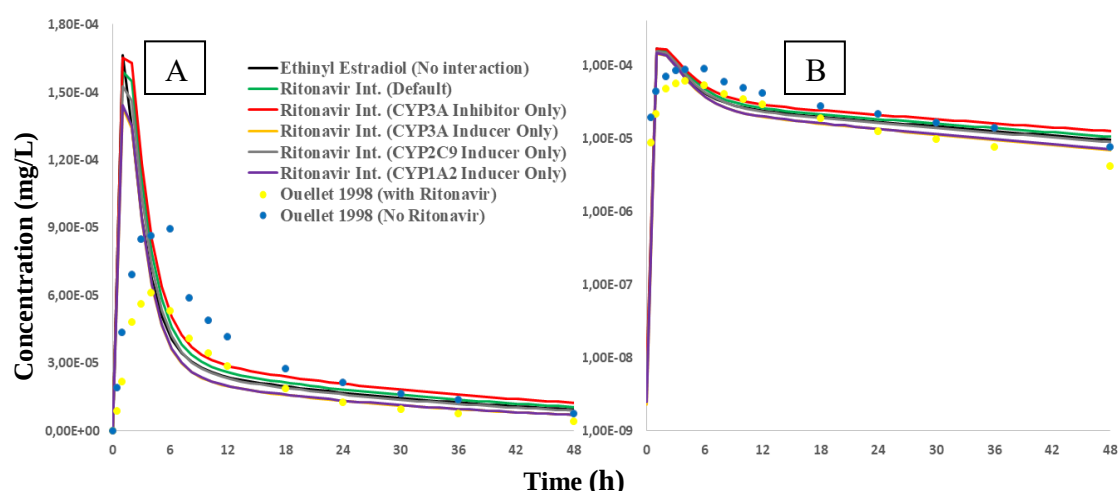


Figure 4.20: Simulated mean plasma concentration-time profiles of ethinyl estradiol (A), and the data plotted with the y-axis on a log scale (B) after single oral dose of 50 μg ethinyl estradiol on its own and preceded by pre-treatment with ritonavir with its dosage escalated to 500 mg twice daily for 16 days. Ritonavir was administered in the model as an inhibitor or inducer of a single CYP with which it interacts as a means of identifying which interaction drives its interaction with ethinyl estradiol. Ethinyl estradiol was then administered on the 14th day, (10 trials of 23 female subjects, 19-45 yrs). Observed data, blue and yellow dots, were extracted from Ouellet. et al. 1998.

Minimal changes were seen in ethinyl estradiol exposure when ritonavir was simulated as only an inhibitor of CYP3A or CYP2C9. As a result, it is clear from the above data that the DDI between ritonavir and ethinyl estradiol is driven by the induction effect of ritonavir administration on CYP3A and CYP1A2 with this effect likely being cumulative given that neither of these interactions on their own resulted in a similar magnitude of reduction in ethinyl estradiol exposure, as that seen in Table 4.20.

4.5 Discussion

4.5.1 Modelling: Ritonavir

A ritonavir model for the Simcyp simulator has recently been published.¹⁷ Since no new discoveries were made during the ritonavir fm,CYP investigation and, as a result, the existing model could not be updated or improved with data generated here, attention instead turned to making use of the available model to investigate and explain some gaps in knowledge in ritonavir DDIs with the use of the mefloquine and proguanil/cycloguanil model developed and verified here since both drugs are metabolized by CYP3A; which is a major source of DDIs typically seen with ritonavir administration.

Since ritonavir is a known mixed inhibitor and inducer of CYP3A, an investigation was conducted to ascertain whether the induction of CYP3A by ritonavir was responsible for the relative lack of interaction seen clinically when ritonavir and mefloquine are co-administered. This investigation revealed that ritonavir's inhibitory effect was significantly stronger than its induction effect on CYP3A in the simulations performed; suggesting that the cause of the lack of clinically observed interaction lay elsewhere. Moreover, the work reported in this Chapter as well as in Chapter 2 suggests that CYP3A-mediated metabolism is not a major route of mefloquine elimination *in vivo* as is widely believed. This finding is supported by the clinical DDI study conducted by Khaliq *et al.* who proposed this possibility as well.²⁵

It has previously been shown that LPV/r administration induced CYP1A2 metabolic activity. Given that the fm,CYP investigation of mefloquine showed and confirmed the involvement of this enzyme in its *in vitro* microsomal metabolism, with this knowledge captured in the mefloquine PBPK model developed and verified here, this was thought to be a possible explanation for the lack of inhibitory effect ritonavir co-administration has on mefloquine exposure. Indeed, a study conducted by Rattanapunya *et al.* showed that LPV/r co-administration with mefloquine resulted in reduced mefloquine exposure instead of the increase one would expect given that both drugs in that combination inhibit CYP3A; the major enzyme previously known to be involved in mefloquine metabolism.²⁴

However, despite the induction effect of LPV/r on CYP1A2 being known, neither the published ritonavir inhibitor file nor a lopinavir inhibitor file available in the Simcyp members areas took into account this induction effect. As a result, to investigate the potential induction effect of LPV/r co-administration on mefloquine metabolism, the available ritonavir and lopinavir inhibitor files would need to be updated and optimized to capture this induction effect.

A review of available literature suggested that the induction of CYP1A2 in the LPV/r combination arises as a result of ritonavir since an investigation into the interaction between lopinavir and several CYP isoforms suggested that lopinavir had no effect on CYP1A2.²¹ Moreover, another study indicated that co-administration of ritonavir with theophylline, a known CYP1A2 substrate, resulted in an overall reduction in theophylline exposure. As a means of accurately capturing the induction effect of CYP1A2 by ritonavir, the available ritonavir inhibitor file was modified by manual optimization of the CYP1A2 Ind_{Max} parameter for this file so as to correctly simulate the aforementioned theophylline and ritonavir DDI study. With an optimized Ind_{Max} value of 82 μ M, the now updated ritonavir inhibitor file was able to accurately predict the reduction in theophylline exposure as a result of co-administration with ritonavir in a simulated DDI study.

In an attempt to verify the accuracy of the optimized CYP1A2 Ind_{Max} parameter, as well as ensure that the induction seen with the ritonavir inhibitor file on its own holds true in combination with the available lopinavir inhibitor file, a simulation was performed with these two files as perpetrators of a DDI with caffeine; another known CYP1A2 substrate. These simulations showed that the optimized ritonavir inhibitor file in conjunction with the lopinavir inhibitor file was able to accurately predict CYP1A2 induction by means of the associated reduction of caffeine exposure.

The optimized ritonavir inhibitor file was then used in an attempt to recover the reduction in mefloquine exposure observed upon co-administration of LPV/r by Rattanapunya *et al.*²⁴ This investigation could predict the observed reduction in mefloquine exposure, although it could not accurately predict the magnitude of this reduction. This is illustrated in the observed vs simulated AUC ratios for this interaction, where a 1.54-fold reduction in AUC was observed whereas only a 1.05-fold reduction in AUC was simulated. Nevertheless, this is an improvement over the published ritonavir inhibitor file which predicted a 1.09-fold increase in mefloquine AUC.

In addition, the importance of the discovery of the involvement of CYP1A2 to mefloquine metabolism and its subsequent incorporation into the mefloquine model was also investigated with the use of the optimized ritonavir file. This was achieved using the mefloquine substrate file, which does not take into account CYP1A2 metabolism of mefloquine, available on the Simcyp members' area. This investigation clearly illustrated the importance of CYP1A2 since

the Simcyp mefloquine file was unaffected by the induction of CYP1A2 by ritonavir and, by extension, could not predict the clinically observed reduction in mefloquine exposure.

Given that, like mefloquine, proguanil is known to be metabolized by CYP3A and that the involvement of CYP1A2 in proguanil metabolism was confirmed in the work done here, the optimized ritonavir inhibitor file was also used to recapture the reduction in proguanil exposure observed by Van Luin *et al.*²⁶ upon co-administration of proguanil with both LPV/r and ATV/r combinations. Only minor reductions in proguanil exposure were predicted in the simulations with the optimized ritonavir inhibitor file. However, it should be noted that the published ritonavir inhibitor file predicted a minor increase in proguanil exposure; likely as a result of the induction of CYP1A2 not being taken into account. In addition, since no cycloguanil plasma concentration-time profiles were reported for these studies, it is difficult to draw any conclusions from the simulated cycloguanil profiles for these simulations. At most, an increase in cycloguanil exposure was simulated once the induction of CYP1A2 was taken into account. However, these increases were minor and are likely not of clinical significance.

An attempt was also made to test the updated ritonavir inhibitor file with an available validated Simcyp substrate file; with ethinyl estradiol being chosen. This investigation again showed the importance of taking into account the induction effect of ritonavir on CYP1A2 for DDI studies with substrates that have their elimination mediated by this enzyme. The clinically observed reduction in ethinyl estradiol exposure was only observed once the induction of ritonavir on CYP1A2 was accounted for in the ritonavir inhibitor file, with subsequent studies showing that this DDI is driven by the induction effect of ritonavir on CYP3A and CYP1A2.

Despite the updated ritonavir inhibitor file being able to more accurately predict this DDI, it could not correctly predict the magnitude of the reduction in ethinyl estradiol exposure seen in literature. One potential reason for this is that the ritonavir inhibitor file does not take into account the known induction effect of ritonavir administration on UGTs.²⁸ Since ethinyl estradiol's elimination is mediated by UGT1A1, along with the other mentioned CYP enzymes, induction of UGTs by ritonavir could result in a more accurate prediction of this DDI.

4.5.2 Modelling limitations: Ritonavir

It should be noted that the ritonavir model enhancement work was not done according to scientific best practice. The optimal method to employ to carry out this work would be to measure both Ind_{max} and IndC_{50} *in vitro* with the use of hepatocytes. However, this is a costly endeavor and neither the necessary method nor materials were available to carry out this work.

As such, the work reported in that section should be considered exploratory. Despite this, the work still retains some scientific value as it serves to underline the importance of gathering this data and incorporating it into the ritonavir inhibitor file. Without doing so, simulated DDI investigations between ritonavir and a drug or new chemical entity that is a substrate of CYP1A2 performed on Simcyp cannot be considered accurate.

4.6 Conclusions: Chapter 4

In this chapter, the *in vitro* microsomal enzyme kinetic parameters of ritonavir were measured. Ritonavir metabolism is well characterized in literature, with it being metabolized rapidly both *in vitro* and *in vivo*. However, perhaps as a result of the difficulties arising from the rapidity of its metabolism, ritonavir's *in vitro* microsomal enzyme kinetic parameters have not been previously determined. An investigation into the *in vitro* fm,CYP of ritonavir was also undertaken, with this investigation confirming the previously known roles of CYP3A, CYP2D6, and CYP2C9 in ritonavir *in vitro* metabolism.

