Pharmacokinetics of artemisinin derivatives in rats, healthy volunteers and patients.

Sofia Birgersson

Department of Pharmacology
Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2015

Pharmacokinetics of artemisinin derivatives in rats, healthy volunteers and patients.

sofia.birgersson@pharm.gu.se
sofia.birgersson@outlook.com

ISBN 978-91-628-9531-0 (print)
ISBN 978-91-628-9532-7 (pdf)
Printed in Gothenburg, Sweden 2015
Ineko, Gothenburg

It always seems impossible until it’s done

Nelson Mandela

Pharmacokinetics of artemisinin derivatives in rats, healthy volunteers and patients.

Sofia Birgersson

Department of Pharmacology, Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

ABSTRACT

Malaria is still a major health problem, killing approximately 1,600 people each day. The most vulnerable patient groups are children under the age of five and pregnant women. Artemisinin-based combination therapy is recommended by the World Health Organization as first-line treatment of uncomplicated *P. falciparum* malaria. The aim of this thesis was to investigate the pharmacokinetic properties of artemisinin derivatives with particular focus on pregnancy. As part of the thesis, a sensitive and accurate bioanalytical method for the quantification of artesunate and dihydroartemisinin in plasma and saliva using tandem mass spectrometry was developed. Furthermore, the population pharmacokinetic properties of artemisinin, artesunate and dihydroartemisinin were characterized in pregnant and non-pregnant rats, healthy volunteers and in pregnant and non-pregnant patients, using nonlinear mixed-effects modelling. In conclusion, a bioanalytical method has been developed for non-invasive saliva sampling in order to support high-quality pharmacokinetic field studies and in populations where invasive sampling is unethical or difficult, e.g. pediatric and pregnant studies. Furthermore, this thesis advances our pharmacokinetic understanding of antimalarial drugs. The pharmacokinetic effects of pregnancy in rats were similar to those seen in humans which imply that this animal model could be useful in translational studies in early pregnancy. The developed pharmacokinetic model in healthy volunteers was validated and could be of use in future drug development studies. A lower antimalarial drug exposure was demonstrated in pregnant women with malaria indicating the need for dose adjustment in this vulnerable patient group.

Keywords: malaria, artemisinin, artesunate, dihydroartemisinin, LC-MS/MS, pharmacometrics, pregnancy

ISBN: 978-91-628-9531-0 (print)

SAMMANFATTNING PÅ SVENSKA

Malaria är fortfarande ett stort hälsoproblem i tropiska länder, speciellt i Afrika söder om Sahara. Malaria orsakas av parasiter av släktet plasmodium som överförs till människan genom ett bett av anopheles-myggan. Enligt den senaste rapporten från Världshälsoorganisationen inträffade det 198 miljoner fall av malaria och 584 000 dödsfall under 2013. Av dessa dödsfall var 90% i Afrika och 78% bland barn under 5 år. Förutom unga barn är gravida kvinnor en särskilt utsatt och känslig grupp när det gäller att bli smittad av malaria men även att utveckla den allvarligare formen av sjukdomen. Den rekommenderade behandlingen av malaria är en kombination av ett artemisininderivat och ett läkemedel med längre verkan, en så kallad artemisininbaserad kombinationsbehandling (ACT). Syftet med denna avhandling var att utveckla en metod för att mäta halten av artemisininderivaten i kroppen. Vidare var syftet att, med hjälp av matematisk och statistisk modellering, beskriva farmakokinetiken (hur läkemedlet rör sig i kroppen och hur det elimineras) för dessa läkemedel i gravida och icke-gravida råttor, och i gravida och icke-gravida kvinnliga patienter. En grupp friska frivilliga män undersöktes även med avseende på en ny läkemedelsformulering (en mikroniserad formulering) storlek på dos och eventuell interaktion med ett annat mer långverkande malarialäkemedel, piperakin. En analysmetod med hjälp av vätskekromatografi kopplad till masspektrometri utvecklades för att bestämma halten av artesunat och dess aktiva metabolit dihydroartemisinin i plasma och saliv. I djurmodellen upptäcktes skillnader mellan de gravida och icke-gravida djuren som kan påverka exponeringen av läkemedlet och därmed påverka dess effekt. Studien i friska frivilliga män visade att en ökad dos påverkade hur lång tid det tog för läkemedlet att tas upp i kroppen. Den mikroniserade formuleringen och interaktionen med piperakin påverkade inte farmakokinetiken. Farmakokinetiken för patienterna visade att de gravida kvinnorna hade en lägre exponering av den aktiva metaboliten än de icke-gravida. Sammanfattningsvis har en analysmetod för haltbestämning av artesunat och dihydroartemisinin utvecklats i plasma och saliv. En modell i råtta har utvecklats som följer resultat man tidigare sett i människa vilket stödjer att detta skulle vara en bra djurmodell för translation till människa. En farmakokinetisk beskrivning av artesunat och dihydroartemisinin har gjorts i gravida och icke-gravida kvinnor och visat att exponeringen minskar under graviditet vilket kan kräva en dosökning. Farmakokinetiken för artemisinin i friska frivilliga har för första gången beskrivits i en populationsmodell. Denna modell kan troligtvis användas i framtida läkemedelsstudier.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Sofia Birgersson, Therese Ericsson, Antje Blank, Cornelia von Hagens, Michael Ashton, & Kurt-Jürgen Hoffmann. A high-throughput LC–MS/MS assay for quantification of artesunate and its metabolite dihydroartemisinin in human plasma and saliva
Bioanalysis. 2014 Sep;6(18):2357-69.

II. Sofia Birgersson, Joel Tarning, Kurt-Jürgen Hoffmann, Michael Ashton, Angela Abelö. Pharmacokinetics of artesunate after intravenous and oral administration in pregnant and non-pregnant rats.
In manuscript

III. Sofia Birgersson, Pham Van Toi, Nguyen Thanh Truong, Nguyen Thi Dung, Michael Ashton, Tran Tinh Hien, Angela Abelö, Joel Tarning. Population pharmacokinetic properties of artemisinin in healthy male Vietnamese volunteers.
Submitted.

IV. Sofia Birgersson, Innocent Valea, Halidou Tinto, Maminata Traore, Laeticia C. Toe, Jean-Pierre Van Geertruyden, Geraint R. Davies, Stephen A. Ward, Umberto D'Alessandro, Angela Abelö, Joel Tarning. Population pharmacokinetics of artesunate in pregnant and non-pregnant women with uncomplicated Plasmodium falciparum malaria in Burkina Faso.
In manuscript

CONTENT

ABBREVIATIONS

ACT	Artemisinin-based combination therapy
CYP	Cytochrome P450
HPLC	High performance liquid chromatography
MS/MS	Tandem mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LLOQ	Lower limit of quantification
OFV	Objective function value
PRED	Population prediction
IPRED	Individual prediction
BLQ	Data below the limit of quantification

DEFINITIONS IN SHORT

Pharmacokinetics	What the body does to the drug [1].
Pharmacodynamics	What the drug does to the body [1].
Bioanalysis	Quantitative analysis of a drug and/or its metabolites in a biological matrix [2].
Pharmacometrics	Branch of science concerned with mathematical models of biology, pharmacology, disease and physiology used to describe and quantify interactions between xenobiotics and patients [3].
Population pharmacokinetics	The study of the variability in plasma drug concentrations between individuals when standard dosage regimens are administered [4].

1 INTRODUCTION

Malaria is one of the world's most deadly infectious diseases still claiming nearly 2000 deaths every day [5]. This thesis focuses on the artemisinin derivatives in treatment of malaria and the characterization of their pharmacokinetic properties with a special focus on pregnancy effects.

The thesis consists of four different research papers. In the first paper, a bioanalytical method for the quantification of artesunate and dihydroartemisinin in plasma and saliva using liquid chromatography coupled to a mass spectrometer was developed (Paper I).
To characterize the effects of pregnancy on the pharmacokinetic properties during early and late pregnancy, a study of artesunate and the active metabolite, dihydroartemisinin, in the rat was performed (Paper II).
The pharmacokinetic properties in healthy male volunteers were investigated for artemisinin using a population modeling approach (Paper III).
In the last paper, the pharmacokinetic properties of artesunate and dihydroartemisinin, with focus on pregnancy effect, were investigated (Paper IV).

The chapters in this thesis are organized as follow. Chapter 1 offers an introduction to the field and familiarizes the reader with the theory behind the methodology used. Chapter 2 presents the broad aim of the thesis. Chapter 3 presents the methodology in detail and chapter 4 and 5 describe the results and discuss the impact of the findings in this thesis, respectively. Chapter 6 and 7 present the main conclusions of the thesis and future perspectives.

1.1 Malaria

Malaria is one of the most deadly diseases in the world, with the highest burden in sub-Saharan Africa [5]. According to the World Health Organization an estimated 3.2 billion people are at risk of being infected, and in 2013 there were 198 million cases of malaria globally. The disease causes an estimated 584 000 deaths yearly where 90% occurs in the African region mostly in children under the age of five (78% of all deaths).

Malaria is an infectious disease caused by the *Plasmodium* parasite. Five species infect humans; *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium falciparum. P. falciparum* causes the most severe infection and the severity is related to the relatively high parasite load during infection and a reduced microcirculatory flow [6], [7]. The latter is related to a process called sequestration, where the parasite infected erythrocytes adhere to the capillary endothelium lining. This results in blockage of the transport of oxygen and nutrients, but it also makes the parasite undetectable in peripheral blood samples and provides an escape from spleen removal.

The parasites are transmitted to humans via the bite of the female *anopheles* mosquito. This dual host parasite has different life-cycles in the human and in the mosquito (Figure 1) [8], [9]. In humans, the cycle starts by the injection of the parasite sporozoites during a blood meal (Figure 1. A). The sporozoites then rapidly invade the liver and the hepatocytes (Figure 1. B), where they grow, divide and mature into schizonts. After rupture of the hepatocyte, merozoites are released into the blood stream (Figure 1. C) where they infect the erythrocytes. Inside the erythrocyte an asexual replication takes place, in which an early ring stage of malaria develops into trophozoites and blood schizonts, which upon rupture of the erythrocyte, releases new merozoites into the blood stream. For *P. falciparum* this asexual stage takes approximately 48 hours and these cycles are responsible for the characteristic fever symptoms of malaria [7].

The released merozoites either enter a new asexual cycle or enter a sexual replication where the merozoite matures into male and female gametocytes. Gametocytes are the sexual form spreading the disease after being consumed by the biting mosquito (Figure 1. D) [8].

Inside the mosquito the parasite enters the sporogonic cycle. Already in the mosquito stomach the male and female gametocytes generates zygotes that becomes ookinetes and sequentially oocytes. From the oocytes sporozoites are released into the salivary glands and the sporozoites can be transferred to a human during a blood meal.

Early symptoms are fever, headache, chills and vomiting but if left untreated the infection can progress to the severe form of malaria. Severe malaria follows a multi-system disorder, with severe anemia, cerebral malaria, renal failure, pulmonary edema, often leading to death [10].

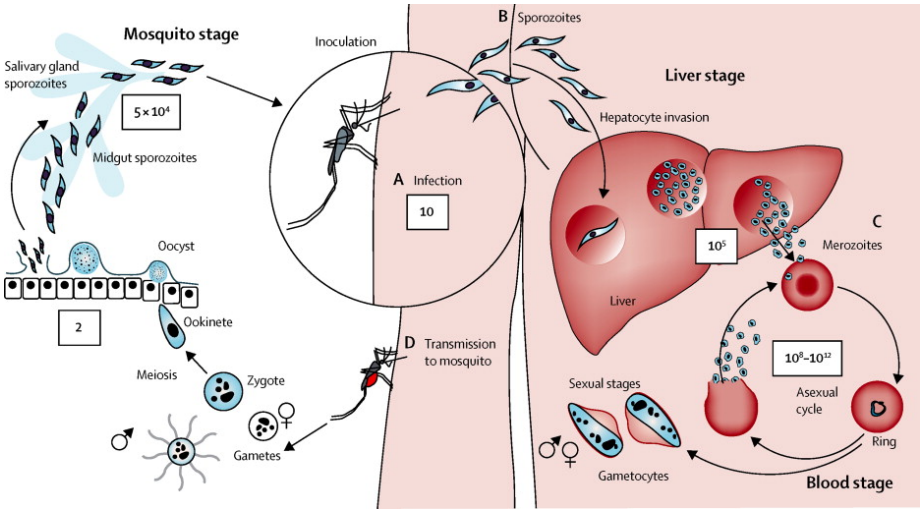

Figure 1. Life cycle of the malaria parasite.
Reprinted from The Lancet, with permission from Elsevier[9] .

1.1.1 Malaria in pregnancy

There are approximately 32 million women getting pregnant each year in sub-Saharan malaria endemic countries [5]. In high-transmission areas of malaria, adults have gradually been exposed to the disease for a long time, causing a semi-immunity to malaria (acquired immunity) [11]. However, during pregnancy the acquired immunity to malaria decreases and these women have an increased risk of contracting malaria but also to progress to the severe state of malaria [12]–[15]. Under the assumption that insecticide treated bed nets are used and the mosquitos bite primarily at night-time, this could partly be explained by behavior changes. These women generally use the bathroom more often at night, which in turn increases the exposure to the mosquitos. During pregnancy the physiology is altered resulting in an increased body temperature and an increased volume of breath which attracts the mosquitos. Pregnant women also have a new organ, the placenta, which does not have the acquired immunity gained during several years of malaria exposure. Thus, primigravida women are at higher risk of acquiring malaria and develop severe malaria, compared to multigravida women [16]. The immune system is also altered during pregnancy to prevent the body from rejecting the fetus [14]. Infection of malaria during pregnancy increases the risk of maternal anemia and the risk of delivering a baby with low birth weight (LBW) which is strongly associated with infant mortality [17]. LBW can be caused by even a single episode of malaria infection during pregnancy and the mechanism can be either intrauterine growth retardation or preterm birth or a combination of both [18].

1.1.2 Resistance

Development of drug-resistance against antimalarial drugs has traditionally started in Western Cambodia. This was previously seen for both chloroquine and sulfadoxine/pyrimethamine [19]. Several reports indicate emerging artemisinin-resistant parasites in Southeast Asia characterized by increased parasite clearance times in patients with *falciparum* malaria [20]–[24]. Attempts have been made to identify a molecular marker for artemisinin resistance, and several Kelch13-propeller gene mutations are suggested to be causally associated with artemisinin resistance [25], [26]. In recent studies, evidence for resistance also to the artemisinin partner drug, piperaquine, was found [26], [27]. In the work by Leang *et al*, a significantly higher proportion of patients with recrudescent malaria were found in the Western Cambodia compared to the eastern parts. Despite this emerging resistance, artemisinin and its derivatives are still effective in Africa and most regions of Southeast Asia. Treatment failure is commonly less than 5% at day 28 when administered in a combination with a longer acting antimalarial drug in *falciparum* malaria but if the resistance would spread it would severely limit our ability to combat malaria resulting in increasing number of severe malaria and deaths [28], [29]. The recommendation by Leang *et al* is to start using triple combinations of artemisinin derivatives and longer acting compounds.