Since a ritonavir PBPK model was published for use in the Simcyp simulator, and no new discoveries were made here to update that model with, this published model was then used to investigate specific gaps in knowledge with reference to ritonavir DDIs. Specifically, the lack of inhibitory effect of ritonavir co-administration with mefloquine was explored. This investigation showed that it was not ritonavir's effect as an inducer of CYP3A that was limiting its ability to inhibit the metabolism of mefloquine by this enzyme. As a result, different avenues were considered.

The induction effect of ritonavir on CYP1A2 was then investigated since this enzyme was found to be involved in the metabolism of mefloquine. Since the published ritonavir inhibitor file did not take into account this induction, this effect was investigated, optimized, and incorporated into the existing file. This was achieved by manually optimizing the CYP1A2 Ind_{Max} parameter in the file so as to correctly predict a DDI between theophylline and ritonavir. An optimized value of 82 μ M was found to achieve this. Next, as a means to verify the accuracy of this value as well as to ensure that the induction effect holds true with ritonavir administered in the model along with lopinavir, a DDI study was simulated where caffeine was co-administered with LPV/r. This study showed that the optimized ritonavir inhibitor file was able to accurately predict CYP1A2 induction in simulated DDI studies when co-administered with lopinavir.

The optimized ritonavir inhibitor file was then used to recapture the reduction in mefloquine exposure upon LPV/r co-administration observed by Rattanapunya *et al.*²⁴ These simulations were able to predict the observed reduction in mefloquine exposure, though not the magnitude of this induction. This is in contrast to the published ritonavir file which predicted an increase in mefloquine exposure. This illustrates the importance of CYP1A2 induction in this interaction.

Moreover, the importance of incorporating CYP1A2-mediated metabolism of mefloquine into the mefloquine model was also investigated. This was done using the mefloquine substrate file available in the Simcyp members' area, which does not take CYP1A2 metabolism into account. As a result of this, the Simcyp mefloquine model could not predict the reduction on mefloquine exposure upon co-administration of LPV/r. This underlined the importance of this discovery as well as the incorporation of CYP1A2 induction into the ritonavir inhibitor file.

In addition to this, an attempt was made to recapture the reduction in proguanil exposure observed by Van Luin *et al.* upon co-administration of proguanil with both LPV/r and ATV/r combinations since the involvement of CYP1A2 in the metabolism of proguanil was confirmed here.²⁶ Overall, only minor changes were seen in these DDI simulations. However, a key difference was noted between the published and optimized ritonavir inhibitor files; that being that the optimized file predicted a reduction in proguanil AUC whereas the published file predicted an increase. However, these findings are unlikely to carry clinical significance.

The DDI between ritonavir and ethinyl estradiol was also investigated with the use of the available ethinyl estradiol substrate file as well as the updated ritonavir inhibitor file. This showed the importance of taking into account CYP1A2 induction by ritonavir as well as the need for the ritonavir inhibitor file to be updated to reflect its status as an inducer of UGTs. Doing so will likely yield improved predictions of DDIs between ritonavir and drugs that are substrates of any UGTs.

4.7 References

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Chapter 5 Summary, Conclusions and Future work

5.1 Summary and Conclusions

For two of the three clinically used drugs investigated here, *in vitro* metabolism studies were used to inform the development of PBPK models of these drugs with the use of the Simcyp simulator. In the case of mefloquine, these *in vitro* metabolism studies uncovered and confirmed the previously unreported contribution of CYP1A2 to mefloquine metabolism. This knowledge was then incorporated into the PBPK model developed for mefloquine through a middle-out PBPK modelling approach. The model was subsequently verified with the use of a number of available literature sources where it was shown that the model could successfully be used to simulate mefloquine exposures across different types of clinical studies.

Upon verification, this model was applied to a variety of novel scenarios of interest where it was able to show the potential for inhibitors of CYP1A2 to increase mefloquine exposure, suggesting that care should be taken if a CYP1A2 inhibitor is co-prescribed with mefloquine. In addition, the induction effect of smoking on CYP1A2 was also investigated. A notable difference in mefloquine exposure was observed between smokers and non-smokers when this effect was simulated. Given that a similar effect has previously been observed with quinine, a drug structurally related to mefloquine, and cigarette smoking, this could have clinical significance. However, since no significant relationship between cigarette smoking and blood concentrations of hydroxychloroquine, another drug structurally similar to mefloquine, or its main metabolite desethylchloroquine were found in a previous study, this might not be especially relevant for malaria patients. On the other hand, individuals travelling to malaria endemic areas and taking the commonly prescribed mefloquine as a prophylactic, may need to consider this effect if they are heavy smokers.

The model also showed evidence of a DDI between mefloquine and efavirenz, a drug which may be co-prescribed with mefloquine in HIV-infected patients. This potential interaction has been suggested in literature, but has not been investigated clinically. The work done here suggests a near 2-fold reduction in mefloquine exposure which, if this reduction in exposure could result in treatment failure or an increase in mefloquine resistance, should be definitively addressed with an *in vivo* study.

Lastly, the PBPK model confirmed the observation that mefloquine exposure is not affected by polymorphisms in CYP3A5 or by ethnic differences in Caucasian and black South African

populations. Malaria infection may be a more important contributor to the variability seen in the pharmacokinetics of mefloquine.

In the case of proguanil, the *in vitro* metabolism investigation identified the involvement of a previously unreported enzyme to proguanil metabolism; namely CYP2D6. It also confirmed the involvement of CYP1A2, which was previously suggested but not confirmed with the use of rCYP studies. This knowledge was then incorporated into a PBPK model developed for both proguanil as well as cycloguanil as its metabolite through a middle-out PBPK modelling approach. Both the proguanil and cycloguanil portions of the model were subsequently verified for single and multiple dose as well as DDI studies, despite the tendency of the model to overpredict cycloguanil exposure outside of studies where only single dose proguanil administration took place.

The developed and verified proguanil/cycloguanil model was subsequently applied to novel scenarios of interest. While proguanil exposure remained relatively stable in all application simulations, these runs showed the potential for CYP1A2 genotype to have an effect similar, but not of the same magnitude to, that of CYP2C19 on proguanil exposure. In addition, these simulations predicted a notable increase in cycloguanil exposure in a representative black South African population, indicating that a chief difference in the Simcyp Healthy Volunteers population file and the modified black South African population file is potentially an increase in clearance as a result of increased CYP expression or activity in the latter file. This effect could have clinical significance but would need to be explored *in vivo* on the African continent.

However, in general, the model application simulations illustrated the relative lack of interaction between proguanil/cycloguanil and factors that affect the activity of the CYP enzymes, outside of CYP2C19, involved in the conversion of proguanil to cycloguanil; whether it be co-administration with inhibitors or inducers or administration to individuals who have polymorphisms in these enzymes that encode for increased or decreased proguanil metabolism. This is likely as a result of the number of enzymes involved in proguanil metabolism with the involvement of five CYP enzymes making it difficult to elucidate the importance of changes in the genotype or the effect potential inducers or inhibitors of any one of the five may have on proguanil or cycloguanil exposure. Although, this does point to a potential strategy in future drug development. Given the potential negative effects DDIs have on drug regulatory approval, designing a drug that is metabolized in relatively equal amounts by a number of enzymes could circumvent this issue. This is of course incredibly difficult to do, especially in early drug

development where efficacy and tolerability are paramount. But, if DDIs do become an issue in regulatory approval for a potential new drug, shifting its metabolism away from any one enzyme could provide a way to avoid said DDIs.

In contrast to mefloquine and proguanil, the ritonavir *in vitro* metabolism investigation did not uncover a new CYP enzyme involved in its microsomal metabolism, although a bioanalytical method wherefrom the microsomal enzyme kinetics of ritonavir, namely its microsomal K_M and V_{max} , could be determined was developed. As a result of new discoveries not being made during the *in vitro* metabolism investigation to be used to inform the development of a ritonavir PBPK model, and since a ritonavir model for the Simcyp simulator has recently been published, the published ritonavir model was instead used to to investigate and explain some gaps in knowledge in ritonavir DDIs. This was done with the use of the mefloquine and proguanil/cycloguanil model developed and verified here since both drugs are metabolized by CYP3A; which is a major source of DDIs typically seen with ritonavir administration.

One of the main questions investigated was why ritonavir, despite its status as a potent inhibitor of CYP3A, did not have a stronger inhibitory effect on mefloquine PK, given mefloquine's status as a CYP3A substrate. This investigation, carried out by testing ritonavir's ability to induce CYP3A in Simcyp given its status as a mixed inducer and inhibitor of CYP3A, showed that the induction effect of ritonavir on CYP3A simulated in Simcyp was comparatively minor and could not explain the lack of inhibitory effect.

Given the discovery of the involvement of CYP1A2 in mefloquine metabolism, ritonavir's potential induction effect on this enzyme was next investigated to explain the lack of inhibitory effect ritonavir has on mefloquine PK. This was carried out by initially carrying out a review of available literature which suggested that the known induction effect of LPV/r treatment on CYP1A2 was as a result of ritonavir and not lopinavir. A study, which forms part of the ritonavir product label, was found which showed that ritonavir co-administration with theophylline, a known CYP1A2 substrate, resulted in an overall reduction in theophylline exposure. The published ritonavir inhibitor file was then optimized to recapture this reduction by optimizing its induction effect on CYP1A2. The accuracy of this simulated induction effect was then verified, along with lopinavir administration, by recapturing an increase in CYP1A2 activity as a result of LPV/r administration using caffeine, another known CYP1A2 substrate, as a probe. Upon verification, the ritonavir inhibitor file was updated with the optimized CYP1A2 Ind_{Max} parameter.

The optimized ritonavir inhibitor file was then used in an attempt to recover the reduction in mefloquine exposure observed upon co-administration of LPV/r by Rattanapunya *et al.* This investigation could predict the observed reduction in mefloquine exposure, although it could not accurately predict the magnitude of this reduction. Nevertheless, this is an improvement over the published ritonavir inhibitor file which predicted an increase in mefloquine AUC and highlights the importance of capturing the induction effect on CYP1A2 of ritonavir in these PBPK models.

In addition to the above, the importance of the discovery of the involvement of CYP1A2 in mefloquine metabolism and its subsequent incorporation into the mefloquine model was also investigated with the use of the optimized ritonavir file. This was achieved through the use of the mefloquine substrate file, which does not take into account CYP1A2 metabolism of mefloquine, available on the Simcyp members' area. This investigation clearly illustrated the importance of CYP1A2 since the Simcyp mefloquine file was unaffected by the induction of CYP1A2 by ritonavir and, by extension, could not predict the clinically observed reduction in mefloquine exposure. The above findings highlights the importance of capturing the induction effect on CYP1A2 of ritonavir in these PBPK models as well as the discovery of the involvement of CYP1A2 in mefloquine metabolism. Since these drugs could be routinely prescribed together in HIV patients being treated with malaria, having knowledge of this interaction and understanding its potential effects is of great clinical significance.

Proguanil is also known to be metabolized by CYP3A while the involvement of CYP1A2 in proguanil metabolism was confirmed in the work done here. As a result the optimized ritonavir inhibitor file was also used in an attempt to recapture the reduction in proguanil exposure observed by Van Luin *et al.* upon co-administration of proguanil with both LPV/r and ATV/r combinations. Only minor reductions in proguanil exposure were predicted in the simulations with the optimized ritonavir inhibitor file. However, it should be noted that the published ritonavir inhibitor file predicted a minor increase in proguanil exposure; likely as a result of the induction of CYP1A2 not being taken into account. In addition, since no cycloguanil plasma concentration-time profiles were reported for these studies, it is difficult to draw any conclusions from the simulated cycloguanil profiles for these simulations. At most, an increase in cycloguanil exposure was simulated once the induction of CYP1A2 was taken into account. However, these increases were minor and are likely not of clinical significance.

5.2 Potential future work

Important discoveries were made in the development, verification and application of the mefloquine PBPK model. However, the model was not able to accurately predict mefloquine absorption at dosages over 1000 mg. Remeasuring parameters related to mefloquine absorption could allow for the more accurate prediction of mefloquine absorption kinetics, thereby extending the range of doses the model is able to be used for as well as increasing the potential applications of the model as well. Although outside the scope of this work, an attempt can be made to model the effect of food on mefloquine disposition since it is known that mefloquine bioavailability is altered by the consumption of a high-fat breakfast. This will further increase the viability of the model for different applications.

Ultimately, the work done here was able to answer some questions that exist within mefloquine pharmacokinetics, specifically why a larger inhibitory effect is not seen with ritonavir co-administration as well as why a reduction in mefloquine exposure is observed with LPV/r administration instead of the expected increase in exposure. It was also confirmed that ethnicity has a minimal effect on mefloquine PK. However, the one main source of variability in mefloquine PK that was not investigated here remains to be studied; namely the effect of disease state on mefloquine exposure. Studying the physiological effects that malaria infection has on patients and incorporating them into a PBPK population file, as was done with the black South African TB file discussed in earlier chapters, could see PBPK modelling be used to investigate this effect. This will be a large undertaking but will be necessary in reducing variability in mefloquine PK and achieving better treatment outcomes.

In addition, mefloquine is prescribed as a hydrochloride salt of its two erythro-enantiomers. However, it has been shown in literature that the (+)-enantiomer has favourable PK as compared to its (-)-enantiomer counterpart, such as a shorter half-life resulting in a more rapid achievement of steady-state as well as kinetics not being time or concentration dependent as a result of a lack of enterohepatic recycling which is present with the (-)-enantiomer.¹ Given these improved PK characteristics, this opens the door for mefloquine to be administered exclusively as its (+)-erythro enantiomer. This should be further studied, with the model developed and verified here being a good base from which to predict potential *in vivo* exposures for this enantiomer. Moreover, the (+)-enantiomer has shown activity against *Mycobacterium avium* in mice.² If similar activity is seen in *Mycobacterium tuberculosis*, (+)-erythro-mefloquine could also potentially be repositioned as an anti-TB agent, where its long half-life and extended exposure could be a boon to TB treatment.

The proguanil model worked reasonably well and could accurately predict a variety of outcomes from proguanil administration. However, the model did show a resistance to the effect of inhibitors or inducers of CYP enzymes, or when simulations were done with individuals who had CYP polymorphisms different from the wild-type. This can potentially be overcome by properly accounting for the CYP mediated conversion of proguanil to CPB in the model. Doing so could also potentially improve predictions of cycloguanil exposure under certain conditions, such as when proguanil is administered to individuals who are CYP2C19 poor metabolizers.

Ritonavir is well studied, but developing PBPK models to predict its effects as a perpetrator of DDIs remains a major challenge, likely as a result of its ability to both inhibit and induce its metabolism and transport into and efflux out of cells given its status as a substrate of P-gp. In saying that, the recently published Simcyp PBPK models have been accurately verified for the stated applications. However, the work done here shows that ritonavir's induction effect on CYP1A2 needs to be accounted for to more accurately predict future DDIs. An investigation into measuring its induction of other CYPs and clinically important drug metabolizing enzymes as well as transporters and incorporating that knowledge into future models will be of great significance. Particularly to predicting potential DDIs for new medications which are to be used on the African continent where HIV infection, and by extension ritonavir administration, is, sadly, all too common.

5.3 References

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Chapter 6 Materials and Methods

6.1 Materials

Mefloquine, proguanil, cycloguanil, ritonavir, and all internal standards were sourced from Sigma-Aldrich (St Louis, MO, USA). Carboxymefloquine was kindly donated by our collaborators at Roche (Basel, Switzerland). Reagents, namely magnesium chloride, potassium mono- and dibasic, were sourced from Sigma-Aldrich (St Louis, MO, USA) as well. Purities were defined by the manufacturer as >98%. Solvents, namely acetonitrile, methanol, and 2-Propranol, were all LC-MS grade and were sourced from Honeywell Burdick & Jackson (Muskegon, MI, USA). Human liver microsomes (HLM) were sourced from Sekisui Xenotech (Kansas City, KS, USA). Purified isoforms of recombinant human CYPs, in the form of high reductase batosomes (CYP enzymes co-expressed in *Escherichia coli*), were obtained from Sekisui Xenotech (Kansas City, KS, USA) as well.

All analytical methods were conducted by LC-MS/MS. The specific instrumentation used was an Agilent HPLC system coupled to an AB Sciex 4500 QTRAP MS (ABSciex, Johannesburg, South Africa). Information regarding columns used, mobile phase composition, and gradient conditions are summarized in the relevant Chapters.

Stocks were stored in 1.5 mL polypropylene Eppendorf™ microcentrifuge tubes. These were also used to prepare calibration curves when necessary. All incubations were conducted in 2 mL 96 deep-well plates. After the incubation procedure was carried out, samples were transferred to 96-well microtiter plates for LC-MS/MS analysis.

6.2 Preparation of stock solutions

Stock solutions of the substrates were prepared at defined concentration ranges to ensure that the amount of organic solvent would be similar in all incubations. The final organic solvent concentrations were less than 0.5% for all incubations.¹² In all, 8 substrate concentrations and 2 metabolite concentrations were prepared for mefloquine and proguanil, since the metabolite formation approach was employed, and 8 substrate concentrations were used for ritonavir, since the substrate depletion approach was employed. The concentration ranges for the substrates and the exact concentrations used for the metabolites, as well as the solvents used to prepare all these solutions for each compound, are summarized in Table 6.1 below. All substrate stocks were stored at concentrations 200-fold higher than that used in the incubation.

Table 6.1: Summary of the preparation of incubation and calibration curve stock solutions

Approach	Analyte	Solvent	Incubation Stock Conc. Range (mM)	Metabolite Concentrations (mM)
Metabolite Formation	Mefloquine	ACN ^a /H ₂ O 95/5	1-50	-
	Carboxymefloquine	DMSO ^b	-	3.0, 2.4
Metabolite Formation	Proguanil	ACN ^a /H ₂ O 95/5	1-60	-
	Cycloguanil	DMSO ^b	-	3.0, 2.4
Substrate Depletion	Ritonavir	MeOH ^c (100%)	0.025-1	-

^aAcetonitrile, ^bDimethylsulfoxide, ^cMethanol

6.3 Preparation of Carboxymefloquine calibration curve

The standard curve was prepared using 1.5 mL polypropylene Eppendorf™ microcentrifuge tubes. Prior to the addition of the compound into the biological matrix, a working solution (WS) was first prepared in 50/50 water/acetonitrile (H₂O/ACN) for each standard and quality control (QC) sample. This was done by serially diluting the metabolite stock solutions, seen in Table 6.1 above, into the relevant WS for the standards and QCs, respectively. Subsequent to this, 50 µL of the corresponding WS was added to 950 µL of blank microsomal matrix, at the requisite microsome concentration for the assay in question. 50 µL of the biological matrix containing the compound at the required concentrations was then transferred to a 96 deep-well plate in duplicate and extracted by the addition of 300 µL ice-cold ACN containing the relevant internal standard. The mixture was then placed on a plate shaker for 10 minutes at 750 rpm before being centrifuged at 2630 x rcf for 30 minutes. 200 µL of the supernatant was then transferred to a 96 well analysis plate and submitted for analysis. In Tables 6.2-6.9 below is summarized the process by which the calibration standards and quality controls were prepared for the mefloquine and proguanil incubations.

Table 6.2: Preparation of working solutions for the calibration standards for carboxymefloquine

Working Solution	Source Solution	Volume of Source Solution (µL)	Blank Solvent (µL)	Metabolite Concentration (nM)
WS 1	MSS*	10.0	990	30000
WS 2	WS 1	500	500	15000
WS 3	WS 2	400	600	5000
WS 4	WS 3	400	600	1000
WS 5	WS 4	200	600	250
WS 6	WS 5	100	500	50.0
WS 7	WS 6	100	900	10.0

*Metabolite Stock Solution

Table 6.3: Preparation of calibration standards for carboxymefloquine

Calibration Standard	Volume of Matrix (mL)	Working Solution	Amount spiked (µL)	Final concentration (nM)
STD 1	0,950	WS 1	50,0	1500
STD 2	0,950	WS 2	50,0	750
STD 3	0,950	WS 3	50,0	250
STD 4	0,950	WS 4	50,0	50.0
STD 5	0,950	WS 5	50,0	12.5
STD 6	0,950	WS 6	50,0	2.50
STD 7	0,950	WS 7	50,0	0.50

Table 6.4: Preparation of working solutions for the quality control samples for carboxymefloquine

QC Working Solution	Source Solution	Volume of Source Solution (µL)	Blank Solvent (µL)	Metabolite Concentration (nM)
QCWS 1	MSS*	10	990	24000
QCWS 2	QCWS 1	500	500	12000
QCWS 3	QCWS 2	100	500	2000
QCWS 4	QCWS 3	100	900	200
QCWS 5	QCWS 4	100	900	20.0

Table 6.5: Preparation of quality control samples for carboxymefloquine

Quality Control	Volume of Matrix (mL)	Working Solution	Amount spiked (µL)	Final concentration (nM)
QC 1	0,950	QCWS 1	50,0	1200
QC 2	0,950	QCWS 2	50,0	600
QC 3	0,950	QCWS 3	50,0	100
QC 4	0,950	QCWS 4	50,0	10.0
QC 5	0,950	QCWS 5	50,0	1.00

Table 6.6: Preparation of working solutions for the calibration standards for cycloguanil