1.2 Treatment of malaria

Since, the year 2000, malaria mortality rate has decreased by 47% worldwide and this is partly a result of the implementation of artemisinin-based combination therapy (ACT) [5]. The WHO first-line recommendation for treatment of uncomplicated *falciparum* malaria is three days of an ACT, consisting of one artemisinin derivative and one long-acting partner drug [30]. In 2013, a total of 392 million treatments of ACTs were delivered to private and public health care. The artemisinins have a short half-life, they are highly effective and eliminates the majority of the parasite biomass during the first three days of treatment [31]. The partner drugs have a longer elimination half-life and different mechanisms of action compared to the artemisinins. These longer acting drugs eliminate the remaining parasites and minimize the risk of recrudescent malaria. Administering two or more drugs with different mechanisms of action also decreases the probability of resistance development against the artemisinins substantially [32]. The recommended treatment for severe malaria is intravenous artesunate for 24 h or until the patient is able to take oral medication, and then oral ATC for three days.

The focus of this thesis was on artemisinin, artesunate and dihydroartemisinin, and the possible interaction between artemisinin and the partner drug, piperaquine, was also investigated in healthy male volunteers.

1.3 Artemisinins

Artemisinin was first isolated in 1972 from the plant *artemisia annua L*. Since then, several derivatives including artesunate, dihydroartemisinin and artemether have been synthesized [33], [34]. Artemisinin is a sesquiterpene lactone (figure 2A) with poor solubility in both water and oil and with an elimination half-life of 1.4-2.6 h [35]–[37]. Artemisinin has not been commonly used in ACTs due to the pronounced auto-induction of the metabolizing enzymes, cytochrome P450 (CYP) 2B6, 2A6 and 3A4, resulting in a decrease of 70-80% of the exposure to the drug from the first day of dosing to the seventh day of dosing [38]–[41]. Significantly increased activities has also been seen in CYP2C19 and CYP1A2 [39]–[41]. Nevertheless, the efficacy of a short, two-day combination-treatment, have shown to be comparable to first-line recommended ACTs [42]. Artesunate (figure 2B) is a water-soluble hemisuccinate ester derivative of artemisinin with a half-life of less than 15 min [43], [44]. Artesunate is rapidly converted into the active metabolite, dihydroartemisinin by pre-systemic hydrolysis, systemic esterases and by CYP2A6 [33], [45]. Dihydroartemisinin is a reduced lactol (figure 2C) with an estimated half-life of 0.5-1.0 h [43], [44]. It is metabolized by glucuronidation in the gastrointestinal tract and in the liver by UDP-glucuronosyltransferase (UGT) 1A9 and 2B7 [46]. Both artesunate and dihydroartemisinin are highly efficient with a rapid parasite clearance [47].

Several different mechanisms of action for the artemisinins have been proposed. However, it is generally accepted that the endoperoxide bridge is the essential core structure responsible for the mechanism of action [48]. Golenser *et al*, states, that the crucial mechanism is considered to be artemisinin interference with the plasmodial sarcoplasmic/endoplasmic calcium ATPase (SERCA). The drug thereby disturbs the calcium-mediated signaling and the expression of an important protein, PfATP6. Artemisinin radicals are thereupon derived and cause inactive plasmodium enzymes with subsequent parasite death.

Susceptibility to antimalarial drugs changes as the parasite matures. However, the late ring and the early trophozoite stages were found to be the most sensitive, with the highest efficacy of the artemisinins [49].

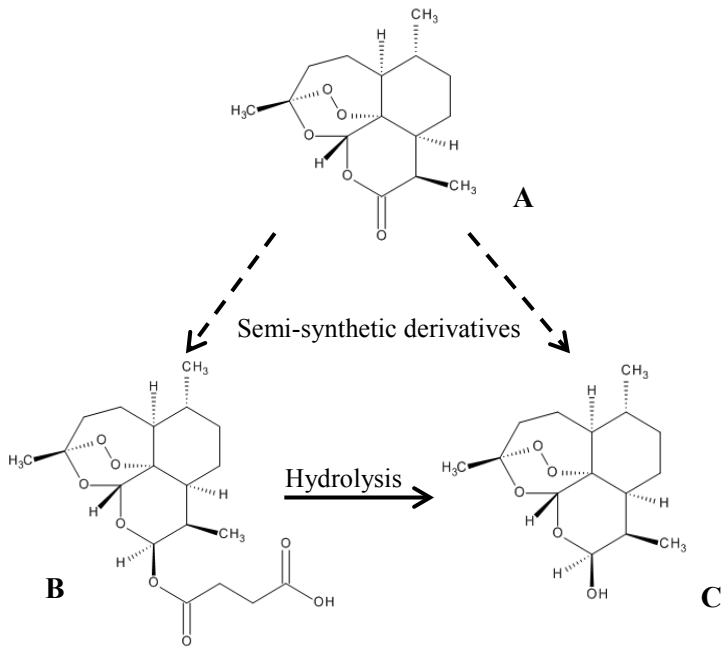

Figure 2. Chemical structures of artemisinin (A) and its semi-synthetic derivatives (dashed arrows) artesunate (B) and dihydroartemisinin (C). Artesunate is rapidly metabolized (solid arrow) to its active metabolite dihydroartemisinin.

1.4 Bioanalysis

Accurate and precise bioanalytical methods are crucial in order to perform high standard pharmacokinetic studies. Traditionally, it has been difficult to quantify the artemisinins due to the lack of a structure cromophore making ultraviolet detection inadequate. Both post-column on-line derivatization before ultraviolet detection and reductive mode electrochemical detection has been employed to quantify the artemisinins. However, these assays suffer from low sensitivity (limit of detection of 5-30 ng/mL) and large sample volumes (up to 1.0 mL) [50]–[55]. Two methods using liquid chromatography mass spectrometry has been described previously in the literature [56], [57]. Naik *et al* developed a method for artesunate and dihydroartemisinin with artemisinin as internal standard using a sample volume of 500 µL and a linear calibration range from 1-600 and 600-3000 ng/mL, respectively. In the method by Hanpithakpong *et al*, only 50 µL of plasma sample was used and the lower limit of quantification was 1.19 and

1.96 for artesunate and dihydroartemisinin, respectively. The breakthrough of liquid chromatography coupled to mass spectrometry and tandem mass spectrometry has dramatically increased the sensitivity of these compounds. However, up to date there has been no method for determining artesunate and dihydroartemisinin in the non-invasive matrix of saliva.

1.5 Pharmacokinetic Data analysis

1.5.1 Non-compartmental analysis

In non-compartmental analysis, the area under the curve is calculated using the linear trapezoidal method for ascending concentrations and the linear or logarithmic trapezoidal method for descending concentrations. This measurement of total exposure is used to determine the pharmacokinetic parameters. The accuracy of this analysis is highly dependent on the sampling schedule of the compound of interest and rich data is needed. The non-compartmental analysis is a highly useful and rapid approach for describing the pharmacokinetic properties of a drug. No assumptions regarding the shape of the concentration-time profile are made and consequently there is no risk of model misspecifications. However, there are limitations, for example when a mechanistic understanding is needed, when metabolite data are present, or when covariate relationships need to be evaluated. It is also difficult to assess pharmacokinetic-pharmacodynamic relationships with a model-independent approach and it is not possible to use the generated results for clinical trial simulations.

1.5.2 Population pharmacokinetic and pharmacodynamic modeling

The field of population pharmacokinetics was introduced in the 1970s and has since had an increased importance in the drug development process. When population pharmacokinetics was extended with pharmacodynamics the discipline of pharmacometrics was introduced. The aim of pharmacometrics is to describe and quantify interactions between a biological system and one or more drugs. Mathematical and statistical models are used to describe these processes of pharmacology, disease and physiology. Pharmacometric data are usually analyzed with a non-linear mixed effects modeling approach, containing both fixed effects and random effects [58]. The most commonly used software's are NONMEM, Monolix and ADAPT [59]. A general non-linear mixed effects model contains of three components: a structural model, a statistical model and a covariate model (figure 3) [60]. The covariate model could be excluded dependent on the data available.

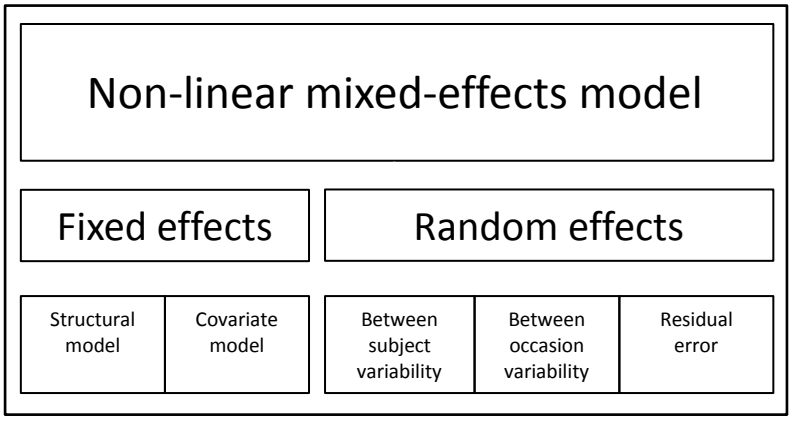

Figure 3. Schematic figure of a nonlinear mixed-effects model components.

Structural model

The simplest representation of a structural model is a one-compartment model after an intravenous bolus dose (equation 1).

$$C_p = \frac{Dose}{V}\, e^{\left(-\frac{CL}{V} * t\right)} \qquad \textit{Equation 1.}$$

Where Cp is the plasma concentration predicted for the typical patient based on the given dose, the volume of distribution (V) and the elimination clearance (CL) over time (t).

Statistical model

The statistical model consists of the between-subject variability, the between-occasion variability and the residual variability. The between-subject variability describes the differences in exposure between one individual and the population mean. The between-subject variability is most often described as an exponential model (equation 2):

$$\theta_i = \theta_{TV} * e^{\eta_i} \qquad \textit{Equation 2.}$$

Where θ_i is the value of parameter θ for individual i. θ_{TV} is the typical value of the parameter and η_i is the between-subject variability for individual i. η_i is drawn from a normal distribution with zero mean and variance ω^2 ($\eta_i \sim N(0, \omega^2)$) and will result in an individual parameter which is log-normally distributed.

The between-occasion variability explains the differences in the same patient at different occasions, commonly implemented as variability between dosing occasions. By ignoring these errors the parameter estimates can be biased [61]. Between occasion variability is exemplified in equation 3.

$$\theta_{ik} = \theta_{TV} \cdot e^{\eta_i + K_k} \qquad \textit{Equation 3.}$$

Where K_k is the between-occasion variability for occasion k. K_k is drawn from a normal distribution with mean 0 and variance π^2.

The residual variability is the unexplained variability, including model misspecification, error in sampling or error in the chemical analysis. The residual variability can be implemented in different ways, e.g. additive error (equation 4), proportional error (equation 5), or a combination of both (equation 6).

$$y_{ij} = IPRED_{ijk} + \varepsilon_{ij} \qquad \textit{Equation 4.}$$

$$y_{ij} = IPRED_{ijk} + IPRED \cdot \varepsilon_{ij} \qquad \textit{Equation 5.}$$

$$y_{ij} = IPRED_{ijk} + IPRED \cdot \varepsilon_1 + \varepsilon_2 \qquad \textit{Equation 6.}$$

Where $IPRED_{ijk}$ is the predicted value (e.g. concentration) for individual i at observation j and occasion k. ε_{ij} is the difference between the true observation and the predicted value for individual i at observation j.

Model validation commonly includes biological plausibility, goodness-of-fit diagnostics, (observed concentrations vs population predicted concentrations, observed concentrations vs individually predicted concentrations, conditionally weighted residuals vs predicted concentrations and conditionally weighted residuals vs time), parameter precision and confidence intervals from bootstrap methodology and visual predictive checks (observed vs simulated concentrations from the final model).

2 AIM

The overall aim of this thesis was to evaluate the pharmacokinetic properties of artemisinin and its derivatives, with particular focus on pregnancy, using population pharmacokinetic modeling and simulation.

Specific objectives

1. To develop and validate a sensitive and robust LC–MS/MS method for the simultaneous determination of artesunate and dihydroartemisinin in human plasma and saliva to enable detailed pharmacokinetic studies (Paper I).
2. To describe the pharmacokinetic properties of artesunate and its active metabolite, dihydroartemisinin, in rats after two different doses and routes of administration during two periods of pregnancy with non-pregnant rats as control (Paper II).
3. To describe the population pharmacokinetic properties of artemisinin in healthy Vietnamese volunteers, and to determine the effect of different formulations, doses and interaction with piperaquine (Paper III).
4. To describe the population pharmacokinetic properties of artesunate and its active metabolite, dihydroartemisinin, in pregnant and non-pregnant women with uncomplicated *P. falciparum* malaria and to determine potential pregnancy effect (Paper IV).

3 MATERIALS AND METHODS

3.1 Bioanalytical method development (Paper I)

3.1.1 Instrumentation

The LC-system was a PE-200 LC-pump connected to a sample injector equipped with temperature-controlled Peltier tray set at 8°C (Perkin Elmer, Waltham, MA, USA). Artesunate, dihydroartemisinin and internal standard (artemisinin) were analyzed on a BETASIL phenyl-hexyl 50x2.1mm, 5 µm ThermoHypersil column protected by a BETASIL phenyl-hexyl 150x2.1 mm, 5 µm ThermoHypersil guard cartridge (Thermo Scientific, Waltham, USA). A mobile phase consisting of acetonitrile-ammonium acetate 10 mM pH 4.0 (50:50, v/v) at a flow rate of 200 µL/min was used. An API 3000 triple quadrupole mass spectrometer (AB Sciex, MA, United States) with an electrospray ionization source (ESI) operated in the positive ion mode was used for the multiple reaction monitoring (MRM) LC-MS/MS analysis. Data acquisition and quantification were performed using Analyst 1.4.2 (AB Sciex, MA, United States).

3.1.2 Optimization

The composition of the mobile phase was evaluated in different degree of acetonitrile, pH (acetic acid) and concentration of ammonium acetate. Configurations for the mass spectrometer were tuned by infusing each substance directly into the mass spectrometer. Further optimization was performed by infusing the previous standard solution (10 µL/min) via a "T" connector after the column into the mobile phase (flow 200 ml/min). The ESI temperature was maintained at 225 °C and the ESI voltage was set to 5500V. Declustering potential was optimized to 10, 9 and 15 V for artesunate, dihydroartemisinin and internal standard, respectively, focusing potential to 60, 70 and 65 V, collision potential to 14, 12 and 15 V, and collision exit potential to 6, 6 and 4 V, respectively. The entrance potential was set to 5 V for all three compounds. High purity nitrogen was used as nebulizer (15 psi), curtain (10 psi) and collision gas (4 psi). These potentials for ESI^+ were used for detecting the ammonium adduct ($M+NH_4^+$) ions of the analytes. Quantification was performed using multiple reaction monitoring (MRM) at transitions m/z 402.5-267.1, 302.4-267.3 and 300.4-209.2 for artesunate, dihydroartemisinin and artemisinin as internal standard, respectively.