Working Solution	Source Solution	Volume of Source Solution (µL)	Blank Solvent (µL)	Metabolite Concentration (nM)
WS 1	MSS*	10.0	990	30000
WS 2	WS 1	500	500	15000
WS 3	WS 2	400	600	3000
WS 4	WS 3	400	600	600
WS 5	WS 4	200	600	200
WS 6	WS 5	100	500	80.0
WS 7	WS 6	200	800	20.0

Table 6.7: Preparation of calibration standards for cycloguanil

Calibration Standard	Volume of Matrix (mL)	Working Solution	Amount spiked (µL)	Final concentration (nM)
STD 1	0,950	WS 1	50,0	1500
STD 2	0,950	WS 2	50,0	750
STD 3	0,950	WS 3	50,0	150
STD 4	0,950	WS 4	50,0	30.0
STD 5	0,950	WS 5	50,0	10.0
STD 6	0,950	WS 6	50,0	4.00
STD 7	0,950	WS 7	50,0	1.00

Table 6.8: Preparation of quality control samples for cycloguanil

QC Working Solution	Source Solution	Volume of Source Solution (µL)	Blank Solvent (µL)	Metabolite Concentration (nM)
QCWS 1	MSS*	20	980	24000
QCWS 2	QCWS 1	200	800	4800
QCWS 3	QCWS 2	200	800	960
QCWS 4	QCWS 3	200	1000	160
QCWS 5	QCWS 4	200	600	40

Table 6.9: Preparation of quality control samples for cycloguanil

Quality Control	Volume of Matrix (mL)	Working Solution	Amount spiked (µL)	Final concentration (nM)
QC 1	0,950	QCWS 1	50,0	1200
QC 2	0,950	QCWS 2	50,0	240
QC 3	0,950	QCWS 3	50,0	48.0
QC 4	0,950	QCWS 4	50,0	8.00
QC 5	0,950	QCWS 5	50,0	2.00

A calibration curve was prepared for each incubation in which metabolite concentration was determined.

6.4 General incubation conditions

Phosphate buffer (0.1 mM, pH, 7.4) was prepared fresh monthly, or as needed, from 100 mM mono- and dibasic potassium phosphate stock solutions which were prepared fresh on the day the buffer was made. This was then stored at 8°C. Human liver microsomes (HLMs) were stored at -80°C and thawed on ice immediately before use. Each frozen stock was used once and left-over material was discarded. Magnesium chloride (MgCl₂) and NADPH solutions were prepared fresh on the day of use. Stock solutions of substrates and metabolites were

prepared in solvent for method development then prepared fresh for the kinetics studies as shown above. They were stored at 8°C for duration of use.

6.5 Incubation Procedure

Specific incubation conditions for each assay, such as microsomal or enzyme concentration and incubation duration, are defined in the relevant compound Chapter. In general, the assay procedure was initiated by thawing microsomes on ice, while pre-heating 0.1mM phosphate buffer (PB) (pH, 7.4) for a minimum of 10 minutes at 37°C. The incubation pre-mix was then made by diluting the now thawed microsomes from 20mg/ml to the appropriate microsomal concentration for the assay in question and diluting the MgCl₂ solution, prepared using PB, to 3.3 mM in pre-warmed PB. The eight substrate concentrations used for the relevant assay were then added to a 96 deep-well plate and the incubation pre-mix solution was added to each of these wells diluting the substrate concentrations two hundred times.

The deep-well plate containing the substrate and incubation pre-mix was then pre-heated at 37°C in a water bath for 5 minutes. After pre-heating, 45 µL of this pre-mix was transferred to each incubation plate, 96 deep-well plates marked for the relevant time points for the assay, in duplicate. The reactions were commenced by the addition of 5 µL of 10 mM NADPH, a solution prepared using PB, to each plate. Each plate was then incubated at 37°C for the allotted time.

Once the time allotted for each incubation was completed, the reactions were stopped with the addition of 300 µL ice-cold acetonitrile containing the relevant internal standard. Once reactions were completed, the incubation plates were placed in a -20°C fridge for 15 minutes. Subsequent to this, the plates were centrifuged for 30 minutes at 2630 x rcf with the centrifuge kept at 8°C. After centrifugation, 200µL of all samples were transferred to a 96-well analysis plate and submitted for LC-MS/MS analysis.

Other methodologies, such as those for linearity determination and substrate inhibition assays, are discussed in the relevant subsections of each Chapter for every compound.

Appendices

Appendix A: Mefloquine

A.1 Mefloquine Method Partial Validation

In order to have added confidence in the consistency, reproducibility, and robustness of the data generated here, a partial method validation plan was set out to create methods in the spirit of good laboratory practice. This validation was done for the metabolites for each reaction for which the metabolite formation approach was employed since the rate of metabolite formation was used to determine enzyme kinetics. As such, the following validation plan and validation criteria was set out prior to the commencement of the validation process:

Inter- and intra-run reproducibility, accuracy, and precision of the calibration curve of each method would be evaluated over the course of 3 days. Accuracy and precision, for both intra- and inter-day assessment, would need to be within a range of 85-115% of the theoretical concentration and a %CV of no more than 15% respectively for all calibration standards and QC samples with the exception of the lowest standard and QC; where these parameters are expanded to 80-120% and a %CV of no more than 20% respectively. In terms of intra-day reproducibility, at least 67% of all calibration standard and QC samples need to fall within the previously mentioned accuracy and precision criteria. For the inter-day evaluation, at least 67% of the replicates for each standard and QC concentration level need to fall within the accuracy and precision criteria. This translates to at least 4 of the 6 replicates for each standard and QC over the 3 days.

A representative calibration curve is shown in Figure A.1. This representative calibration curve was constructed using 7 calibration standards, in duplicate, over a range of 0.5-1500 nM. The procedure used to prepare this curve is described in Chapter 6. The curve shown was fitted to the regression which best fit the data in the case of both carboxymefloquine and cycloguanil, this was a quadratic regression with a weighting of $1/x$.

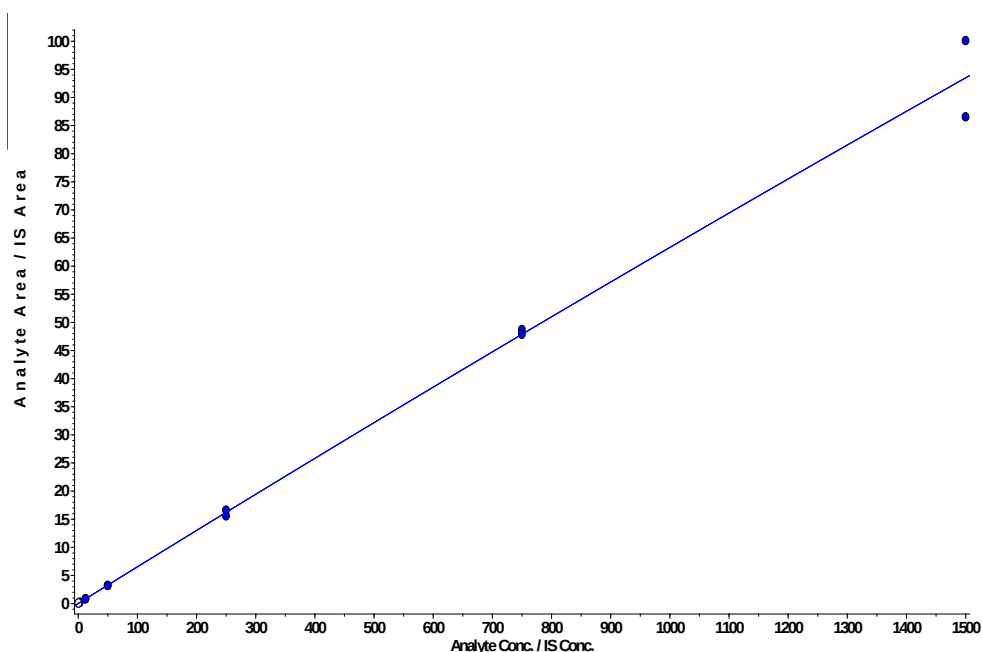


Figure A.1: Representative calibration curve of 7 duplicate calibration standards with a range of 0.5-1500 nM for carboxymefloquine

The calibration coefficient (r) represents the strength of the correlation between the analyte concentration and the peak area ratio (analyte peak area/IS peak area). This method performed well over the 3 validation days with the r value remaining at or above 0.99.

A.1.1 Mefloquine Intra-batch accuracy and precision: Day 1

Table A.1: Summary of back calculated standard concentrations for carboxymefloquine on validation day 1

	STD 1 1500 nM	STD 2 750 nM	STD 3 250 nM	STD 4 50 nM	STD 5 12.5 nM	STD 6 2.5 nM	STD 7 0.5 nM
Observed Mean Concentrations [nM]							
n = 2	1500	757	248	48.7	12.4	2.59	0.504
SD	2	2	2	2	2	2	1*
%CV	114	6.78	7.96	0.25	0.31	0.01	0.00
Accuracy	7.63	0.90	3.21	0.51	2.52	0.37	0.00
	99.8	101	99.1	97.4	99.0	103	101

*Value/s excluded as they did not meet acceptance criteria

Table A.2: Summary of back calculated quality control concentrations for carboxymefloquine on validation day 1

	STD 1 1200 nM	STD 2 600 nM	STD 3 100 nM	STD 4 10 nM	STD 5 1 nM
Observed Mean Concentrations [nM]					
n = 2	1120	597	97.2	10.5	0.979
SD	2	2	2	2	1*
%CV	45.9	35.8	3.02	0.52	0.00
Accuracy	4.11	5.99	3.11	4.98	0.00
	93.2	99.5	97.2	105	97.9

*Value/s excluded as they did not meet acceptance criteria

A.1.2 Mefloquine Intra-batch accuracy and precision: Day 2

Table A.3: Summary of back calculated standard concentrations for carboxymefloquine on validation day 2

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
	1500 nM	750 nM	250 nM	50 nM	12.5 nM	2.5 nM	0.5 nM
Observed Mean Concentrations [nM]							
	1510	729	258	52.7	13.2	2.56	0.429
n = 2	2	2	2	2	2	2	2
SD	47.27	41.71	0.42	0.67	0.01	0.07	0.01
%CV	3.13	5.72	0.16	1.27	0.09	2.78	2.10
Accuracy	101	97.2	103	105	106	102	85.8

Table A.4: Summary of back calculated quality control concentrations for carboxymefloquine on validation day 2

	STD 1	STD 2	STD 3	STD 4	STD 5
	1200 nM	600 nM	100 nM	10 nM	1 nM
Observed Mean Concentrations [nM]					
	1120	574	102	9.56	0.951
n = 2	2	2	2	2	2
SD	4.56	32.0	0.39	0.37	0.140
%CV	0.41	5.57	0.38	3.90	14.7
Accuracy	93.3	95.7	102	95.6	95.1

A.1.3 Mefloquine Intra-batch accuracy and precision: Day 3

Table A.5: Summary of back calculated standard concentrations for carboxymefloquine on validation day 3

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
	1500 nM	750 nM	250 nM	50 nM	12.5 nM	2.5 nM	0.5 nM
Observed Mean Concentrations [nM]							
	1490	765	244	48.1	12.6	2.44	0.532
n = 2	2	2	2	2	2	2	2
SD	113	40.0	6.75	1.51	0.44	0.12	0.05
%CV	7.56	5.23	2.77	3.13	3.50	5.05	9.02
Accuracy	99.6	102	97.7	96.2	101	97.5	106

Table A.6: Summary of back calculated quality control concentrations for carboxymefloquine on validation day 3

	STD 1	STD 2	STD 3	STD 4	STD 5
	1200 nM	600 nM	100 nM	10 nM	1 nM
Observed Mean Concentrations [nM]					
	1220	587	94.3	8.81	1.04
n = 2	1*	2	2	2	2
SD	0.00	38.3	8.71	0.24	0.12
%CV	0.00	6.52	9.24	2.71	11.5
Accuracy	102	97.8	94.3	88.1	104

*Value/s excluded as they did not meet acceptance criteria

A.1.4 Mefloquine Inter-batch accuracy and precision

Table A.7: Summary of back calculated standard concentrations for carboxymefloquine across the three validation days

Validation Day	Replicate	STD 1 1500 nM	STD 2 750 nM	STD 3 250 nM	STD 4 50 nM	STD 5 12.5 nM	STD 6 2.5 nM	STD 7 0.5 nM
Observed Concentrations [nM]								
1	1	1610	763	256	48.9	12.1	2.60	0,504
	2	1380	750	240	48.4	12.7	2.58	0.708*
2	1	1460	688	258	53.3	13.2	2.49	0,438
	2	1560	771	258	52.0	13.2	2.63	0,420
3	1	1610	805	251	49.6	13.0	2.56	0,580
	2	1380	725	237	46.6	12.2	2.31	0,484
n = 6		6	6	6	6	6	6	5*
Average		1500	750	250	49.8	12.7	2.53	0.462
SD		97.0	36.9	8.41	2.25	0.46	0.10	0.034
%CV		6,46	4.92	3.36	4.51	3.65	4.13	7.34
Accuracy		100	100	100	99.6	102	101	92.3

*Value/s excluded as they did not meet acceptance criteria

Table A.8: Summary of back calculated quality control concentrations for carboxymefloquine across the three validation days

Validation Day	Replicate	STD 1 1200 nM	STD 2 600 nM	STD 3 100 nM	STD 4 10 nM	STD 5 1 nM
Observed Concentrations [nM]						
1	1	1160	633	100	9.95	0.979
	2	1070	561	94.2	11.0	2.03*
2	1	1120	542	102	9.19	1.09
	2	1110	606	102	9.93	0.801
3	1	1220	625	103	9.05	1.16
	2	1000*	548	85.6	8.57	0.920
n = 6		5*	6	6	6	5*
Average		1140	586	97.8	9.61	0.990
SD		49.6	36.7	6.17	0.78	0.126
%CV		4.36	6.26	6.31	8.17	12.8
Accuracy		94.8	97.6	97.8	96.1	99.0

*Value/s excluded as they did not meet acceptance criteria

Overall, accuracy for all standards and QCs was within a range of 88.1-106%, well within the criteria. Precision for these methods, expressed as %CV, did not exceed 14%. This is, in part, as a result of a number of standards and QC's being excluded for being outside of acceptance criteria. However, at least 67% of each standard and QC were within acceptance criteria, thus being within the criteria in the pre-determined validation plan. Moreover, the low %CV illustrates the reproducibility of this method. This method was then considered to be precise and accurate within a concentration range of 0.5-1500 nM.

A.2 Mefloquine Model Verification

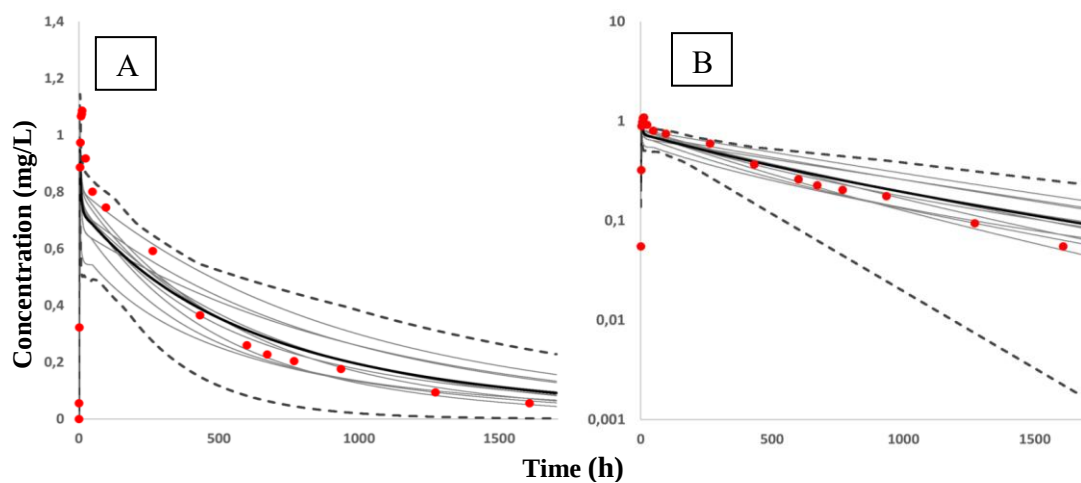


Figure A.2: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after a single oral dose of 750 mg mefloquine (A), (10 trials of 5 male subjects, 24-28 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Boudreau et al., 1990. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population

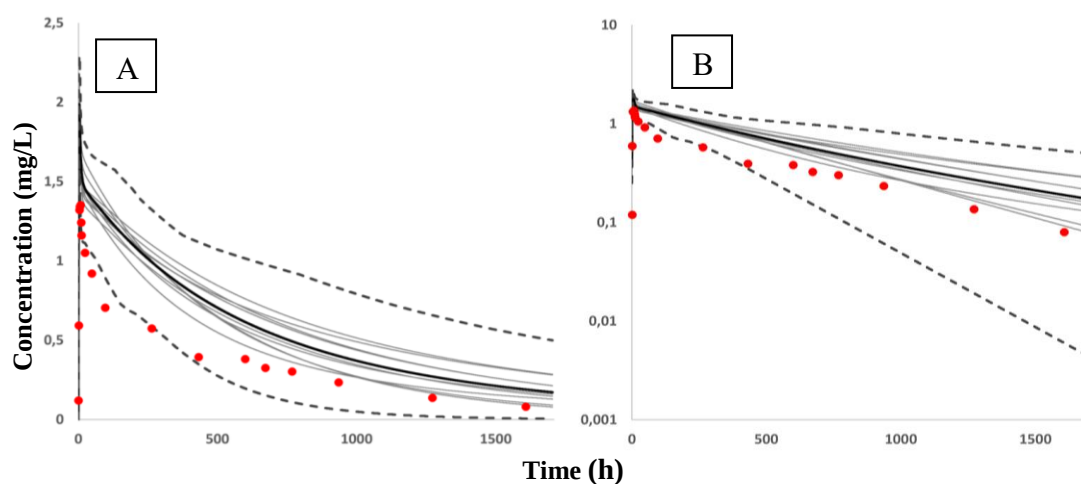


Figure A.3: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after a single oral dose of 1500 mg mefloquine (A), (10 trials of 5 male subjects, 24-35 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Boudreau et al., 1990. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population

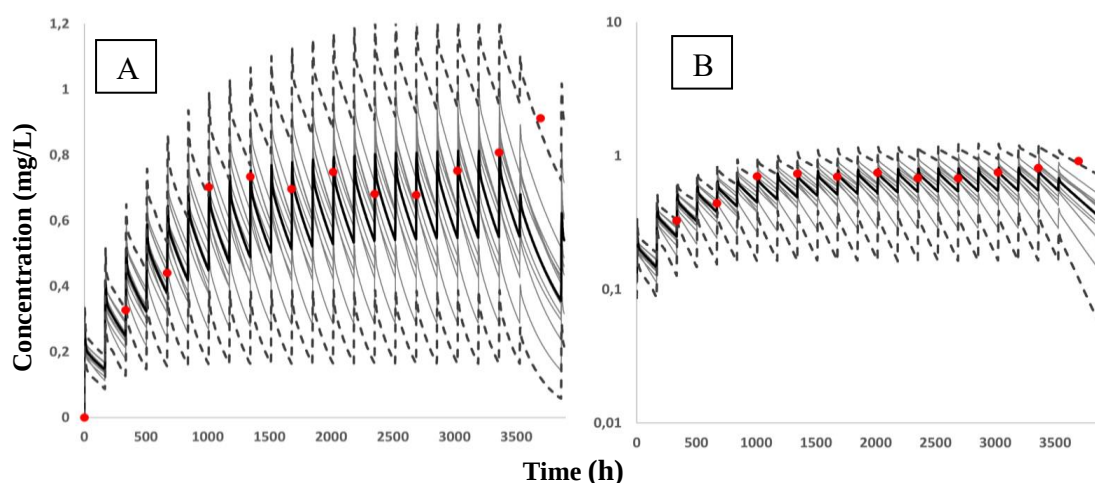


Figure A.4: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after 21 weekly 250 mg doses followed by 125 mg dose for one week then 250 mg in week 24 (A), (10 trials of 5 male subjects, 26-39 yrs) and the data plotted with the y-axis on a log scale scale (B). Observed data were extracted from Mimica et al., 1983. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

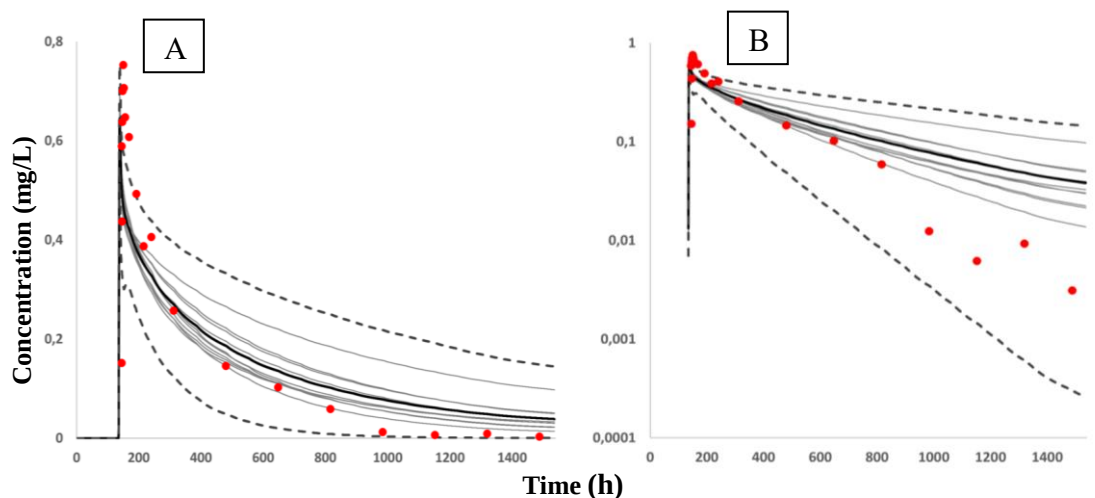


Figure A.5: Simulated (black line) and observed (red data points) mean plasma concentration-time profiles of mefloquine after single oral dose of 500 mg preceded by pre-treatment with 600 mg rifampicin, taken once daily for 7 consecutive days before mefloquine dose and 7 consecutive days after mefloquine dose (A), (10 trials of 7 male subjects, 24-35 yrs) and the data plotted with the y-axis on a log scale (B). Observed data were extracted from Ridditid et al., 2000. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

Appendix B: Proguanil

B.1 Proguanil Method Partial Validation

Partial validation of the proguanil analytical method followed the same validation plan as described above.