3.1.3 Sample preparation

For the preparation of samples, 150 µL ice-cold internal standard working solution (3 µg/mL) was added to 300 µL aliquots of thawed plasma or saliva, standard or quality control sample (final internal standard concentration in extracted samples, 1000 ng/mL) using a Brand HandyStep® pipette. To extract artesunate, dihydroartemisinin and internal standard from the biological matrix, solid phase extraction (SPE) was utilized using a HyperSep *Retain PEP* 96-well plate (Thermo Scientific, PA, USA). The SPE plate was initially activated and conditioned with methanol (1000 µL) followed by water (1000 µL). Biological matrix samples, standard and quality control samples (300 µL, reduced volume to minimize sample bench time) were loaded onto the SPE plate and a low vacuum applied. The SPE wells were washed with water (1000 µL), using a medium vacuum before full vacuum was applied briefly and the SPE column tips wiped dry with tissue paper. The analytes were finally eluted at low vacuum using methanol-acetonitrile (90:10, v/v, 2x250 µL) followed by water (500 µL). Combined elution volumes were thoroughly agitated before being transferred to glass microvials, and injected (20 µL) onto the LC-MS/MS system. All biological samples were processed within 30 minutes after thawing on ice.

3.1.4 Validation

Validation was carried out according to FDA guidelines for accuracy and precision of the calibration curve and the lower limit of quantification, selectivity, intra- and inter-day precision, recovery and matrix effects and stability [62].

3.2 Animal study (Paper II)

Non-pregnant and pregnant rats at gestation day 10 and 20 were administered single doses of artesunate either intravenously or orally at either of the two dose levels, 20 mg/kg and 100 mg/kg. The experimental procedures used in this study were approved by the Ethics board for animal research, Gothenburg Sweden (152/2008). The rats were anesthetized by inhalation of isoflurane, and a catheter inserted to the left jugular vein. For rats receiving intravenous dose, an additional catheterization of the right carotid artery was performed. All catheters were tunneled subcutaneously to emerge at the back of the neck. All animals were allowed to recover for at least 12 h after surgery before dosing. Solutions for both oral and intravenous administration were made fresh every day. An appropriate amount of artesunate was dissolved in sodium bicarbonate to give a drug concentration of 25 and 100 mg/mL for low and high dosing, respectively. Pre-dose blood samples were

drawn as control samples and an additional eight samples were obtained at 5, 15, 30, 45, 60, 90, 120 and 180 minutes after dose in each animal. Plasma samples were analyzed with a validated LC-MS/MS method for both parent compound and its active metabolite [57].

3.3 Ethics and study designs (Paper III and IV)

In paper III, 15 healthy Vietnamese male volunteers received four different dose regimens of a single dose of artemisinin as a conventional formulation (160 mg and 500 mg) and as a micronized test formulation (160 mg alone and in combination with piperaquine phosphate, 360 mg) with a washout period of three weeks between each period (i.e. four-way cross-over). Venous plasma samples were collected frequently up to twelve hours after dose in each period. The clinical trial protocol was approved by the internal Scientific and Ethical Committee of the Hospital for tropical diseases, Ho Chi Minh City and the Oxford Tropical Research Ethics Committee (OxTREC 019-06), University of Oxford, Oxford, United Kingdom. Artemisinin was quantified in plasma using liquid chromatography coupled with tandem mass spectrometry [63].

In paper IV, 24 women in their second (n=12) and third (n=12) trimesters of pregnancy and 24 paired non-pregnant women were enrolled in the study, all with uncomplicated *P. falciparum* malaria. Treatment was a standard fixed-dose combination of oral artesunate and mefloquine once daily over three days [30], [64]. Frequent blood samples were collected pre-dosing and at scheduled time points. The study was approved by the National Health Ethics Committee, Ministry of Health, Burkina Faso and by both the Institute of Tropical Medicine and the Ethics Committee of the University Hospital, Antwerp, Belgium. The study was registered at www.clinicaltrials.gov (identifier:NCT00701961). Samples were extracted by solid phase extraction and quantified for both artesunate and dihydroartemisinin using a validated LC-MS/MS method [57].

3.4 Non-compartmental analysis (Paper II)

In paper II, individual plasma concentration-time data of artesunate and dihydroartemisinin was first analyzed with a non-compartmental approach as implemented in Phoenix WinNonlin version 5.0 (Pharsight, Certara, St. Louis, USA). Complete *in vivo* conversion of artesunate into its active metabolite dihydroartemisinin by the hydrolysis of the ester group was

assumed, and the administered dose of dihydroartemisinin was calculated using the relative difference in molecular weights. Total exposure up to the last measured concentration ($AUC_{0\text{-last}}$) was calculated using the linear trapezoidal method for ascending concentrations and the logarithmic trapezoidal method for descending concentrations. The terminal elimination half-life was estimated by log-linear regression of at least six observed concentrations in the terminal phase. Total exposure was extrapolated to infinity by C_{LAST}/λ_Z for each individual to compute total drug exposure (AUC_∞). Maximum concentration (C_{MAX}) and time to C_{MAX} (T_{MAX}) were extracted directly from the observed data. Differences between non-pregnant animals and the two groups of pregnant animals were evaluated by descriptive statistics and a nonparametric Kruskal-Wallis test, for all pharmacokinetic parameters using SPSS version 20 (SPSS Inc., Chicago IL). The impact of dose levels on pharmacokinetic parameters was also investigated by the same statistical test.

3.5 Population pharmacokinetic modeling (Paper II-IV)

A population pharmacokinetic modeling approach was used for data evaluation in paper II-IV as described below. Plasma concentrations were transformed into their natural logarithms and concentration-time data was characterized using nonlinear mixed-effects modeling in NONMEM (version 7.1.2; ICON Development Solutions, MD) [65]. Post-processing and diagnostics were performed using Pearl-speaks-NONMEM (PsN) (version 3.4.2) [66]; Pirana (version 2.4.0) [67] and Xpose (version 4.0) [68] package in R (version 2.13.1; The R Foundation for Statistical Computing).

In paper III, artemisinin concentration-time profiles were available. In paper II and IV, artesunate and dihydroartemisinin concentration-time profiles were available and modeled simultaneously (complete conversion of artesunate into dihydroartemisinin was assumed). In paper II the model included both intravenous and oral route of administration enabling an estimation of the absolute oral bioavailability of artesunate. A pre-systemic conversion of artesunate into dihydroartemisinin (i.e. an estimated fraction of dihydroartemisinin was absorbed directly from the dosing compartment) was also evaluated in this paper.

In paper IV, a pre-systemic conversion of artesunate into dihydroartemisinin was evaluated as first-order absorption of dihydroartemisinin from both the dose compartment and the transit compartment into the central compartment of the metabolite.

The first-order conditional estimation (FOCE) method was applied in the model building process. A Laplacian estimation method was used when censored data, (below the lower limit of quantification), was implemented with the M3-method (paper II and IV) [69]. Model discrimination was performed using basic goodness-of-fit graphical evaluation and the objective function value (OFV; computed by NONMEM as proportional to minus two times the log likelihood of data) [70]. For nested models with one parameter difference, ΔOFV of 3.84, 6.63 and 10.83 corresponds to a *p*-value of 0.05, 0.01 and 0.001, respectively. Structural models with one-, two- and three-disposition compartments were fitted to the data, for parent compound and in paper II and IV also for the active metabolite. The absorption phases were evaluated with a first order absorption model with and without lag time, zero-order absorption, sequential zero- and first-order absorption and with a flexible transit compartment model with a fixed number of 1-10 transit compartments [71].

Inter-individual variability was added exponentially as illustrated below (Eq. 7).

$$\theta_i = \theta_{TV} \times \exp(\eta_i) \qquad \textit{Equation 7.}$$

where θ_i is the individually estimated parameter value for the i^{th} patient and θ_{TV} is the typical value for the population. η_i is the inter-individual variability, assumed to be normally distributed around zero and with a variance ω^2. The residual random variability was modeled as additive error models on log-transformed concentrations being essentially equivalent to an exponential residual error on an arithmetic scale. In paper II and IV, two additive error models were implemented for artesunate and dihydroartemisinin, respectively.

Different approaches to handle data below the lower limit of quantification (LLOQ) were evaluated to avoid bias in parameter estimates. Initially, the data were omitted (M1-method) as in the case of paper III where only 5% of the data were below the LLOQ (all within 30 minutes of dosing). In paper II and IV this data was modelled as categorical data (M3-method) and in paper IV also as LLOQ/2 (M5-method) [69], [72]. Simulation-based diagnostics were used to discriminate between the M1 and M3-method (i.e. fraction of simulated and observed data below the limit of quantification).

3.5.1 Covariate analysis

Covariates were investigated with a stepwise covariate methodology. Stepwise forward inclusion (p<0.05) were used for both continuous and

categorical covariates followed by a stepwise backward exclusion (p<0.01). The covariates were tested with a linear, power and exponential relationship. Bodyweight, centered on the population median weight, was evaluated as an allometric function on all clearance and volume parameters, where clearance were scaled to mass to a power of 0.75 and where the volume was scaled to mass to the power of one [73]–[75].

In paper III and IV, a full-covariate approach (i.e. the covariate of interest was added simultaneously as a categorical covariate on all estimated fixed effects) was also implemented. These full covariate models were analyzed using 500 re-sampled datasets (bootstrap) and the 90% confidence interval of the covariate effects calculated to investigate the impact on each covariate on the pharmacokinetic properties. A covariate related change in the parameter estimates of more than 20% was assumed to be of clinical relevance.

3.5.2 Model evaluation

Basic goodness-of-fit characteristics were evaluated by plotting observed drug concentrations against individually predicted and population predicted drug concentrations and by plotting conditional weighted residuals against population predicted drug concentrations and time [76]. Eta and epsilon shrinkages were calculated to evaluate the reliability of the goodness-of-fit diagnostics [77]. Visual predictive checks (prediction corrected) were performed using 2000 simulations at each concentration time point (protocol time points were used for binning) [78]. Bootstrap diagnostics (1000 re-sampled datasets) were performed for the final models to obtain standard errors for parameter estimates and non-parametric confidence intervals around these parameters.

4 RESULTS AND DISCUSSION

4.1 Bioanalytical method development (Paper I)

4.1.1 Optimization

In the optimization of the LC–MS/MS properties, the highest abundance of the ions was found with the ammonium adduct [MNH4+]. Therefore, the following precursor–product ion pairs, *m/z* 402.5–267.1, 302.4–267.3 and 300.4–209.2 were chosen for artesunate, dihydroartemisinin and internal standard, respectively. Only the α-epimer of dihydroartemisinin was quantified. Previously published data have also demonstrated higher analytical response of α-dihydroartemisinin compared with the response of β-dihydroartemisinin [56], [79]. Using the current experimental conditions, this could be due to steric reason in the formation of the ammonium adduct as the precursor ion used in this method. The signal intensity and the baseline for artesunate were much lower than those for dihydroartemisinin and internal standard. Optimal chromatographic conditions were found with acetonitrile–ammonium acetate 10 mM pH 4.0 (50:50, v/v).

4.1.2 Sample preparation

Artesunate undergoes both biological and chemical hydrolysis, the latter accounting for approximately 80% of the total hydrolysis in clinical plasma samples [80]–[82]. The use of fluoride/oxalate tubes during sampling aimed to counteract the biological instability of artesunate by inhibiting the enzyme mediated *ex-vivo* hydrolysis. A range of different SPE products and experimental conditions were tested to optimize the preparation of plasma and saliva samples used in the current study. A HyperSep Retain PEP 96-well plate, containing polymeric material modified with urea containing functional groups, was selected based on excellent performances in terms of analyte recovery and reproducibility. With this sorbent, problems with column drying often associated with traditional silica-based SPE materials, were eliminated.

4.1.3 Validation

Validation according to FDA guidelines was completed successfully.

The LLOQ was set to 5 ng/ml for both artesunate and dihydroartemisinin in plasma and saliva, respectively, providing adequate accuracy and precision (table 1 and 2) and with a signal-to-noice ratio of five or above.

Table 1. *Intra-day and inter-day accuracy and precision for artesunate (ARS) and dihydroartemisinin (DHA) in human plasma.*

		Intra-day (n=5)			Inter-day (n=15)		
Analyte nominal concentration (ng/mL)		Calculated concentration (ng/mL)	Accuracy (%)	%CV	Calculated concentration (ng/mL)	Accuracy (%)	%CV
ARS	5	4.98±0.05	99.6	0.9	4.97±0.21	99.5	4.2
DHA	5	5.03±0.07	101	1.4	5.05±0.12	101	2.5
ARS	15	14.6±0.45	97.4	3.1	14.6±1.08	97.4	7.4
DHA	15	15.0±0.26	100	1.7	15.1±0.30	100	2
ARS	300	307±6.87	102	2.2	298±12.3	99.2	4.1
DHA	750	787±35.5	105	4.5	763±37.2	101	4.9
ARS	750	763±17.3	102	2.3	737±35.1	98.3	4.8
DHA	1500	1506±60.2	100	4	1412±111	94.1	7.8

Calculated concentrations (ng/mL) are presented as mean ± SD and precision represented by the %CV.

Table 2. Intra-day and inter-day accuracy and precision for artesunate (ARS) and dihydroartemisinin (DHA) in human saliva.

		Intra-day (n=5)			Inter-day (n=15)		
Analyte nominal concentration (ng/mL)		Calculated concentration (ng/mL)	Accuracy (%)	%CV	Calculated concentration (ng/mL)	Accuracy (%)	%CV
ARS	5	4.99±0.06	99.8	1.3	4.97±0.09	99.5	1.9
DHA	5	5.01±0.07	100	1.3	5.0±0.07	100	1.4
ARS	15	15±0.10	100	0.7	15.0±0.15	100	1
DHA	15	15±0.11	100	0.7	15.0±0.17	99.9	1.1
ARS	300	301±5.30	100	1.8	301±4.67	100	1.6
DHA	750	751±13.6	100	1.8	747±16.5	99.6	2.2
ARS	750	759±9.10	101	1.2	741±23.2	98.8	3.1
DHA	1500	1518±26.8	101	1.8	1496±25.6	99.7	1.7

Calculated concentrations (ng/mL) are presented as mean ± SD and precision represented by the %CV.

For the first time a bioanalytical assay for determination of artesunate and dihydroartemisinin in human saliva has been described. This sensitive and high-throughput LC-MS/MS method was validated according to FDA guidelines for artesunate and dihydroartemisinin in both plasma and saliva.

4.2 Animal study (Paper II)

4.2.1 Non-compartmental modeling

The model-independent analysis demonstrated no significant pregnancy-related differences for artesunate or dihydroartemisinin after oral doses of artesunate. However, after intravenous doses of artesunate there was a significant increase in both artesunate and dihydroartemisinin clearance and an increased volume of distribution for dihydroartemisinin in pregnant animals. The dose adjusted exposure (AUC/Dose) decreased in pregnant animals for both artesunate and dihydroartemisinin after intravenous doses of artesunate. There were no significant differences in T_{MAX} or half-life parameters for either artesunate or dihydroartemisinin.
A dose dependent increase in clearance and a decrease in both half-life and AUC were found for dihydroartemisinin irrespectively of route of administration. There were no significant dose dependent effects on artesunate pharmacokinetic parameters.
Estimated median parameters from the non-compartmental analysis were carried forward as initial parameter estimates for the nonlinear mixed-effects modelling.