A representative calibration curve of cycloguanil is shown in Figure B.1. This representative calibration curve was constructed using 7 calibration standards, in duplicate, over a range of 1-1500 nM. The procedure used to prepare this curve is described in Chapter 6.

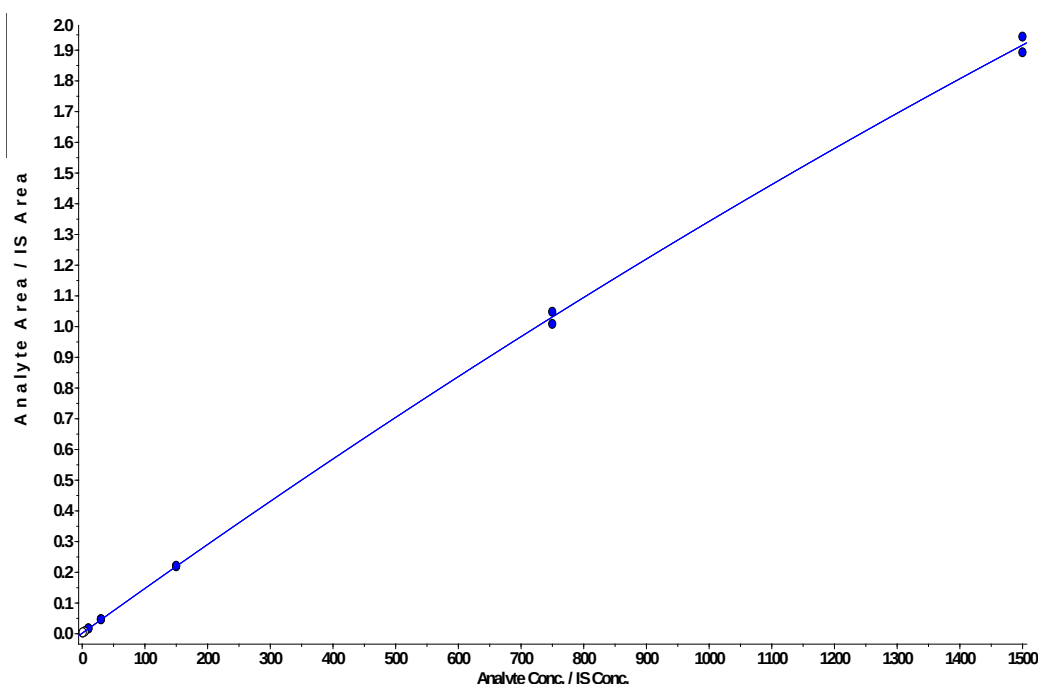


Figure B.1: Representative calibration curve of 7 duplicate calibration standards with a range of 0.5-1500 nM for cycloguanil

B.1.1 Proguanil Intra-batch accuracy and precision: Day 1

Table B.1: Summary of back calculated standard concentrations for cycloguanil on validation day 1

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
	1500 nM	750 nM	150 nM	30 nM	10 nM	4 nM	1 nM
Observed Mean Concentrations [nM]							
	1510	738	153	32.1	10.2	3.72	0.869
n = 2	2	2	2	2	2	1*	1*
SD	49.9	14.3	0.23	2.05	0.23	0.00	0.00
%CV	3.31	1.93	0.15	6.37	2.29	0.00	0.00
Accuracy	101	98.4	102	107	102	93.1	86.9

*Value/s excluded as they did not meet acceptance criteria

Table B.2: Summary of back calculated quality control concentrations for cycloguanil on validation day 1

	STD 1	STD 2	STD 3	STD 4	STD 5
	1200 nM	240 nM	48 nM	8 nM	2 nM
	Observed Mean Concentrations [nM]				
	1260	251	51.9	8.11	1.74
n = 2	2	2	1*	2	1*
SD	19.4	6.52	0.00	0.24	0.00
%CV	1.54	2.60	0.00	2.95	0.00
Accuracy	105	105	108	101	86.8

*Value/s excluded as they did not meet acceptance criteria

B.1.2 Proguanil Intra-batch accuracy and precision: Day 2

Table B.3: Summary of back calculated standard concentrations for cycloguanil on validation day 2

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
	1500 nM	750 nM	150 nM	30 nM	10 nM	4 nM	1 nM
	Observed Mean Concentrations [nM]						
	1500	747	150	30.7	10.2	3.77	0.965
n = 2	2	2	2	2	2	1*	1*
SD	23.5	15.5	0.61	0.08	0.11	0.00	0.00
%CV	1.57	2.08	0.40	0.27	1.10	0.00	0.00
Accuracy	100	99.7	100	102	102	94.1	96.5

*Value/s excluded as they did not meet acceptance criteria

Table B.4: Summary of back calculated quality control concentrations for cycloguanil on validation day 2

	STD 1	STD 2	STD 3	STD 4	STD 5
	1200 nM	240 nM	48 nM	8 nM	2 nM
	Observed Mean Concentrations [nM]				
	1190	233	50.2	7.93	1.74
n = 2	2	2	2	1*	2
SD	40.5	10.9	0.92	0.00	0.08
%CV	3.40	4.67	1.84	0.00	4.81
Accuracy	99.2	97.1	105	99.2	86.8

*Value/s excluded as they did not meet acceptance criteria

B.1.3 Proguanil Intra-batch accuracy and precision: Day 3

Table B.5: Summary of back calculated standard concentrations for cycloguanil on validation day 3

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
	1500 nM	750 nM	150 nM	30 nM	10 nM	4 nM	1 nM
	Observed Mean Concentrations [nM]						
	1480	780	137	28.5	10.0	4.23	1.05
n = 2	2	2	2	2	2	2	2
SD	32.3	13.4	1.81	0.84	0.52	0.15	0.13
%CV	2.18	1.71	1.32	2.96	5.19	3.63	12.47
Accuracy	98.9	104	91.3	95.0	100	106	105

Table B.6: Summary of back calculated quality control concentrations for cycloguanil on validation day 3

	STD 1 1200 nM	STD 2 240 nM	STD 3 48 nM	STD 4 8 nM	STD 5 2 nM
Observed Mean Concentrations [nM]					
	1210	248	47.7	8.41	2.25
n = 2	2	2	2	2	1*
SD	11.2	5.95	1.04	0.46	0.00
%CV	0.93	2.40	2.18	5.42	0.00
Accuracy	100	103	99.3	105	113

*Value/s excluded as they did not meet acceptance criteria

B.1.4 Proguanil Inter-batch accuracy and precision

Table B.7: Summary of back calculated standard concentrations for cycloguanil across the three validation days

Validation Day	Replicate	STD 1 1500 nM	STD 2 750 nM	STD 3 150 nM	STD 4 30 nM	STD 5 10 nM	STD 6 4 nM	STD 7 1 nM
Observed Concentrations [nM]								
1	1	1460	752	154	30.1	9.96	3.03*	0.869
	2	1560	724	153	34.2	10.4	3.72	0.206*
2	1	1480	763	150	30.8	10.4	3.77	0.965
	2	1530	732	151	30.6	10.1	3.12*	1.47*
3	1	1450	766	139	27.6	10.6	4.08	0.916
	2	1520	793	135	29.3	9.53	4.38	1.18
n = 6		6	6	6	6	6	4*	4*
Average		1500	755	147	30.4	10.2	3.99	1.00
SD		38.3	23.0	7.19	1.97	0.35	0.27	0.13
%CV		2.56	3.05	4.90	6.46	3.40	6.66	12.8
Accuracy		99.8	101	97.9	101	102	99.7	100

*Value/s excluded as they did not meet acceptance criteria

Table B.8: Summary of back calculated quality control concentrations for cycloguanil across the three validation days

Validation Day	Replicate	STD 1 1200 nM	STD 2 240 nM	STD 3 48 nM	STD 4 8 nM	STD 5 2 nM
Observed Concentrations [nM]						
1	1	1240	257	89.0*	7.87	1.11*
	2	1280	244	51.9	8.35	1.74
2	1	1150	222	49.2	5.60*	1.65
	2	1230	244	51.1	7.93	1.82
3	1	1220	242	46.6	8.86	2.25
	2	1200	254	48.7	7.95	2.46*
n = 6		6	6	5*	5*	4
Average		1220	244	49.5	8.19	1.86
SD		39.9	11.3	1.86	0.37	0.23
%CV		3.28	4.62	3.75	4.57	12.4
Accuracy		102	102	103	102	93.2

*Value/s excluded as they did not meet acceptance criteria

Accuracy for all standards and QCs was within a range of 86.8-113%, within the validation criteria set here. Precision for these methods, expressed as %CV, did not exceed 12.8%. This is, as with mefloquine, in part as a result of a number of standards and QC's being excluded for being outside of acceptance criteria. However, at least 67% of each standard and QC were within acceptance criteria, thus being within the criteria in the pre-determined validation plan. Moreover, the low %CV illustrates the reproducibility of this method. This method was then considered to be precise and accurate within a concentration range of 1-1500 nM.