4.2.2 Population pharmacokinetic model

The changes in artesunate and dihydroartemisinin plasma concentrations over time were best described with a two-compartment disposition model for both compounds (figure 4). Artesunate absorption was best described by a transit compartment model with one fixed compartment. In the final model, inter-individual variability was retained on all parameters. The population-derived pharmacokinetic estimates with relative standard errors are presented in table 3. Modeling the data below the limit of quantification as categorical data (M3-method) improved the diagnostics of the model compared to the conventional method of omitting these data (figure 5).

Pregnancy as a continuous covariate had a significant impact on several pharmacokinetic parameters. From gestation day 0 to gestation day 20, artesunate and dihydroartemisinin clearance increased by 20.2% and 102%, respectively, with proportional decreases in total drug exposure. In patients, this could have severe consequences resulting in a higher risk of treatment failures and the development of drug resistant parasites. This increase in clearance values could be related to a pregnancy-induced increase in enzymatic activity, which has also been found in pregnant women [83]. Volume of distribution was also affected by pregnancy for both artesunate and dihydroartemisinin, with an increase from gestation day 0 to gestation day 20 of 50.0% and 14.9%, respectively. These changes are commonly seen in pregnant women, due to increased blood volume and changes in plasma proteins [83].

The pregnancy effects found here are well in agreement with previous findings in pregnant women indicating that this can be a suitable animal model to further study the impact of pregnancy on antimalarial drugs [84], [85].

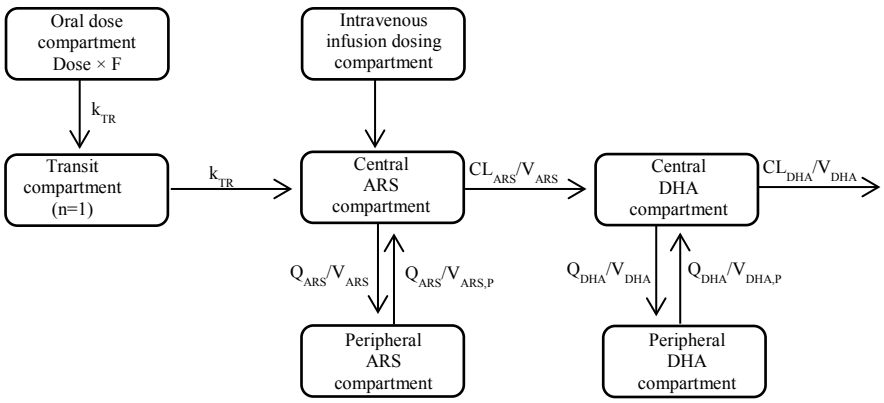

Figure 4. Structural representation of the final model describing artesunate (ARS) and dihydroartemisinin (DHA) pharmacokinetics in pregnant and non-pregnant rats receiving single intravenous infusion and oral doses of artesunate. k_{TR}, absorption rate constant; CL, elimination clearance; V, central volume of distribution; Q, inter-compartment clearance; $V_{P,}$ peripheral volume of distribution; F, absolute oral bioavailability of artesunate.

Table 3. *Parameter estimates of the final population pharmacokinetic model describing artesunate (ARS) and dihydroartemisinin (DHA) in pregnant and non-pregnant rats receiving single intravenous infusion and oral doses of artesunate.*

Parameter	Parameter estimate (RSE%)	CI 95%	IIV CV% (RSE%)	CI 95%
CL_{ARS} (L/h)	3.84 (7.42)	3.00-4.06	24.0 (15.6)	18.8-37.8
V_{ARS} (L)	0.453 (9.66)	0.31-0.45	282 (16.0)	108-395
Q_{ARS} (L/h)	0.105 (9.05)	0.083-0.12	12 (20.7)	6.56-18.5
$V_{ARS,P}$ (L)	0.081 (7.53)	0.070-0.095	171 (27.1)	65.7-184
CL_{DHA} (L/h)	1.03 (6.70)	0.88-1.23	20.3 (17.9)	10.7-20.7
V_{DHA} (L)	0.158 (5.64)	0.14-0.19	1.50 (16.7)	1.00-1.7
Q_{DHA} (L/h)	0.610 (7.17)	0.51-0.69	16.4 (18.2)	11.5-21.2
$V_{DHA,P}$ (L)	0.201 (6.79)	0.17-0.24	17.0 (26.8)	13.0-24.6
F (%)	5.41 (10.3)	4.78-7.43	89.0 (15.2)	41.6-116
MTT (h)	0.144 (9.24)	0.10-0.15	75.0 (19.5)	48.5-103
Nr of trans comp	1 *(fixed)*			
$\sigma_{ARS,IV}$	1.20 (10.3)	0.89-1.49		
σ_{DHAIV}	0.132 (13.7)	0.10-0.18		
PREG on CL_{ARS} (%)	1.35 (25.7)	0.19-1.70		
PREG on V_{ARS} (%)	5.77 (9.26)	4.56-6.90		
PREG on CL_{DHA} (%)	0.49 (58.2)	0.09-1.21		
PREG on V_{DHA} (%)	2.40 (15.2)	1.41-2.99		

Parameter estimates for artesunate (ARS) and dihydroartemisinin (DHA). CL, elimination clearance; V, central volume of distribution; V_P, peripheral volume of distribution, Q, inter-compartment clearance, MTT, mean transit time of the absorption phase; F, oral bioavailability; Nr. trans comp, number of transit compartments in the absorption model; σ, variance of the additive residual errore. PREG on CL or V; factor of percental increase in CL or V per increase in pregnancy gestational day. RSE is the relative standard error calculated as 100x standard deviation/mean. CV% is the coefficient of variation calculated as
$100 * \mathrm{SQRT}(e^{\mathrm{variance}} - 1)$ for inter-individual variability (IIV). Parameter estimates are based on population mean values from NONMEM, RSE% and CI values are based on 307 successful bootstrap runs (out of 320).

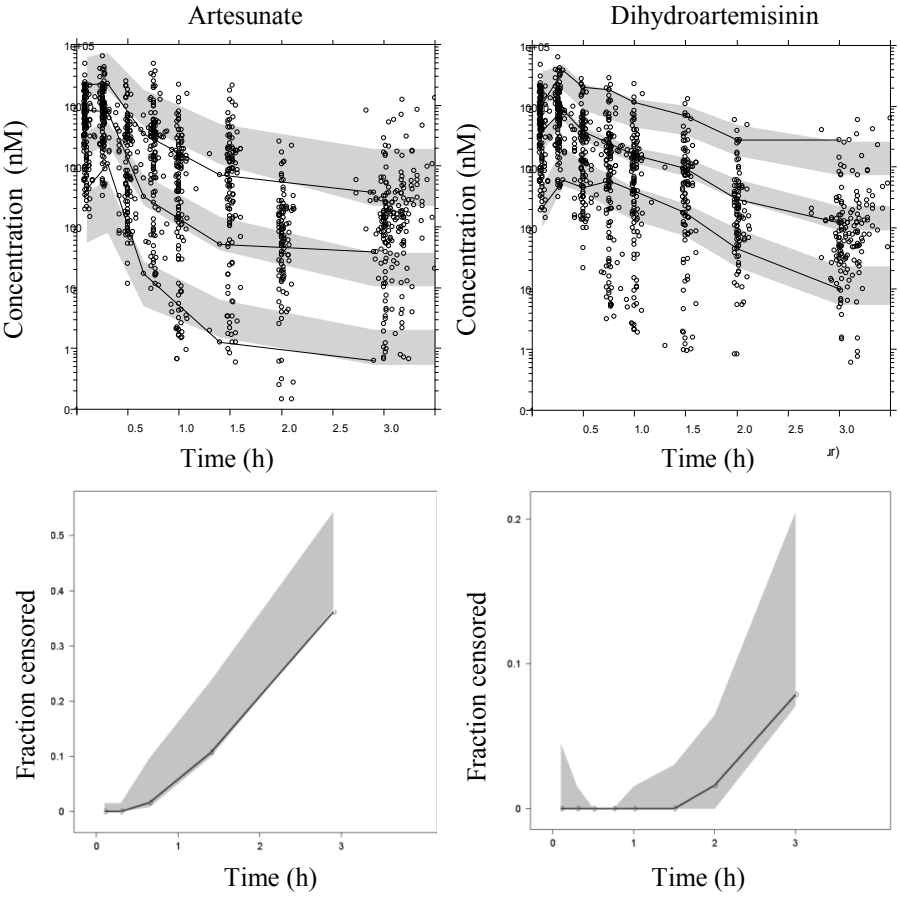

Figure 5. Prediction corrected visual predictive checks of the final population pharmacokinetic model of artesunate and dihydroartemisinin in pregnant and non-pregnant rats receiving single intravenous infusion and oral doses of artesunate. Upper panel: open circles represents the observations, the broken lines are the 5th and 95th percentiles of the observations and the solid line is the median of the observations. Shaded areas represent the 95% confidence interval of simulated 5th, 50th and 95th percentiles. Lower panel: the shaded area represents the simulated 95% confidence intervals of the fraction of BQL data. The black solid line represents the observed fraction of BQL data.

4.3 Artemisinin pharmacokinetics in healthy volunteers (Paper III)

The pharmacokinetics of artemisinin was best characterized by a one-compartment disposition model with seven transit compartments in the absorption phase (figure 6). Data from all four regimens were successfully modeled simultaneously, including between-dose occasion variability. A transit-compartment absorption model was significantly better than all other absorption models tested. The population-derived pharmacokinetic estimates with relative standard errors are presented in table 4 and 5. Parameter estimates in this work in healthy volunteers were in agreement with those previously reported in patients by Sidhu et al. after taking into account the differences in bodyweight [86].

The stepwise covariate approach did not result in any significant covariates in the final model.

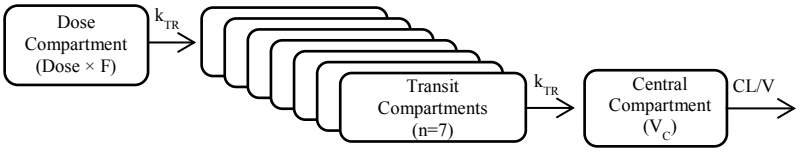

Figure 6. Structural representation of the final model describing artemisinin population pharmacokinetics in healthy male Vietnamese subjects. k_{TR}, absorption rate constant; CL, elimination clearance; V_C, volume of distribution of the central compartment; F, relative oral bioavailability.

Table 4. Parameter estimates of the final model describing artemisinin population pharmacokinetics in healthy male Vietnamese subjects.

Parameter	Population estimate (RSE%)	CI 95%	IIV/IOV* CV% (RSE%)	CI 95%
CL/F (L/h)	417 (9.32)	350-501	17.1* (34.3)	11.1-22.6
V/F (L)	1210 (9.02)	1030-1450	-	-
Nr of trans comp	7 *(fixed)*	-	-	-
MTT (h)	0.787 (5.97)	0.702-0.891	53.9* (20.3)	41.6-66.9
F	1 *(fixed)*	-	34.3 (52.3)	17.3-50.5
σ (CV%)	51.6 (5.84)	44.9-58.1	-	-

CL/F, apparent elimination clearance; *V/F*, apparent volume of distribution; *Nr. trans comp*, number of transit compartments in the absorption model; *MTT*, mean transit time of the absorption phase; *F*, relative oral bioavailability; σ, additive residual error. RSE is the relative standard error calculated as $100x\ standard\ deviation/mean$. CV% is the coefficient of variation calculated as $100 * SQRT(e^{variance} - 1)$ for inter-individual variability (IIV) and inter-occasion variability (IOV). CI 95%, 95% confidence intervals calculated as the 2.5 and 97.5 percentiles of bootstrap estimates. Parameter estimates are based on population mean values from NONMEM, RSE% and CI values are based on 954 successful bootstrap runs (out of 1000).

Table 5. Secondary parameters of the final model describing artemisinin population pharmacokinetics in healthy male Vietnamese subjects

Parameter	Treatment 1	Treatment 2	Treatment 3	Treatment 4
C_{MAX} (ng/mL)	111 [45.2-183]	96.7 [52.1-169]	244 [133-479]	144 [58-200]
T_{MAX} (h)	1.41 [0.762-2.06]	1.09 [0.773-2.28]	1.72 [1.12-3.65]	0.992 [0.628-1.90]
$AUC_{0-\infty}$ (ng*h/mL)	441 [472-146]	349 [181-642]	994 [468-2040]	467 [192-761]
AUC_{0-12} (ng*h/mL)	461 [144-651]	342 [178-624]	956 [462-1973]	462 [189-744]
$t_{1/2}$ (h)	1.97 [1.64-3.37]	1.80 [1.46-3.20]	1.93 [1.71-2.43]	2.02 [1.64-2.42]

Secondary parameters estimated from the final model and values are presented as median [range]. C_{max} is the maximum concentration and T_{max} is the time to reach C_{max}. AUC_{∞} is the accumulated area under the concentration-time curve from time zero extrapolated to infinity and AUC_{0-12} is the accumulated area under the concentration-time curve from time zero to 12 h after dose. $t_{1/2}$ is the estimated terminal elimination half-life. Treatment 1 was administrated as 160 mg micronized artemisinin, treatment 2 was 160 mg of the reference formulation of artemisinin, treatment 3 was 500 mg of the reference formulation of artemisinin and treatment 4 was 160 mg micronized artemisinin and 720 mg of piperaquine phosphate.

In the full covariate approach, mean–transit-time increased by a median of 69.1% with increasing dose size (160 mg vs 500 mg, figure 7B). None of the other parameters were influenced by artemisinin formulation (figure 7A), dose (figure 7B) or concomitant piperaquine administration (figure 7C), although there was a trend towards an increasing volume of distribution with increasing doses. The data in the present study was previously used in a non-compartmental analysis by Hien *et al* [87]. In that analysis the two different formulations were concluded to not fulfill the criteria for bioequivalence due to large variability, according to the FDA guidelines [88]. However, the clinical impact was considered to be negligible. The full covariate population modeling approach used in the present study showed similar results to what Hien *et al* found with no absorption-related pharmacokinetic differences between the reference formulation and the micronized powder formulation. The study design with two different dose sizes for the conventional formulation was due to earlier findings suggesting dose-dependent artemisinin pharmacokinetics [38], [89]. However, dose differences did not change the relative bioavailability although the mean transit time increased substantially at higher doses in the present study. Possible explanations for this prolonged absorption could be the low solubility or elongated dissolution of the drug powder. It has previously been shown that artemisinin has a high permeability via passive diffusion, therefore, transportation across the intestinal membranes is not likely to be a rate-limiting step increasing mean transit time [90].

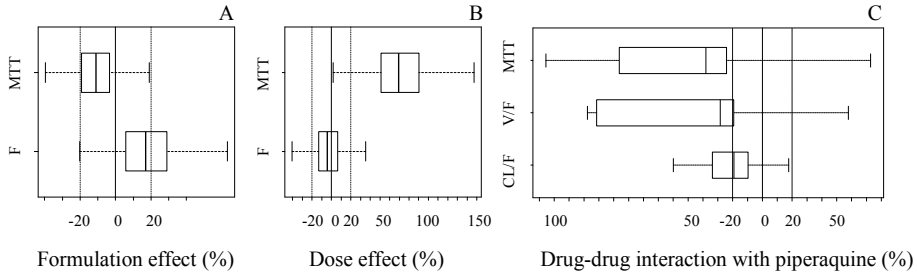

Figure 7. Box (25th and 75th percentile) and whisker (1.5×interquartile range) plots of the full covariate models. The solid black zero-line represents no covariate effect (and the dotted black lines represent a covariate effect of ±20%). Formulation (A), dose (B) and the potential drug-drug interaction with piperaquine (C) were investigated as covariates. MTT is the mean transit time, F is the relative oral bioavailability, V/F is the apparent volume of distribution and CL/F is the apparent elimination clearance. All covariates were added as categorical functions.

Concentration-time profiles for three groups, healthy volunteers, adult patients [86] and pediatric patients [91], were simulated after receiving identical dosages (weight adjusted) of artemisinin as in the present study (figure 8). The time to reach maximum concentration occurred at nearly the same time in all three groups, although both groups of patients showed lower maximum concentrations compared to healthy volunteers. The absorption models differ between healthy volunteers and patients, most likely due to sparse sampling in the absorption phase in patients. Children seem to have a lower exposure compared to the other groups which indicates that dose adjustment by allometric scaling would be preferable to adjusting merely by body weight. The developed population pharmacokinetic model showed good predictive performance, as illustrated in the visual predictive check, and could therefore be suitable for population based simulations and clinical trial design (figure 9).

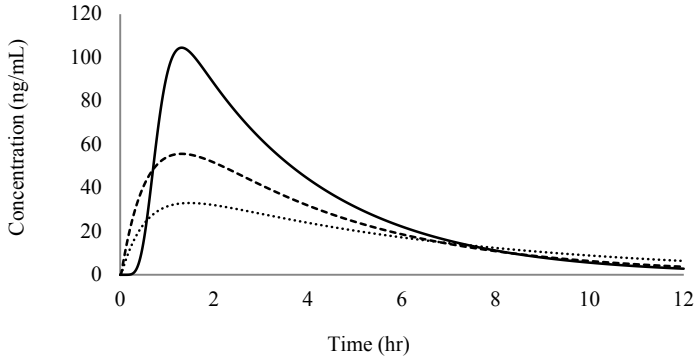

Figure 8. Simulated mean concentration-time profiles of healthy male Vietnamese volunteers (solid line, present study), adult patients (dashed line, [86]*) and pediatric patients (dotted line,* [91]*).*

This is the first population pharmacokinetic characterization of artemisinin in healthy volunteers. Increasing the dose resulted in a significant increase in the mean transit absorption time but the micronized formulation or concomitant piperaquine administration did not affect the pharmacokinetic properties of artemisinin.

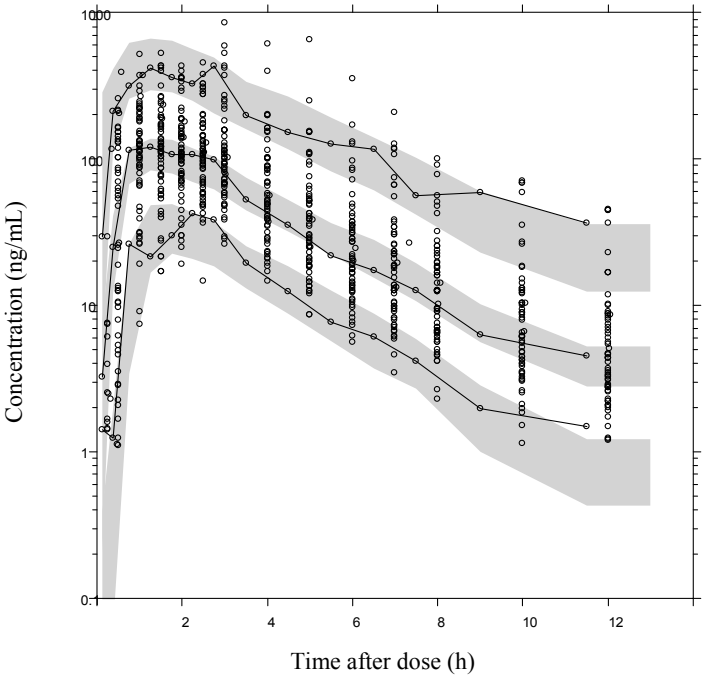

Figure 9. Prediction corrected visual predictive check of the final model describing artemisinin population pharmacokinetics in healthy male Vietnamese subjects. Open circles represents the observations, the broken lines are 5th and 95th percentiles of the observations and the solid line is the median observed concentrations (nmole/L). Shaded areas represent the 95% confidence interval of simulated 5th, 50th and 95th percentiles.

4.4 Pregnant patient population (Paper IV)

In this study, a population pharmacokinetic model was developed for artesunate and the active metabolite, dihydroartemisinin, in pregnant and non-pregnant women in Burkina Faso, treated with a fixed-dose combination of artesunate and mefloquine. The final model was a one-compartment disposition model for both artesunate and dihydroartemisinin, where artesunate absorption was described with a transit compartment model (n=3) (figure 10). Allowing for inter-individual variability in the relative bioavailability of artesunate significantly improved the model fit. The best performing model was obtained when data below the LLOQ was modeled as categorical data (i.e. the M3-method) (figure 11). Final parameter estimates and

secondary parameter estimates are summarized in table 6 and 7, respectively.
Pregnancy as a categorical covariate had a significant impact on apparent oral clearance of dihydroartemisinin, resulting in a 24% increased elimination clearance of dihydroartemisinin in pregnant women as compared to non-pregnant women. This finding was in agreement with the study by Morris *et al* where pregnant and non-pregnant women with *Plasmodium falciparum* malaria received oral artesunate. A pregnancy-induced increase of 42% in the elimination clearance of dihydroartemisinin was found [84].
No other covariates were significant in the stepwise covariate approach.
When further evaluating the effect of pregnancy in a full covariate model approach, the same result as in the step-wise covariate search was found. Dihydroartemisinin clearance increased (21.6%) with pregnancy (figure 12). There was a strong trend towards a decreased mean transit absorption time in pregnant women. This could indicate an effect in the absorption phase that possibly would be significant in a larger patient study.

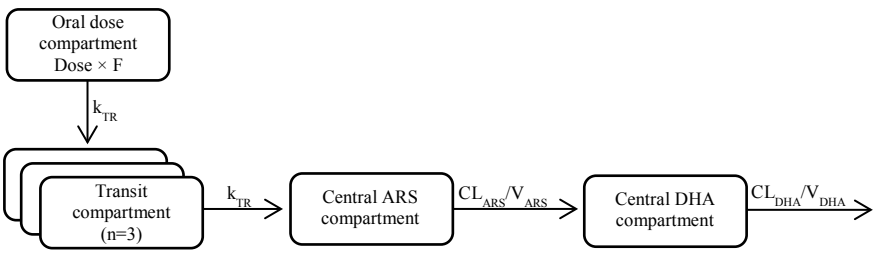

Figure 10. Structural representation of the final model describing artesunate (ARS) and dihydroartemisinin (DHA) population pharmacokinetics in pregnant and non-pregnant women. k_{TR}, absorption rate constant; CL/F, elimination clearance; V/F, volume of distribution of the central compartment; F, oral bioavailability.

To further investigate the possible effect of malaria on the pharmacokinetics, the data was divided into two subsets (i.e. only pregnant or only non-pregnant patients). Parasite load (number of parasites on a logarithmic scale) was added as a continuous covariate effect in a stepwise manner. In the subset of only pregnant patients, a 25% increase in the bioavailability per log parasite load increase was found to be significant. Adding this disease effect as a fixed effect to the

final model resulted in a larger increase in dihydroartemisinin clearance with pregnancy which indicates that disease severity attenuates the effect of pregnancy. Thus, the overall pharmacokinetic effect of pregnancy in this study might be underestimated due to an imbalance in the parasitemia at enrollment

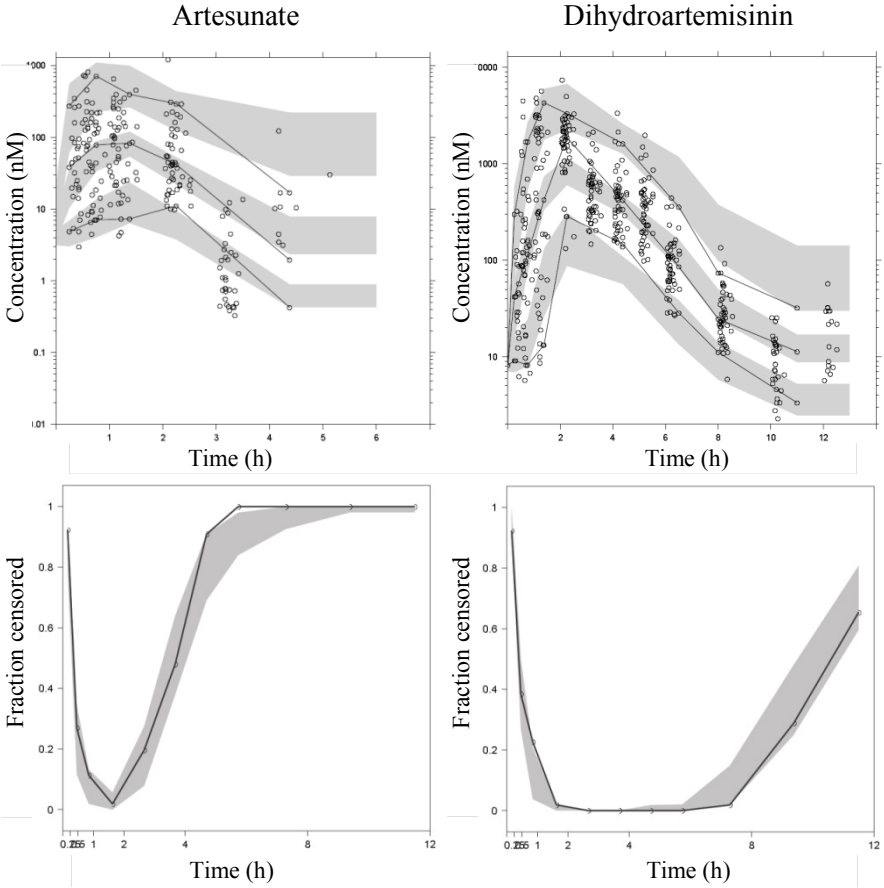

Figure 11. Visual predictive check of the final model describing artesunate and dihydroartemisinin population pharmacokinetics in pregnant and non-pregnant women. Upper panel: Open circles represents the observations, and the solid line is the median observed concentrations. Shaded areas represent the 95% confidence intervals of simulated 5th, 50th and 95th percentiles. Lower panel: the shaded area represents the simulated 95% confidence intervals of the fraction of BQL data. The black solid line represents the observed fraction of BQL data.

The pregnancy-induced increase in clearance could be as high as 31%, taking into account the different parasite biomass in the two patient groups. These opposite effects of disease severity and pregnancy are in

accordance with a recent study by Kloprogge *et al* [92]. In that study pregnant women with uncomplicated *Plasmodium falciparum* malaria were treated with intravenous and oral artesunate. The result showed that malaria and pregnancy demonstrated opposite effects on bioavailability of orally administered artesunate. Kloprogge *et al* found that malaria increased the bioavailability by 87% while pregnancy decreased oral bioavailability by 23%.
The final model of pregnant and non-pregnant patients showed satisfactory predictive performance indicating the model to be suitable for simulations (figure 11).

Table 6. Parameter estimates of the final model describing artesunate (ARS) and dihydroartemisinin (DHA) population pharmacokinetics in pregnant and non-pregnant women.

Parameters	Population estimate (RSE%)	CI 95%	IIV CV% (RSE%)	CI 95%
CL_{ARS}/F (L/h)	3480 (9.30)	2870-4100	26.6 (54.9)	1.34-35.4
V_{ARS}/F (L/h)	1650 (11.1)	1290-2050	-	
CL_{DHA}/F (L/h)	147 (6.50)	129-165	9.6 (47.1)	3.55-12.4
V_{DHA}/F (L/h)	260 (5.60)	236-286	-	
MTT (h)	0.834 (9.75)	0.720-1.00	56.5 (20.6)	46.2-74.1
F (%)	100* *(fixed)*		41.1 (24.1)	32.2-51.2
Nr of trans comp	3 *(fixed)*			
PREG on CL_{DHA}/F	24.2 (24.2)	12.4-36.5		
σ_{ARS}	0.89 (10.8)	0.721-1.07		
σ_{DHA}	0.66 (9.58)	0.528-0.750		

CL/F, apparent elimination clearance; *V/F*, apparent volume of distribution; *MTT*, mean transit time of the absorption phase; *F*, relative oral bioavailability; *Nr. trans comp*, number of transit compartments in the absorption model; PREG on CL_{DHA}/F; factor of percental increase in CL_{DHA}/F with pregnancy; σ, additive residual error as the variance. RSE is the relative standard error calculated as $100x\ standard\ deviation/mean$. CV% is the coefficient of variation calculated as $100 * SQRT(e^{variance} - 1)$ for inter-individual variability (IIV). Parameter estimates are based on population mean values from NONMEM, RSE% and CI values are based on 86 successful bootstrap runs (out of 100).

Table 7. Secondary parameters of the final model describing artesunate and dihydroartemisinin population pharmacokinetics in pregnant and non-pregnant women.

	Non-pregnant	Pregnant	Total
Artesunate			
t1/2 (h)	0.304 (0.260-0.563)	0.334 (0.251-0.656)	0.322 (0.251-0.656)
$AUC_{0-\infty}$ (ng*h/mL)	90 (45.9-238)	140 (45.1-313)	97.8 (45.1-313)
C_{MAX} (ng/mL)	32.8 (11.0-87)	54.1 (10.9-108)	39.8 (10.9-108)
T_{MAX} (h)	1.49 (0.769-2.45)	1.09 (0.505-2.06)	1.23 (0.505-2.45)
Dihydroartemisinin			
t1/2 (h)	1.22 (1.09-1.40)	0.980 (0.905-1.17)	1.13 (0.905-1.40)
$AUC_{0-\infty}$ (ng*h/mL)	1,823 (932-3,034)	1,599 (606-3,600)	1,736 (606-3600)
C_{MAX} (ng/mL)	35.6 (18.4-68.7)	51.6 (19.0-120)	29.5 (8.05-79.9)
T_{MAX} (h)	2.39 (1.55-3,72)	1.91 (1.11-3.08)	2.07 (1.11-3.72)

Secondary parameters estimated from the final model are presented as median (range). C_{max} is the maximum concentration and T_{max} is the time to reach C_{max}. $AUC_{0-\infty}$ is the accumulated area under the concentration-time curve from time zero to infinity. $t_{1/2}$ is the estimated terminal elimination half-life.

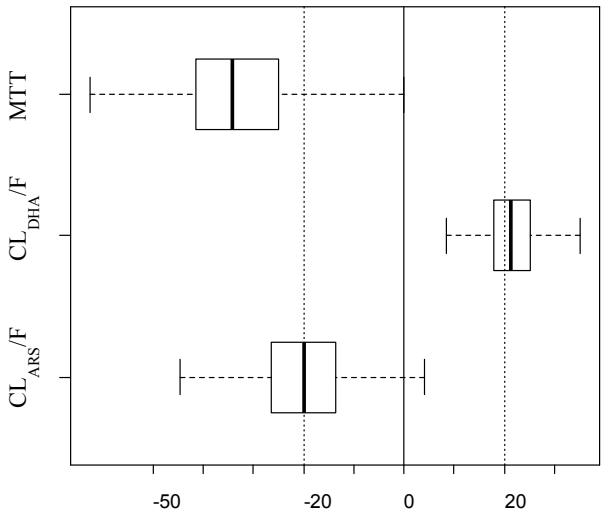

Figure 12. Box (25th and 75th percentile) and whisker (1.5×interquartile range) plot of the full covariate model of pregnancy. The solid black zero-line represents no covariate effect (and the dotted black line represents a covariate effect of ±20. CL_{ARS}/F is the apparent elimination clearance of the parent compound, CL_{DHA}/F is the apparent elimination clearance of the metabolite, MTT is the mean transit time. The covariate was added as a categorical function.