B.2 Proguanil/Cycloguanil Model Verification

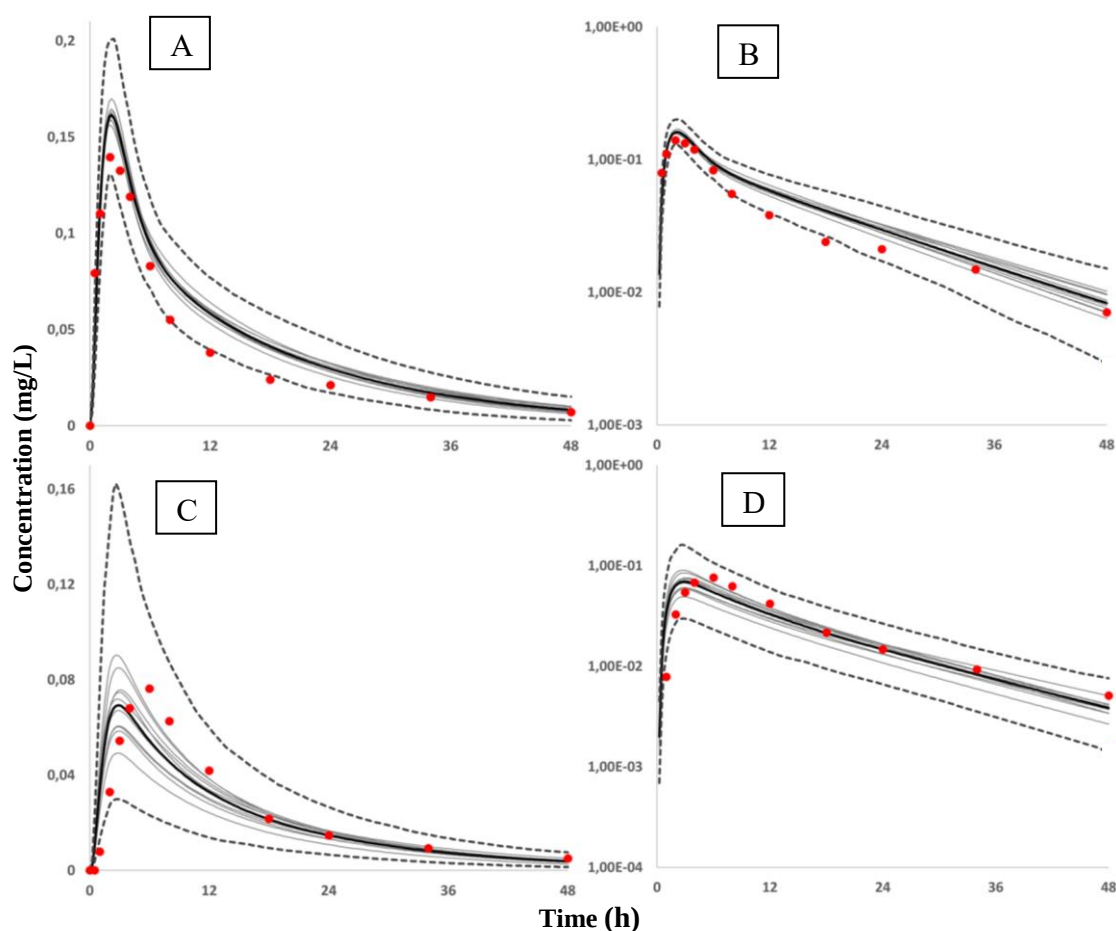


Figure B.2: Simulated (black line) and observed (red dots) mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 12 male subjects, 20-31 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil. Observed data, red dots, were extracted from Funck-Brentano et al. 1997. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

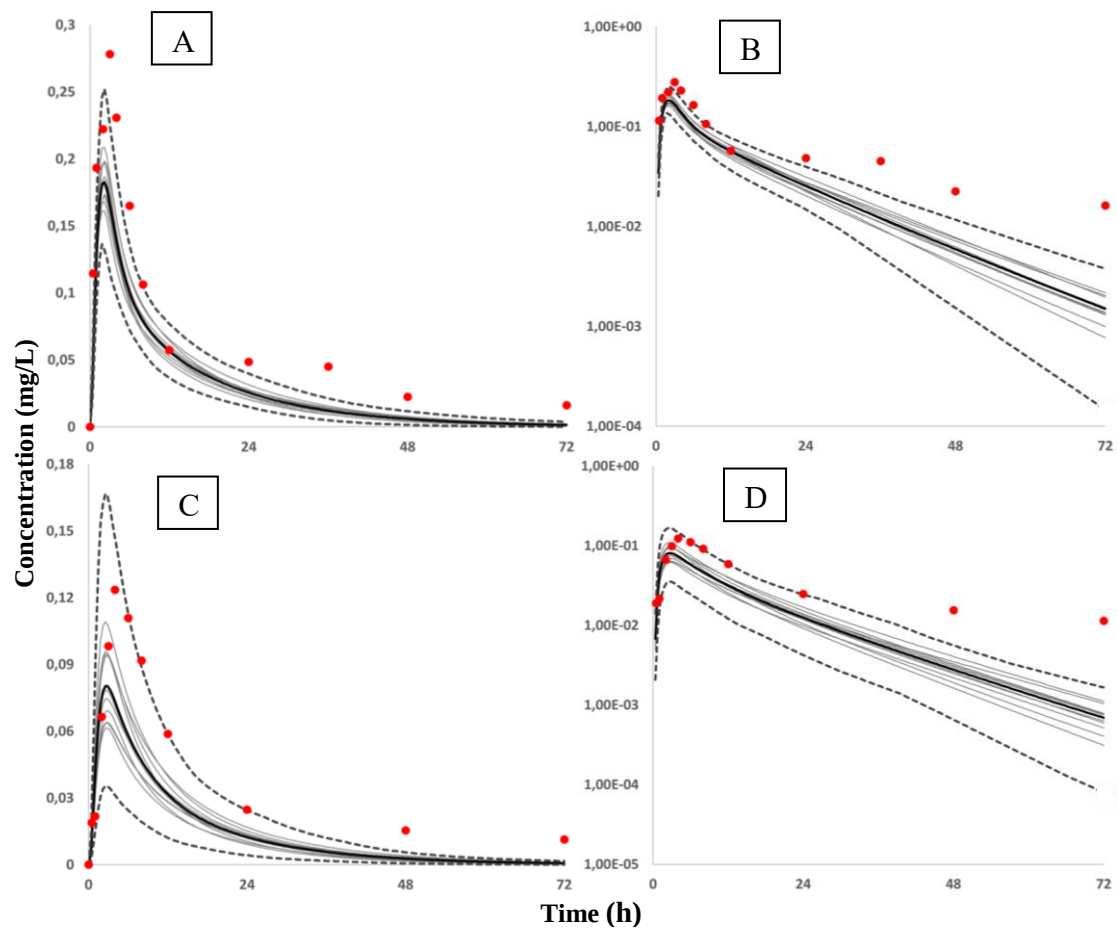


Figure B.3: Simulated (black line) and observed (red dots) mean plasma concentration-time profiles of a single oral dose of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 10 male subjects, 20-30 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil. Observed data, red dots, were extracted from Helsby et al. 1990. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

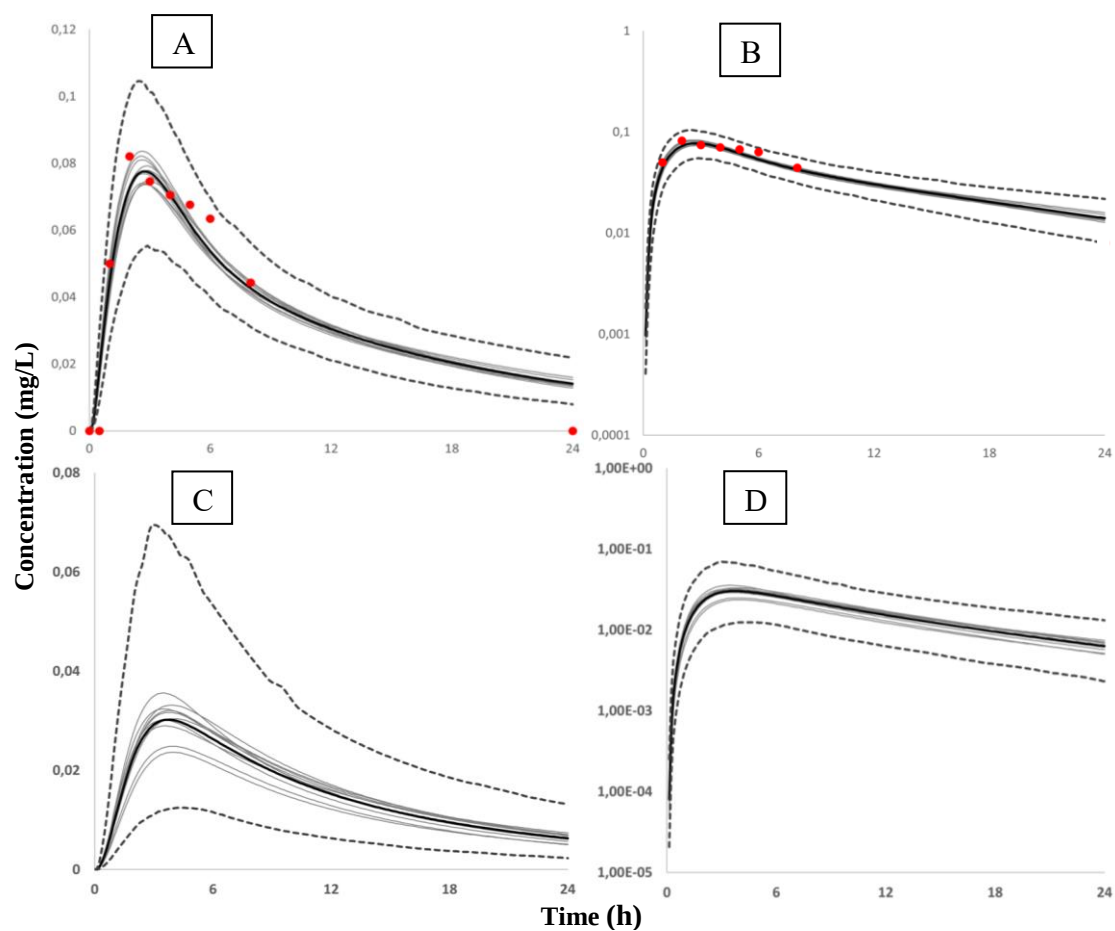


Figure B.4: Simulated (black line) and observed (red dots) mean plasma concentration-time profiles of a single oral dose of 100 mg of proguanil (A), cycloguanil (C) (10 trials of 10 male and 8 female subjects, 20-26 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil. Observed data, red dots, were extracted from Van Luin. et al. 2010. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