5 GENERAL DISCUSSION

In the fight against malaria, WHO states that, the necessary tools are a combination of insecticide treated nets, indoor residual spraying, diagnostic tools and treatment with ACTs [5]. However, the emerging artemisinin resistance is now threatening our ability to control and eliminate malaria. This emphasizes the need for dose-optimization in vulnerable groups in order to prevent sub-therapeutic drug exposures that will undoubtedly result in treatment failures and fuel the development of drug resistant parasites [20]–[22], [93]. Nonlinear mixed-effects modeling can be used to get the most out of the information gained from preclinical and clinical data, and identify patient groups at particular risk of treatment failure and drug resistance development. Validated models can also be used to optimize dose regimens in these groups and to further improve clinical trial designs by stochastic simulations.

In order to conduct high quality pharmacokinetic studies, sensitive bioanalytical methods are essential. The method presented in this thesis was validated for plasma and saliva, offering a non-invasive method for therapeutic drug monitoring and pharmacokinetic field studies. Concentrations in saliva are usually lower compared to those in plasma and the equilibrium between the two matrices is dependent on the physiochemical properties of the drug. However, if the distribution is rapid and pH-independent, the concentration in saliva could correspond to the unbound concentration of the drug in plasma [94], [95]. Gordi *et al*, showed comparable levels of artemisinin in saliva as in unbound plasma concentrations using high performance liquid chromatography [51]. Considering that artesunate is highly hydrophilic (pKa 4.6, >99% ionized in plasma, pH 7.4) and that dihydroartemisinin is relatively lipophilic (pKa 12.1, almost unionized in plasma), dihydroartemisinin will be more likely to be transported by passive diffusion [90], [96]. Dihydroartemisinin is therefore more likely to show a correlation between concentrations in saliva and unbound concentrations in plasma. Indeed, saliva concentrations versus unbound concentrations in plasma of dihydroartemisinin were found to have a higher correlation than that of artesunate when using the developed and validated bioanalytical method to analyze plasma and saliva samples from a clinical study in cancer patients treated with artesunate [97], [98]. Saliva concentrations as a proxy for unbound plasma concentrations of dihydroartemisinin could also be highly advantageous in malaria treatment monitoring and subsequent evaluations of the dose-response relationship since dihydroartemisinin is an active metabolite of artesunate and mainly

responsible for the parasite elimination after oral administration of artesunate. The assay was validated for artesunate and dihydroartemisinin in both plasma and saliva according to FDA guidelines, and has proven to be highly useful in clinical sample analysis, with future applicability in malaria treatment evaluations.

Several of the artemisinin derivatives are today used in combination therapies in the treatment of malaria. Artemisinin itself, has previously not been commonly used in ACTs as a consequence of the auto-induction of its own metabolizing CYP-enzymes [39]–[41]. However, in a short two-day treatment the auto-induction will be less prominent although with effectiveness comparable to a three-day artesunate-mefloquine treatment [42], [99]. In addition, a lower incidence of adverse events in the gastrointestinal tract was seen after administration of artemisinin-piperaquine as compared to after administration of dihydroartemisinin-piperaquine. A shorter treatment course and more favorable safety profile would undoubtable result in increased compliance. However, a shorter treatment course should be considered carefully before clinical implementation due to the possible resistance development. Another concern is the relatively low bioavailability of artemisinin when used clinically and different attempts have been made to increase the bioavailability of the drug. Micronized formulations are known to increase the solubility and thereby the bioavailability of drugs, and it was therefore hypothesized to be a viable approach to increase the bioavailability of artemisinin [100].

It was concluded from the pharmacokinetic analysis that increasing the dose resulted in a significant increase of the mean transit absorption time but the micronized formulation or concomitant piperaquine administration did not affect the pharmacokinetic properties of artemisinin. Therefore, the micronized formulation will not result in an enhanced absorption of the drug as hoped. The developed final model may still be an important tool to investigate new dosing regimens *in silico* and to be implemented in clinical trial simulations for informative design of future clinical trials.

Malaria during pregnancy is a major health concern for both the mother and the fetus. ACTs are recommended by the WHO as first-line treatment of uncomplicated *P. falciparum* malaria in the second and third trimesters [30]. However, ACTs are not recommended to women in their first trimester of pregnancy. This restriction is based on studies in animals where the tested drugs (artemisinin, artesunate, dihydroartemisinin, artemether and artemotil) have shown strong embryotoxic effects both in rats and monkeys and teratogenic effects in rats [101]–[103]. Clinical studies during pregnancy are

generally rare and often small in size due to the ethical and practical concerns of recruiting pregnant women. In the first trimester, only a few clinical studies exists for artesunate and dihydroartemisinin [104], [105]. In the second and third trimester, there are more extensive studies of these drugs, but the results are somewhat contradictive [84], [106], [107]. Therefore, it would be of interest to have a simple and scalable animal model in order to evaluate this in more detail. The pharmacokinetic properties of artesunate and dihydroartemisinin have successfully been described in the pregnant and non-pregnant rat and in the pregnant and non-pregnant patient in this thesis. Identified pregnancy effects in the rat correlated with effects found in pregnant women, which indicates that this could be a suitable animal model for studies in early pregnancy with translation to human.

The pharmacokinetic evaluation of artesunate and dihydroartemisinin in pregnant and non-pregnant rats demonstrated a substantial pregnancy-induced increase in volume of distribution and clearance during pregnancy. An increase in volume of distribution is commonly seen in pregnant women due to an increased blood volume and altered plasma protein levels. This could result in a lower initial concentration and/or a longer half-life, which is less likely in this case due to the simultaneous increase in clearance. Several metabolizing enzymes are also altered during pregnancy, which could explain the increase in clearance.

The last project of this thesis focused on describing the pharmacokinetic effects of pregnancy in pregnant and non-pregnant patients with uncomplicated *P. falciparum* malaria after receiving oral artesunate treatment. Pregnant patients showed a significantly increased elimination clearance for dihydroartemisinin but no other alteration to the pharmacokinetics. Dihydroartemisinin is metabolized via glucuronidation by the enzymes, UGT 1A9 and 2B7, and this increase in clearance could be related to increased levels of enzymes during pregnancy. An increase in clearance with subsequent lower exposure of the drug could have serious consequences as it increases the risk of treatment failure. Sub-therapeutic exposures and treatment failures will undoubtedly fuel the development of drug resistant malaria parasites.

This thesis has focused on evaluating and describing the pharmacokinetic properties of the artemisinins. A bioanalytical method is presented here for the measurement of artesunate and dihydroartemisinin in saliva, a non-invasive and field-adapted sampling method, as well as in plasma for the use in pharmacokinetic studies or therapeutic drug monitoring. A micronized formulation of artemisinin was evaluated, but the increase in bioavailability

was not considered to be of clinical importance. The pregnant rats showed similar pharmacokinetic properties to pregnant women, indicating that this could be a suitable animal model for translational studies from rat to pregnant women. Pregnant patients receiving oral artesunate might need a higher treatment dose since dihydroartemisinin exposure decreased compared to non-pregnant patients.

6 CONCLUSION

This thesis has investigated the pharmacokinetic properties of artemisinin and its derivatives, artesunate and dihydroartemisinin using nonlinear mixed-effects modeling. The following conclusions can be drawn:

- For the first time an accurate and sensitive bioanalytical assay for determination of artesunate and dihydroartemisinin in human saliva has been described.

- A pregnancy-related increase in the volume of distribution and in clearance was found for artesunate and dihydroartemisinin in the rat. This is in agreement with what has been found in pregnant women, indicating that this animal model could be suitable for translation to human data.

- A micronized formulation of artemisinin or concomitant piperaquine administration did not affect the pharmacokinetic properties of artemisinin in healthy male volunteers. This conclusion was in agreement with the results in the traditional bioequivalence study using non-compartmental analysis.

- A pregnancy-related increase in the elimination of dihydroartemisinin was found in patients. This could indicate a decreased treatment effect and possibly the need of increased dosing of artesunate in pregnant women with malaria.

7 FUTURE PERSPECTIVES

Since the year 2000, malaria mortality has decreased by nearly 50% worldwide. The main reason for this substantial improvement is the implementation of the highly effective artemisinin-based combination treatment and the roll out of bed nets. Despite this decrease in mortality, there are still roughly 2000 deaths every day and drug resistance against the artemisinins has now been established in Southeast Asia. However, the artemisinins are still the recommended first-line therapy against malaria and still shows excellent cure rates in most parts of the world, especially in Africa where the highest burden is. It is therefore important to characterize the pharmacokinetics of the artemisinins and optimize dosing regimens in all patient groups in order to maximize the therapeutic life-span of this treatment.

Pharmacometrics is a highly useful tool to maximize the information gained from pre-clinical and clinical trials and to identify particular patient groups at risk of treatment failure and resistance development. A pharmacometric framework was used in this thesis to evaluate the influence of pregnancy but also the influence of two different formulations, doses, and drug-drug interactions on the exposure to artemisinin and its derivatives. The findings presented in this thesis (similar pharmacokinetic properties in the pregnant rat as in pregnant women, lower exposure of dihydroartemisinin in pregnant women, not sufficient effect of micronized formulation, no dose-dependency or drug-drug interactions) advance our knowledge substantially about the pharmacokinetic properties of artemisinins. Furthermore, the thesis present validated and accurate population-based models for future clinical trial design simulations of new drug combination studies (possibly triple combinations), further evaluation of the pharmacokinetic properties in early pregnancy but also for establishing an evidence-based dose recommendation for artesunate in pregnant women.

ACKNOWLEDGEMENT

The work on this thesis was carried out at the unit of pharmacokinetics and drug metabolism, department of pharmacology, institute of neuroscience and physiology, Sahlgrenska academy, University of Gothenburg, with visits to the pharmacology department at the Mahidohl-Oxford Tropical Medicine Research Unit, Bangkok, Thailand.

I want to thank my supervisor Michael Ashton, for introducing me to the field of pharmacokinetics, it has been a true pleasure to be part of the unit and I have learned so much…you still haven't taught me to enjoy the Ale, though…

My co-supervisor Angela Äbelö, for all the time you put in to teach me about the art of modeling! Thanks for long discussions about modeling, models and life in general. Keep up the good teaching spirit, and looking forward to future discussions.

My co-supervisor Joel Tärning, for introducing me to research in general and pharmacokinetics and pharmacometrics in particular.

My mentor in the world of mass spectrometry, Kurt-Jürgen. You have taught me so much more than fragments, ester hydrolysis, mass spectra and ion counts, having you in the lab have always brightened up my days!

To my co-authors; Michael Ashton, Angela Äbelö, Joel Tärning, Kurt-Jürgen Hoffmann, Innocent Valea, Halidou Tinto, Maminata Traore, Laeticia C. Toe, Jean-Pierre Van Geertruyden, Geraint R. Davies, Stephen A. Ward, Umberto D'Alessandro, Pham Van Toi, Nguyen Thanh Truong, Nguyen Thi Dung Tran Tinh Hien, Therese Ericsson, Antje Blank, Cornelia von Hagens, this would not have been possible without you.

To all the participants of the clinical studies, both patients and healthy volunteers and the medical staff.

To the great people in the unit of PKDM of Gothenburg.
Carl, my partner in crime for a long time and lovely traveling companion. Dinko, I have had so much fun working with you, and the "pyjamas-story" is the best! Therese, thanks for a nice collaboration and always an invitation to join your exercise. Eddie, you are a true happy spirit! Jesper, newest member of the unit, enjoy your time. Elin, our newest triathlete member in the unit, thanks for nice discussions about science and training and lots more. To all the colleagues I have had the pleasure to work with in the group: Emile, Betty, Maria, Eugenia, Rasmus, Daniel, Sara and Sofia Friberg-Hietala, you have all meant a lot to me. Christina Roth, we started as master students at the unit and that's where it all began. I had so much fun working with you, thanks for always cheering me up!
Richard, the engineer (??!), always a helping hand for me and everyone else in the unit. I am so happy about our friendship, and look forward to visit you in Bangkok.

To my master-thesis students, Jennie, Frida and Christian. Thanks for all your help.

To all my friends and colleagues at the department of pharmacology.

To everyone at the MORU unit in Bangkok, the atmosphere you have at the office is unique and you made me feel very welcome.

To Niklas Lindegårdh, for inviting me to Bangkok, and being a great source of inspiration. Rest in peace.

To my dear and most beloved family, thank you for always supporting me although I have "stayed in school" for a long time. To my dad who always wondered why I didn't become a physician, studying for such a long time; Pappa, jag kommer inte att bli läkare men nu är jag doktor!

And my extended family of relatives and dear friends, I receive so much love from you all. Caroline D and Caroline R, especially for getting me into study pharmacy and for everlasting support. Thanks.

Jonas, last but not least. You are my partner in life, my best friend and loved one. This would not have been possible without your solid support. You and Gustav mean the world to me.
Med dig är jag stark, just idag!

REFERENCES

[1] L. Benet, *Pharmacokinetics: Basic principles and its use as a tool in drug metabolism.* 1984.

[2] Paul B. David B. Troy, *Remington: The Science and Practice of Pharmacy*. Lippincott Williams & Wilkins, 2006.

[3] J. S. Barrett, M. J. Fossler, K. D. Cadieu, and M. R. Gastonguay, "Pharmacometrics: a multidisciplinary field to facilitate critical thinking in drug development and translational research settings.," *J. Clin. Pharmacol.*, vol. 48, no. 5, pp. 632–649, 2008.

[4] L. Aarons, "Population pharmacokinetics: theory and practice.," *Br. J. Clin. Pharmacol.*, vol. 32, no. 6, pp. 669–670, 1991.

[5] World Health Organization, "WHO World Malaria Report 2014," 2014.

[6] A. M. Dondorp, "Clinical significance of sequestration in adults with severe malaria.," *Transfus. Clin. Biol.*, vol. 15, no. 1–2, pp. 56–7, Jan. .

[7] L. H. Miller, M. F. Good, and G. Milon, "Malaria pathogenesis.," *Science*, vol. 264, no. 5167, pp. 1878–83, Jun. 1994.

[8] C.-C. for D. C. and Prevention, "CDC - Malaria - About Malaria - Biology."

[9] N. J. White, S. Pukrittayakamee, T. T. Hien, M. A. Faiz, O. A. Mokuolu, and A. M. Dondorp, "Malaria.," *Lancet*, vol. 383, no. 9918, pp. 723–35, Feb. 2014.

[10] D. of C. of T. D. World Health Organization, "Severe and Complicated Malaria," *Trans. R. Soc. Trop. Med. Hyg.*, vol. 84, no. Supplement 2, pp. 1 – 65, 1990.

[11] P. C. Bull, B. S. Lowe, M. Kortok, C. S. Molyneux, C. I. Newbold, and K. Marsh, "Parasite antigens on the infected red cell surface are

targets for naturally acquired immunity to malaria.," *Nat. Med.*, vol. 4, no. 3, pp. 358–60, Mar. 1998.

[12] J. Smereck, "Malaria in pregnancy: update on emergency management.," *J. Emerg. Med.*, vol. 40, no. 4, pp. 393–6, Apr. 2011.

[13] J. Schantz-Dunn and N. M. Nour, "Malaria and pregnancy: a global health perspective.," *Rev. Obstet. Gynecol.*, vol. 2, no. 3, pp. 186–92, Jan. 2009.

[14] E. T. Abrams and S. R. Meshnick, "Malaria during pregnancy in endemic areas: a lens for examining maternal-fetal conflict.," *Am. J. Hum. Biol.*, vol. 21, no. 5, pp. 643–50, Jan. .

[15] S. Lindsay, J. Ansell, C. Selman, V. Cox, K. Hamilton, and G. Walraven, "Effect of pregnancy on exposure to malaria mosquitoes.," *Lancet*, vol. 355, no. 9219, p. 1972, Jun. 2000.

[16] M. Fried, F. Nosten, A. Brockman, B. J. Brabin, and P. E. Duffy, "Maternal antibodies block malaria.," *Nature*, vol. 395, no. 6705, pp. 851–2, Oct. 1998.

[17] H. L. Guyatt and R. W. Snow, "Impact of Malaria during Pregnancy on Low Birth Weight in Sub-Saharan Africa," vol. 17, no. 4, pp. 760–769, 2004.

[18] M. J. Rijken, A. T. Papageorghiou, S. Thiptharakun, S. Kiricharoen, S. L. M. Dwell, J. Wiladphaingern, M. Pimanpanarak, S. H. Kennedy, F. Nosten, and R. McGready, "Ultrasound evidence of early fetal growth restriction after maternal malaria infection," *PLoS One*, vol. 7, no. 2, pp. 1–8, 2012.

[19] J. Verdrager, "Epidemiology of the emergence and spread of drug-resistant falciparum malaria in South-East Asia and Australasia.," *J. Trop. Med. Hyg.*, vol. 89, no. 6, pp. 277–89, Dec. 1986.

[20] C. Amaratunga, S. Sreng, S. Suon, E. S. Phelps, K. Stepniewska, P. Lim, C. Zhou, S. Mao, J. M. Anderson, N. Lindegardh, H. Jiang, J. Song, X. Z. Su, N. J. White, A. M. Dondorp, T. J. Anderson, M. P. Fay, J. Mu, S. Duong, and R. M. Fairhurst, "Artemisinin-resistant Plasmodium falciparum in Pursat province, western Cambodia: a

parasite clearance rate study," *Lancet Infect Dis*, vol. 12, no. 11, pp. 851–858, 2012.

[21] A. M. Dondorp, F. Nosten, P. Yi, D. Das, A. P. Phyo, J. Tarning, K. M. Lwin, F. Ariey, W. Hanpithakpong, S. J. Lee, P. Ringwald, K. Silamut, M. Imwong, K. Chotivanich, P. Lim, T. Herdman, S. S. An, S. Yeung, P. Singhasivanon, N. P. Day, N. Lindegardh, D. Socheat, and N. J. White, "Artemisinin resistance in Plasmodium falciparum malaria," *N Engl J Med*, vol. 361, no. 5, pp. 455–467, 2009.

[22] H. Noedl, Y. Se, K. Schaecher, B. L. Smith, D. Socheat, M. M. Fukuda, and A. S. Consortium, "Evidence of Artemisinin-Resistant Malaria in Western Cambodia," *N. Engl. J. Med.*, vol. 359, no. 24, pp. 2619–2620, 2008.

[23] K. Na-Bangchang and J. Karbwang, "Emerging artemisinin resistance in the border areas of Thailand.," *Expert Rev. Clin. Pharmacol.*, vol. 6, no. 3, pp. 307–22, May 2013.

[24] D. L. Saunders, P. Vanachayangkul, and C. Lon, "Dihydroartemisinin-piperaquine failure in Cambodia.," *N. Engl. J. Med.*, vol. 371, no. 5, pp. 484–5, Jul. 2014.

[25] F. Ariey, B. Witkowski, C. Amaratunga, J. Beghain, A.-C. Langlois, N. Khim, S. Kim, V. Duru, C. Bouchier, L. Ma, P. Lim, R. Leang, S. Duong, S. Sreng, S. Suon, C. M. Chuor, D. M. Bout, S. Ménard, W. O. Rogers, B. Genton, T. Fandeur, O. Miotto, P. Ringwald, J. Le Bras, A. Berry, J.-C. Barale, R. M. Fairhurst, F. Benoit-Vical, O. Mercereau-Puijalon, and D. Ménard, "A molecular marker of artemisinin-resistant Plasmodium falciparum malaria.," *Nature*, vol. 505, no. 7481, pp. 50–5, Jan. 2014.

[26] J. Straimer, N. F. Gnadig, B. Witkowski, C. Amaratunga, V. Duru, A. P. Ramadani, M. Dacheux, N. Khim, L. Zhang, S. Lam, P. D. Gregory, F. D. Urnov, O. Mercereau-Puijalon, F. Benoit-Vical, R. M. Fairhurst, D. Menard, and D. A. Fidock, "K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates," *Science (80-.).*, vol. 347, no. 6220, pp. 428–31, Dec. 2014.

[27] R. Leang, W. R. J. Taylor, D. M. Bouth, L. Song, J. Tarning, M. C. Char, S. Kim, B. Witkowski, V. Duru, A. Domergue, N. Khim, P. Ringwald, and D. Menard, "Evidence of Plasmodium falciparum

Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment.," *Antimicrob. Agents Chemother.*, vol. 59, no. 8, pp. 4719–26, Aug. 2015.

[28] N. J. White, "Antimalarial drug resistance," *J Clin Invest*, vol. 113, no. 8, pp. 1084–1092, 2004.

[29] J. Zwang, E. A. Ashley, C. Karema, U. D'Alessandro, F. Smithuis, G. Dorsey, B. Janssens, M. Mayxay, P. Newton, P. Singhasivanon, K. Stepniewska, N. J. White, and F. Nosten, "Safety and efficacy of dihydroartemisinin-piperaquine in falciparum malaria: a prospective multi-centre individual patient data analysis," *PLoS One*, vol. 4, no. 7, p. e6358, 2009.

[30] World Health Organization, "WHO | Guidelines for the treatment of malaria. Third edition," 2015.

[31] F. Nosten and N. J. White, "Artemisinin-based combination treatment of falciparum malaria," *Am. J. Trop. Med. Hyg.*, vol. 77, no. SUPPL. 6, pp. 181–192, 2007.

[32] N. J. White, "Delaying antimalarial drug resistance with combination chemotherapy.," *Parassitologia*, vol. 41, no. 1–3, pp. 301–8, Sep. 1999.

[33] V. Navaratnam, S. M. Mansor, N. W. Sit, J. Grace, Q. Li, and P. Olliaro, "Pharmacokinetics of artemisinin-type compounds.," *Clin. Pharmacokinet.*, vol. 39, no. 4, pp. 255–70, Oct. 2000.

[34] D. L. Klayman, "Qinghaosu (artemisinin): an antimalarial drug from China.," *Science*, vol. 228, no. 4703, pp. 1049–1055, 1985.

[35] D. D. Duc, P. J. de Vries, X. K. Nguyen, B. Le Nguyen, P. A. Kager, and C. J. van Boxtel, "The pharmacokinetics of a single dose of artemisinin in healthy Vietnamese subjects.," *Am. J. Trop. Med. Hyg.*, vol. 51, no. 6, pp. 785–90, Dec. 1994.

[36] M. Ashton, T. Gordi, N. H. Trinh, V. H. Nguyen, D. S. Nguyen, T. N. Nguyen, X. H. Dinh, M. Johansson, and D. C. Le, "Artemisinin pharmacokinetics in healthy adults after 250, 500 and 1000 mg single

oral doses," *Biopharm Drug Dispos*, vol. 19, no. 4, pp. 245–250, 1998.

[37] P. T. Giao, T. Q. Binh, P. A. Kager, H. P. Long, N. Van Thang, N. Van Nam, and P. J. de Vries, "Artemisinin for treatment of uncomplicated falciparum malaria: is there a place for monotherapy?," *Am. J. Trop. Med. Hyg.*, vol. 65, no. 6, pp. 690–5, Dec. 2001.

[38] M. Ashton, D. S. Nguyen, V. H. Nguyen, T. Gordi, N. H. Trinh, X. H. Dinh, T. N. Nguyen, and D. C. Le, "Artemisinin kinetics and dynamics during oral and rectal treatment of uncomplicated malaria," *Clin Pharmacol Ther*, vol. 63, no. 4, pp. 482–493, 1998.

[39] S. Asimus, D. Elsherbiny, T. N. Hai, B. Jansson, N. V Huong, M. G. Petzold, U. S. H. Simonsson, and M. Ashton, "Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects," *Fundam. Clin. Pharmacol.*, vol. 21, no. 3, pp. 307–316, 2007.

[40] U. S. Simonsson, B. Jansson, T. N. Hai, D. X. Huong, G. Tybring, and M. Ashton, "Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9," *Clin Pharmacol Ther*, vol. 74, no. 1, pp. 32–43, 2003.

[41] U. S. Svensson, M. Ashton, N. H. Trinh, L. Bertilsson, X. H. Dinh, V. H. Nguyen, T. N. Nguyen, D. S. Nguyen, J. Lykkesfeldt, and D. C. Le, "Artemisinin induces omeprazole metabolism in human beings," *Clin Pharmacol Ther*, vol. 64, no. 2, pp. 160–167, 1998.

[42] N. X. Thanh, T. N. Trung, N. C. Phong, H. H. Quang, B. Dai, G. D. Shanks, M. Chavchich, and M. D. Edstein, "The efficacy and tolerability of artemisinin-piperaquine (Artequick®) versus artesunate-amodiaquine (Coarsucam^{TM}) for the treatment of uncomplicated Plasmodium falciparum malaria in south-central Vietnam.," *Malar. J.*, vol. 11, p. 217, Jan. 2012.

[43] C. A. Morris, S. Duparc, I. Borghini-Fuhrer, D. Jung, C.-S. Shin, and L. Fleckenstein, "Review of the clinical pharmacokinetics of artesunate and its active metabolite dihydroartemisinin following intravenous, intramuscular, oral or rectal administration.," *Malar. J.*, vol. 10, p. 263, Jan. 2011.

[44] S. R. Meshnick, T. E. Taylor, and S. Kamchonwongpaisan, "Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy.," *Microbiol. Rev.*, vol. 60, no. 2, pp. 301–315, 1996.

[45] X.-Q. Li, A. Björkman, T. B. Andersson, L. L. Gustafsson, and C. M. Masimirembwa, "Identification of human cytochrome P 450 s that metabolise anti-parasitic drugs and predictions of in vivo drug hepatic clearance from in vitro data," *Eur. J. Clin. Pharmacol.*, vol. 59, no. 5–6, pp. 429–442, Sep. 2003.

[46] K. F. Ilett, B. T. Ethell, J. L. Maggs, T. M. Davis, K. T. Batty, B. Burchell, T. Q. Binh, T. A. Thu le, N. C. Hung, M. Pirmohamed, B. K. Park, and G. Edwards, "Glucuronidation of dihydroartemisinin in vivo and by human liver microsomes and expressed UDP-glucuronosyltransferases," *Drug Metab Dispos*, vol. 30, no. 9, pp. 1005–1012, 2002.

[47] N. J. White, "Assessment of the pharmacodynamic properties of antimalarial drugs in vivo.," *Antimicrob. Agents Chemother.*, vol. 41, no. 7, pp. 1413–22, Jul. 1997.

[48] J. Golenser, J. H. Waknine, M. Krugliak, N. H. Hunt, and G. E. Grau, "Current perspectives on the mechanism of action of artemisinins," *Int. J. Parasitol.*, vol. 36, no. 14, pp. 1427–1441, 2006.

[49] F. ter Kuile, N. J. White, P. Holloway, G. Pasvol, and S. Krishna, "Plasmodium falciparum: in vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria.," *Exp. Parasitol.*, vol. 76, no. 1, pp. 85–95, Feb. 1993.

[50] P. O. Edlund, D. Westerlund, J. Carlqvist, B. L. Wu, and Y. H. Jin, "Determination of artesunate and dihydroartemisinine in plasma by liquid chromatography with post-column derivatization and UV-detection.," *Acta Pharm. Suec.*, vol. 21, no. 4, pp. 223–34, Jan. 1984.

[51] T. Gordi, T. N. Hai, N. M. Hoai, M. Thyberg, and M. Ashton, "Use of saliva and capillary blood samples as substitutes for venous blood sampling in pharmacokinetic investigations of artemisinin.," *Eur. J. Clin. Pharmacol.*, vol. 56, no. 8, pp. 561–6, Nov. 2000.

[52] K. T. Batty, T. M. Davis, L. T. Thu, T. Q. Binh, T. K. Anh, and K. F. Ilett, "Selective high-performance liquid chromatographic determination of artesunate and alpha- and beta-dihydroartemisinin in patients with falciparum malaria.," *J. Chromatogr. B. Biomed. Appl.*, vol. 677, no. 2, pp. 345–50, Mar. 1996.

[53] C.-S. Lai, N. K. Nair, S. M. Mansor, P. L. Olliaro, and V. Navaratnam, "An analytical method with a single extraction procedure and two separate high performance liquid chromatographic systems for the determination of artesunate, dihydroartemisinin and mefloquine in human plasma for application in clinical pharmacological stu," *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, vol. 857, no. 2, pp. 308–14, Oct. 2007.

[54] K. Na-Bangchang, K. Congpuong, L. N. Hung, P. Molunto, and J. Karbwang, "Simple high-performance liquid chromatographic method with electrochemical detection for the simultaneous determination of artesunate and dihydroartemisinin in biological fluids.," *J. Chromatogr. B. Biomed. Sci. Appl.*, vol. 708, no. 1–2, pp. 201–7, Apr. 1998.

[55] V. Navaratnam, M. N. Mordi, and S. M. Mansor, "Simultaneous determination of artesunic acid and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies.," *J. Chromatogr. B. Biomed. Sci. Appl.*, vol. 692, no. 1, pp. 157–62, Apr. 1997.

[56] H. Naik, D. J. Murry, L. E. Kirsch, and L. Fleckenstein, "Development and validation of a high-performance liquid chromatography-mass spectroscopy assay for determination of artesunate and dihydroartemisinin in human plasma.," *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, vol. 816, no. 1–2, pp. 233–42, Feb. 2005.

[57] W. Hanpithakpong, B. Kamanikom, A. M. Dondorp, P. Singhasivanon, N. J. White, N. P. Day, and N. Lindegardh, "A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma," *J Chromatogr B Anal. Technol Biomed Life Sci*, vol. 876, no. 1, pp. 61–68, 2008.

[58] L. B. Sheiner, "The population approach to pharmacokinetic data analysis: rationale and standard data analysis methods.," *Drug Metab. Rev.*, vol. 15, no. 1–2, pp. 153–71, Jan. 1984.