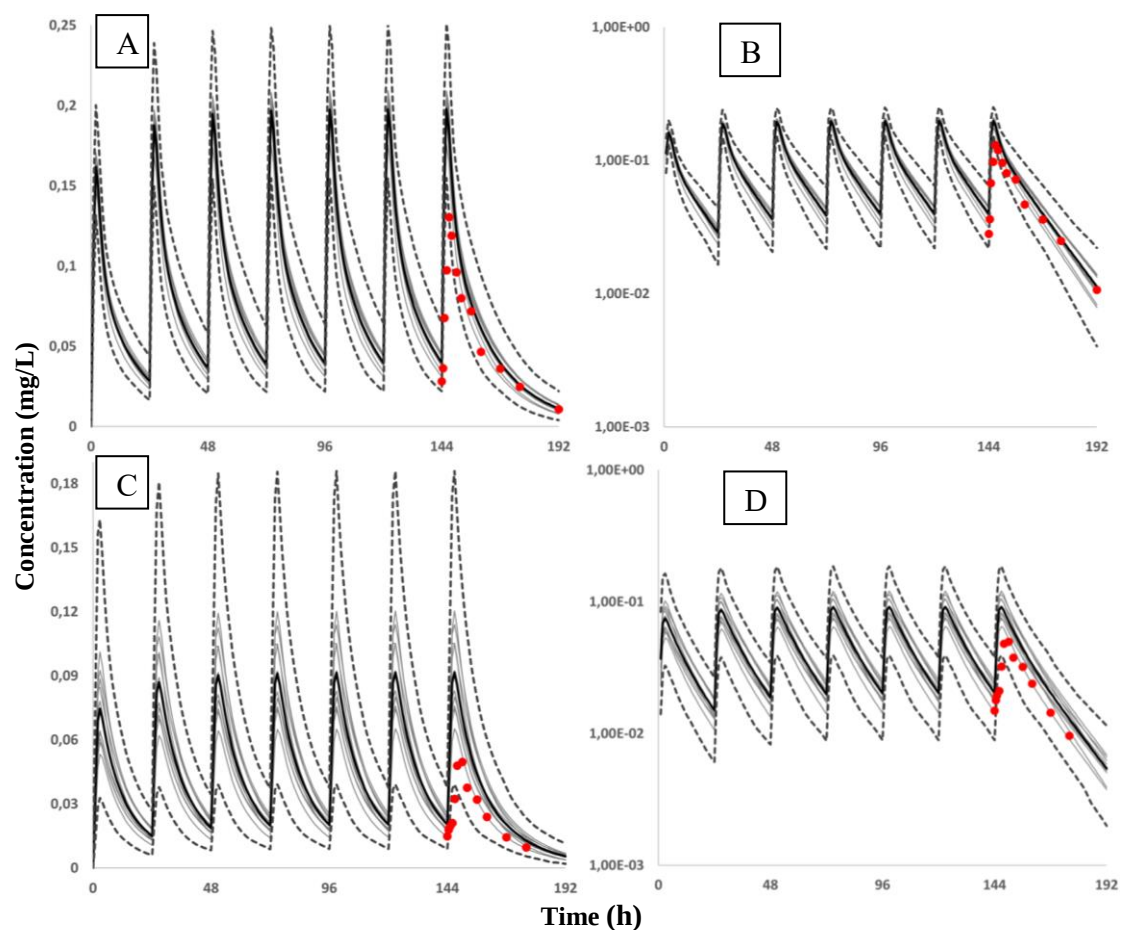


Figure B.5: Simulated (black line) and observed (red dots) mean plasma concentration-time profiles of multiple oral doses of 200 mg of proguanil (A), cycloguanil (C) (10 trials of 6 male subjects, 24-32 yrs) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil. Observed data, red dots, were extracted from Edstein. et al. 1988. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

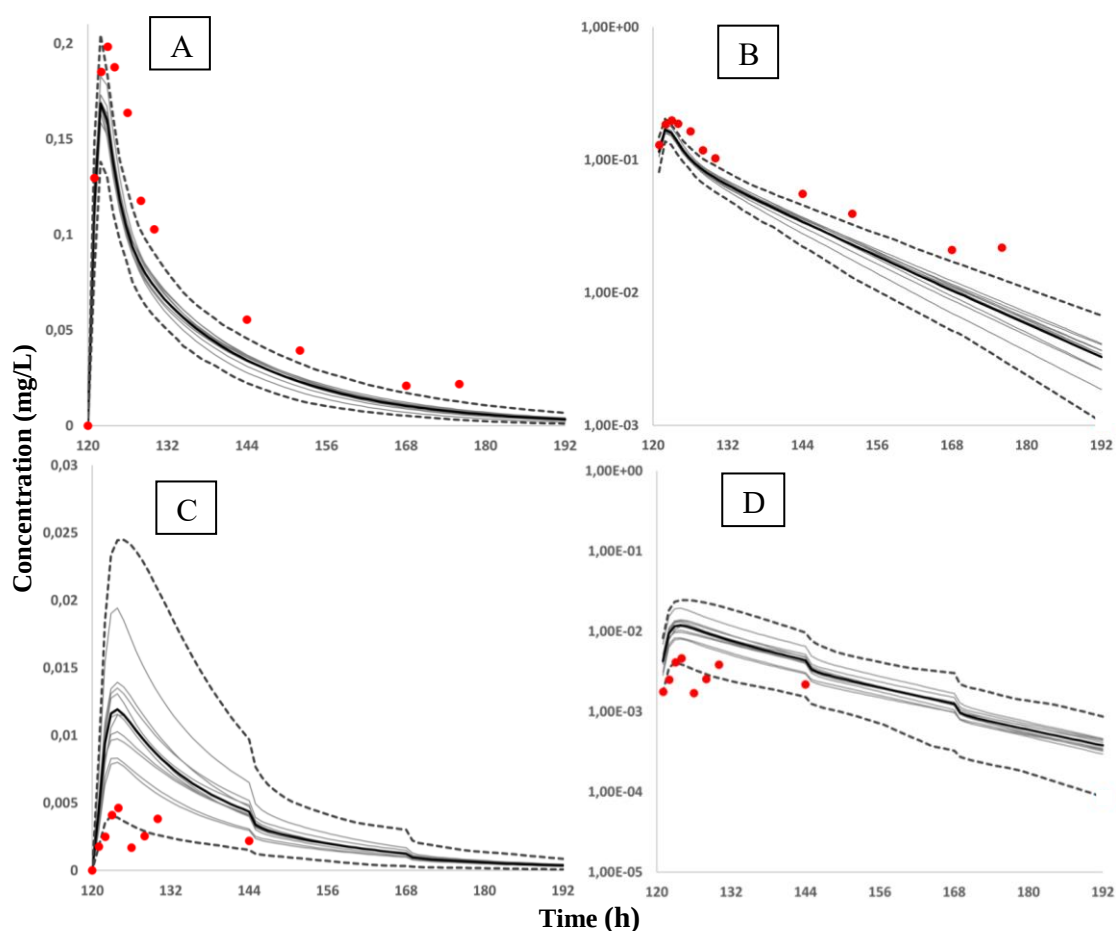


Figure B.6: Simulated (black line) and observed (red dots) mean plasma concentration-time profiles of proguanil (A), cycloguanil (C) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil after single oral dose of 200 mg preceded by pre-treatment with 100 mg of fluvoxamine, taken once a day for 7 consecutive days before proguanil administration. Proguanil was then administered on the 7th day along with the final fluvoxamine dose, (10 trials of 6 male subjects, 22-30 yrs). Observed data, red dots, were extracted from Jeppesen. et al. 1997. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.

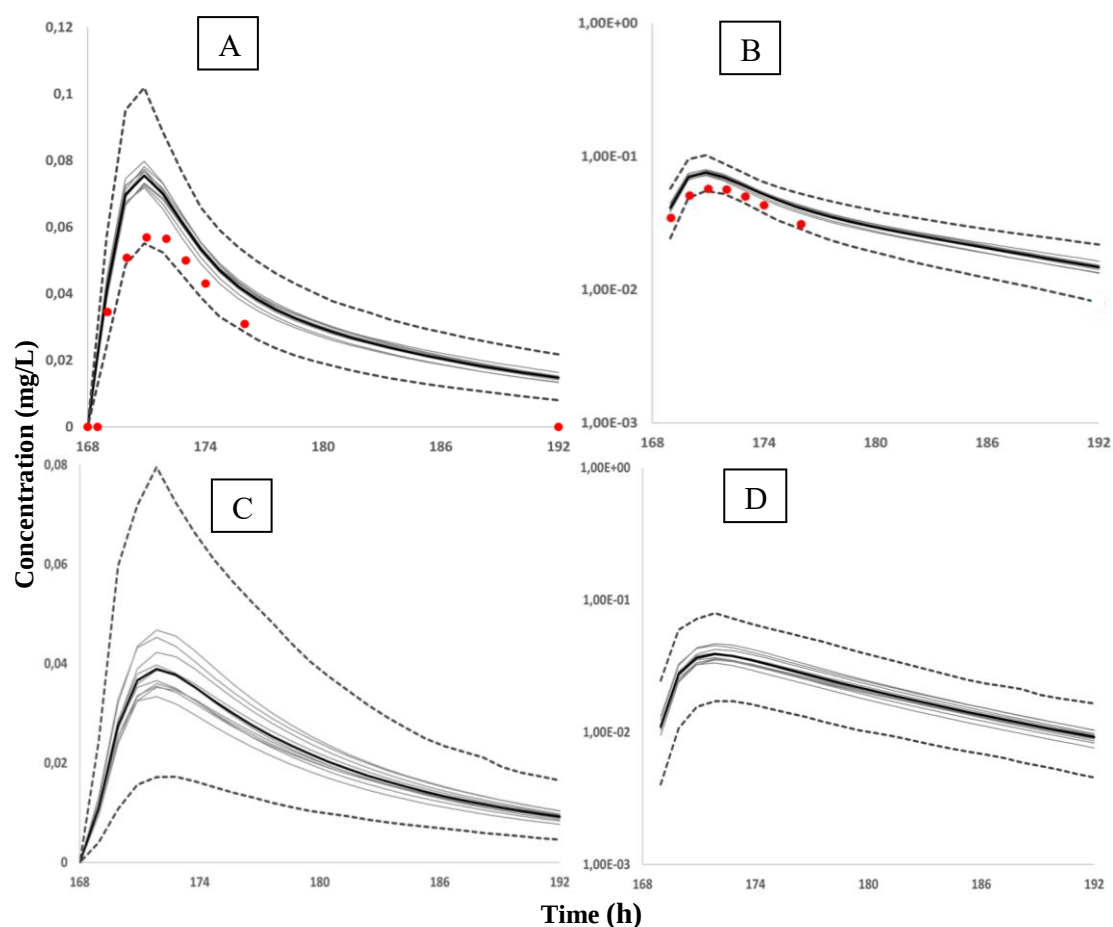


Figure B.7: Simulated (black line) and observed (red dots) mean plasma concentration-time profiles of proguanil (A), cycloguanil (C) and the data plotted with the y-axis on a log scale (B) proguanil, and (D) for cycloguanil after single oral dose of 100 mg preceded by pre-treatment with 600 mg efavirenz, taken once a day for 7 consecutive days before proguanil administration. Proguanil was then administered on the 7th day along with the final efavirenz dose, (10 trials of 18 male and 2 female subjects, 35-60 yrs). Observed data, red dots, were extracted from Van Luin. et al. 2010 with the patients involved in the study receiving chronic efavirenz treatment. The grey lines represent the predictions from individual trials. Dashed lines represent the 5th and 95th percentiles of the total virtual population.