[59] R. J. Bauer, S. Guzy, and C. Ng, "A survey of population analysis methods and software for complex pharmacokinetic and pharmacodynamic models with examples.," *AAPS J.*, vol. 9, no. 1, pp. E60–83, Jan. 2007.

[60] T. H. Grasela and L. B. Sheiner, "Pharmacostatistical modeling for observational data," *J. Pharmacokinet. Biopharm.*, vol. 19, no. S3, p. 25S–36S, 1991.

[61] M. O. Karlsson and L. B. Sheiner, "The importance of modeling interoccasion variability in population pharmacokinetic analyses.," *J. Pharmacokinet. Biopharm.*, vol. 21, no. 6, pp. 735–50, Dec. 1993.

[62] Food and Drug Administration, *Draft Guidance for Industry Bioanalytical Method Validation*, no. September. 2013.

[63] N. Lindegardh, J. Tarning, P. V Toi, T. T. Hien, J. Farrar, P. Singhasivanon, N. J. White, M. Ashton, and N. P. Day, "Quantification of artemisinin in human plasma using liquid chromatography coupled to tandem mass spectrometry," *J Pharm Biomed Anal*, vol. 49, no. 3, pp. 768–773, 2009.

[64] I. Valea, H. Tinto, M. Traore-Coulibaly, L. C. Toe, N. Lindegardh, J. Tarning, J.-P. Van Geertruyden, U. D'Alessandro, G. R. Davies, and S. A. Ward, "Pharmacokinetics of co-formulated mefloquine and artesunate in pregnant and non-pregnant women with uncomplicated Plasmodium falciparum infection in Burkina Faso.," *J. Antimicrob. Chemother.*, vol. 69, no. 9, pp. 2499–507, Sep. 2014.

[65] "NONMEM Users Guide." [Online]. Available: ftp://nonmem.iconplc.com/Public/nonmemVI/guides/intro.pdf. [Accessed: 18-Feb-2015].

[66] L. Lindbom, P. Pihlgren, and E. N. Jonsson, "PsN-Toolkit--a collection of computer intensive statistical methods for non-linear mixed effect modeling using NONMEM," *Comput Methods Programs Biomed*, vol. 79, no. 3, pp. 241–257, 2005.

[67] R. J. Keizer, M. van Benten, J. H. Beijnen, J. H. Schellens, and A. D. Huitema, “Pirana and PCluster: a modeling environment and cluster infrastructure for NONMEM,” *Comput Methods Programs Biomed*, vol. 101, no. 1, pp. 72–79, 2011.

[68] E. N. Jonsson and M. O. Karlsson, “Xpose--an S-PLUS based population pharmacokinetic/pharmacodynamic model building aid for NONMEM,” *Comput Methods Programs Biomed*, vol. 58, no. 1, pp. 51–64, 1999.

[69] S. L. Beal, “Ways to fit a PK model with some data below the quantification limit,” *J. Pharmacokinet. Pharmacodyn.*, vol. 28, no. 5, pp. 481–504, 2001.

[70] S. L. Beal and L. B. Sheiner, “Estimating population kinetics.,” *Crit. Rev. Biomed. Eng.*, vol. 8, no. 3, pp. 195–222, Jan. 1982.

[71] R. M. Savic, D. M. Jonker, T. Kerbusch, and M. O. Karlsson, “Implementation of a transit compartment model for describing drug absorption in pharmacokinetic studies.,” *J. Pharmacokinet. Pharmacodyn.*, vol. 34, no. 5, pp. 711–26, Oct. 2007.

[72] M. Bergstrand and M. O. Karlsson, “Handling data below the limit of quantification in mixed effect models.,” *AAPS J.*, vol. 11, no. 2, pp. 371–380, 2009.

[73] B. J. Anderson and N. H. Holford, “Mechanistic basis of using body size and maturation to predict clearance in humans,” *Drug Metab Pharmacokinet*, vol. 24, no. 1, pp. 25–36, 2009.

[74] B. J. Anderson and N. H. Holford, “Mechanism-based concepts of size and maturity in pharmacokinetics,” *Annu Rev Pharmacol Toxicol*, vol. 48, pp. 303–332, 2008.

[75] S. C. McLeay, G. A. Morrish, C. M. J. Kirkpatrick, and B. Green, “The relationship between drug clearance and body size: systematic review and meta-analysis of the literature published from 2000 to 2007.,” *Clin. Pharmacokinet.*, vol. 51, no. 5, pp. 319–30, May 2012.

[76] A. C. Hooker, C. E. Staatz, and M. O. Karlsson, “Conditional weighted residuals (CWRES): a model diagnostic for the FOCE method,” *Pharm Res*, vol. 24, no. 12, pp. 2187–2197, 2007.

[77] R. M. Savic and M. O. Karlsson, “Importance of shrinkage in empirical bayes estimates for diagnostics: problems and solutions.,” *AAPS J.*, vol. 11, no. 3, pp. 558–69, Sep. 2009.

[78] M. Bergstrand, A. C. Hooker, J. E. Wallin, and M. O. Karlsson, “Prediction-corrected visual predictive checks for diagnosing nonlinear mixed-effects models.,” *AAPS J.*, vol. 13, no. 2, pp. 143–51, Jun. 2011.

[79] W. Cabri, A. Ciogli, I. D’Acquarica, M. Di Mattia, B. Galletti, F. Gasparrini, F. Giorgi, S. Lalli, M. Pierini, and P. Simone, “On-column epimerization of dihydroartemisinin: an effective analytical approach to overcome the shortcomings of the International Pharmacopoeia monograph.,” *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, vol. 875, no. 1, pp. 180–91, Nov. 2008.

[80] K. T. Batty, K. F. Ilett, T. Davis, and M. E. Davis, “Chemical stability of artesunate injection and proposal for its administration by intravenous infusion.,” *J. Pharm. Pharmacol.*, vol. 48, no. 1, pp. 22–6, Jan. 1996.

[81] F. H. Jansen and S. A. Soomro, “Chemical instability determines the biological action of the artemisinins.,” *Curr. Med. Chem.*, vol. 14, no. 30, pp. 3243–59, Jan. 2007.

[82] N. Lindegardh, W. Hanpithakpong, B. Kamanikom, P. Singhasivanon, D. Socheat, P. Yi, A. M. Dondorp, R. McGready, F. Nosten, N. J. White, and N. P. Day, “Major pitfalls in the measurement of artemisinin derivatives in plasma in clinical studies,” *J Chromatogr B Anal. Technol Biomed Life Sci*, vol. 876, no. 1, pp. 54–60, 2008.

[83] G. D. Anderson, “Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach.,” *Clin. Pharmacokinet.*, vol. 44, no. 10, pp. 989–1008, Jan. 2005.

[84] C. a Morris, M. a Onyamboko, E. Capparelli, M. a Koch, J. Atibu, V. Lokomba, M. Douoguih, J. Hemingway-Foday, D. Wesche, R. W. Ryder, C. Bose, L. Wright, A. K. Tshefu, S. Meshnick, and L. Fleckenstein, “Population pharmacokinetics of artesunate and dihydroartemisinin in pregnant and non-pregnant women with malaria.,” *Malar. J.*, vol. 10, no. 1, p. 114, 2011.

[85] J. Tarning, M. J. Rijken, R. McGready, A. P. Phyo, W. Hanpithakpong, N. P. J. Day, N. J. White, F. Nosten, and N. Lindegardh, "Population pharmacokinetics of dihydroartemisinin and piperaquine in pregnant and nonpregnant women with uncomplicated malaria," *Antimicrob. Agents Chemother.*, vol. 56, no. 4, pp. 1997–2007, 2012.

[86] J. S. Sidhu, M. Ashton, N. V Huong, T. N. Hai, M. O. Karlsson, N. D. Sy, E. N. Jonsson, and L. D. Cong, "Artemisinin population pharmacokinetics in children and adults with uncomplicated falciparum malaria," *Br J Clin Pharmacol*, vol. 45, no. 4, pp. 347–354, 1998.

[87] T. T. Hien, W. Hanpithakpong, N. T. Truong, N. T. Dung, P. V Toi, J. Farrar, N. Lindegardh, J. Tarning, and M. Ashton, "Orally formulated artemisinin in healthy fasting Vietnamese male subjects: a randomized, four-sequence, open-label, pharmacokinetic crossover study," *Clin Ther*, vol. 33, no. 5, pp. 644–654, 2011.

[88] US Food and Drug Administration, "Guidance for Industry: Bioavailability and bioequivalence studies for orally administered drug products," 2003.

[89] T. Gordi, D. X. Huong, T. N. Hai, N. T. Nieu, and M. Ashton, "Artemisinin pharmacokinetics and efficacy in uncomplicated-malaria patients treated with two different dosage regimens.," *Antimicrob. Agents Chemother.*, vol. 46, no. 4, pp. 1026–31, Apr. 2002.

[90] P. Augustijns, A. D'Hulst, J. Van Daele, and R. Kinget, "Transport of artemisinin and sodium artesunate in Caco-2 intestinal epithelial cells.," *J. Pharm. Sci.*, vol. 85, no. 6, pp. 577–9, Jun. 1996.

[91] K. T. Batty, S. Salman, B. R. Moore, J. Benjamin, S. T. Lee, M. Page-Sharp, N. Pitus, K. F. Ilett, I. Mueller, F. W. Hombhanje, P. Siba, and T. M. E. Davis, "Artemisinin-naphthoquine combination therapy for uncomplicated pediatric malaria: a pharmacokinetic study.," *Antimicrob. Agents Chemother.*, vol. 56, no. 5, pp. 2472–84, May 2012.

[92] F. Kloprogge, R. McGready, A. P. Phyo, M. J. Rijken, W. Hanpithakpon, H. H. Than, N. Hlaing, N. T. Zin, N. P. J. Day, N. J. White, F. Nosten, and J. Tarning, "Opposite malaria and pregnancy

effect on oral bioavailability of artesunate - a population pharmacokinetic evaluation.," *Br. J. Clin. Pharmacol.*, Apr. 2015.

[93] B. G. Bhat, N. Hosea, A. Fanjul, J. Herrera, J. Chapman, F. Thalacker, P. M. Stewart, and P. A. Rejto, "Demonstration of proof of mechanism and pharmacokinetics and pharmacodynamic relationship with 4'-cyano-biphenyl-4-sulfonic acid (6-amino-pyridin-2-yl)-amide (PF-915275), an inhibitor of 11 -hydroxysteroid dehydrogenase type 1, in cynomolgus monkeys," *J Pharmacol Exp Ther*, vol. 324, no. 1, pp. 299–305, 2008.

[94] W. J. Jusko and R. L. Milsap, "Pharmacokinetic principles of drug distribution in saliva.," *Ann. N. Y. Acad. Sci.*, vol. 694, pp. 36–47, Sep. 1993.

[95] G. Graham, "Noninvasive chemical methods of estimating pharmacokinetic parameters," *Pharmacol. Ther.*, vol. 18, pp. 333–349, 1982.

[96] "Human Metabolome Database: Showing metabocard for Dihydroartemisinin (DHA) (HMDB61088)." [Online]. Available: http://www.hmdb.ca/metabolites/HMDB61088. [Accessed: 23-Aug-2015].

[97] S. Birgersson, T. Ericsson, A. Blank, C. von Hagens, M. Ashton, and K.-J. Hoffmann, "A high-throughput LC-MS/MS assay for quantification of artesunate and its metabolite dihydroartemisinin in human plasma and saliva.," *Bioanalysis*, vol. 6, no. 18, pp. 2357–69, Sep. 2014.

[98] T. Ericsson, A. Blank, C. von Hagens, M. Ashton, and A. Äbelö, "Population pharmacokinetics of artesunate and dihydroartemisinin during long-term oral administration of artesunate to patients with metastatic breast cancer.," *Eur. J. Clin. Pharmacol.*, vol. 70, no. 12, pp. 1453–63, Dec. 2014.

[99] U. S. Svensson, H. Alin, M. O. Karlsson, Y. Bergqvist, and M. Ashton, "Population pharmacokinetic and pharmacodynamic modelling of artemisinin and mefloquine enantiomers in patients with falciparum malaria," *Eur J Clin Pharmacol*, vol. 58, no. 5, pp. 339–351, 2002.

[100] R. J. Jones, A. Rajabi-Siahboomi, M. Levina, Y. Perrie, and A. R. Mohammed, "The influence of formulation and manufacturing process parameters on the characteristics of lyophilized orally disintegrating tablets.," *Pharmaceutics*, vol. 3, no. 3, pp. 440–57, Jan. 2011.

[101] R. L. Clark, A. Arima, N. Makori, Y. Nakata, F. Bernard, W. Gristwood, A. Harrell, T. E. White, and P. J. Wier, "Artesunate: developmental toxicity and toxicokinetics in monkeys," *Birth Defects Res B Dev Reprod Toxicol*, vol. 83, no. 4, pp. 418–434, 2008.

[102] R. L. Clark, T. E. White, A. C. S, I. Gaunt, P. Winstanley, and S. A. Ward, "Developmental toxicity of artesunate and an artesunate combination in the rat and rabbit," *Birth Defects Res B Dev Reprod Toxicol*, vol. 71, no. 6, pp. 380–394, 2004.

[103] R. L. Clark, S. A. Lerman, E. M. Cox, W. E. Gristwood, and T. E. White, "Developmental toxicity of artesunate in the rat: comparison to other artemisinins, comparison of embryotoxicity and kinetics by oral and intravenous routes, and relationship to maternal reticulocyte count," *Birth Defects Res B Dev Reprod Toxicol*, vol. 83, no. 4, pp. 397–406, 2008.

[104] R. McGready, T. Cho, N. K. Keo, K. L. Thwai, L. Villegas, S. Looareesuwan, N. J. White, and F. Nosten, "Artemisinin antimalarials in pregnancy: a prospective treatment study of 539 episodes of multidrug-resistant Plasmodium falciparum.," *Clin. Infect. Dis.*, vol. 33, no. 12, pp. 2009–16, Dec. 2001.

[105] D. Mosha, F. Mazuguni, S. Mrema, E. Sevene, S. Abdulla, and B. Genton, "Safety of artemether-lumefantrine exposure in first trimester of pregnancy: an observational cohort," *Malar. J.*, vol. 13, no. 1, p. 197, 2014.

[106] R. Mcgready, A. P. Phyo, M. J. Rijken, J. Tarning, N. Lindegardh, W. Hanpithakpon, H. H. Than, N. Hlaing, N. T. Zin, P. Singhasivanon, N. J. White, and F. Nosten, "Artesunate/dihydroartemisinin pharmacokinetics in acute falciparum malaria in pregnancy: Absorption, bioavailability, disposition and disease effects," *Br. J. Clin. Pharmacol.*, vol. 73, pp. 467–477, 2012.

[107] J. Tarning, F. Kloprogge, P. Piola, M. Dhorda, S. Muwanga, E. Turyakira, N. Nuengchamnong, F. Nosten, N. P. Day, N. J. White, P. J. Guerin, and N. Lindegardh, "Population pharmacokinetics of Artemether and dihydroartemisinin in pregnant women with uncomplicated Plasmodium falciparum malaria in Uganda," *Malar. J.*, vol. 11, no. 1, p. 293, 2012.