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Clinical Pharmacokinetics
of the Antimalarial Artemisinin
Based on Saliva Sampling

BY

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ABSTRACT

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Artemisinin is the parent compound of a novel family of antimalarials. Repetitive administrations of artemisinin to both healthy volunteers and malaria patients have been shown to result in decreased plasma concentrations of the compound, most probably due to an autoinduction of different CYP450 enzymes. The aim of this thesis was to investigate the clinical pharmacokinetics and efficacy of different dosage regimens of the drug, and study the kinetics of the enzyme induction. Moreover, the putative interaction of the compound with blood components was investigated in vitro.

Artemisinin was found to distribute into red blood cells, competing with oxygen for binding to hemoglobin. The compound was stable in plasma and, in contrast to previous reports, did not bind to red blood cell membranes.

To circumvent the logistical and ethical problems associated with plasma sampling, suitability of saliva as substitute was investigated. Moreover, due to the large number of collected samples, an HPLC method, enabling a direct injection of saliva and plasma samples, was developed.

Saliva artemisinin concentrations were found to correlate with its unbound plasma levels, making saliva a suitable body fluid for pharmacokinetic studies of the compound. Based on saliva samples, artemisinin was shown to exhibit a dose-dependent kinetics and efficacy in malaria patients, with a possible sex-effect on the metabolism of the compound during the first treatment day. Moreover, the time-dependent kinetics of the compound was observed in both malaria patients and healthy subjects. A physiological approach was utilized to model the autoinduction in the latter group. A model with a feedback mechanism of enzymes was able to describe the data, with estimations of the half-lives of induction (3.15 hrs) and elimination of enzymes (32.9 hrs), as well as pharmacokinetic parameters of artemisinin.

In conclusion, artemisinin was found to exhibit a fast induction of enzymes, with time- and dose-dependent drug kinetics and dose-dependent antimalarial efficacy.

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توانا بود هر که دانا بود
وزارت آموزش و پرورش
هوشنگ گرجی

Powerful would be the one who possesses knowledge

Ministry of Education and Upbringing

Hooshang Gargari

To My Patients

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1. PAPERS DISCUSSED

This thesis is based on the following papers, which will be referred to by their Roman numerals (I-V):

I. Bakhshi H., Gordi T., and Ashton M. (1997) In-vitro interaction of artemisinin with intact human erythrocytes, erythrocyte ghosts, hemoglobin and carbonic anhydrase. *Journal of Pharmacy and Pharmacology*, 49:223-226

II. Gordi T., Nielsen E., Yu Z., Westerlund D., and Ashton M. (2000) Direct analysis of artemisinin in plasma and saliva using coupled-column high performance liquid chromatography with a restricted-access material pre-column. *Journal of Chromatography B, Biomedical Science Applications*, 742:155-162

III. Gordi T, Hai T. N., Hoai N. M., Thyberg M., and Ashton M. (2000) Use of saliva and capillary blood samples as substitutes for venous blood sampling in pharmacokinetic investigations of artemisinin. *European Journal of Clinical Pharmacology* 56:561-566

IV. Gordi T., Huong D. X., Hai T. N., Nieu N. T., and Ashton M. Artemisinin pharmacokinetics and efficacy in uncomplicated malaria patients treated with two different dosage regimens. *Antimicrobial Agents and Chemotherapy*. Submitted

V. Gordi T., Huong N. V., Huong D. X., Xie R., Karlsson M. O. and Ashton M. Mixed effect modeling of the time-course of artemisinin autoinduction in healthy subjects. Manuscript

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2. ABBREVIATIONS

AUC_{0-t}	Area under the plasma/saliva concentration-time curve from the time of administration to the last observed time-point
$AUC_{0-\infty}$	Area under the plasma/saliva concentration-time curve from the time of administration to infinity
AUMC	Area under the first moment curve
MRT	Mean residence time
C_{vu}, C_{cu}	Unbound concentration in venous and capillary plasma, respectively
$CI_{95\%}$	95% confidence interval
CL/F	Oral clearance
C_{max}	Maximum observed drug concentration
FST	Fever subsidence time
K_{enz}	Enzyme elimination rate constant
K_{pen}	Enzyme production rate constant, from enzyme precursor
PAUC	Area under the number of remaining parasites vs. time curve from the time of drug administration until total disappearance
PCR	Polymerize Chain Reaction
PK/PD	Pharmacokinetic/Pharmacodynamic
PMRT	Parasite mean residence time
$t_{1/2}$	Elimination half-life
t_{max}	Time of maximum drug concentration
v	Volume
V_d/F	Oral volume of distribution at steady state
PCT	Parasite Clearance Time
pK_a	Acid dissociation constant

3. Introduction

3.1. Malaria infection

Malaria ranks among the major health and development challenges of the world. The disease is prevalent in almost 100 countries, accounting for 40% of the world's population. It affects an estimated three to five hundred million people, causing more than a million deaths per year. Malaria kills one child every 30 seconds. In absolute numbers, malaria kills 3000 children younger than five years of age per day (117). Although the disease is spread in most tropical countries, Sub-Saharan Africa, with more than 80% of the world's malaria cases, is the focus of most efforts in combating the disease due to the high morbidity and mortality rates. Southeast Asia and South America are of interest because of the early development of drug resistance among malaria parasites in these regions. Over time, the human immune system adjusts to combating the malaria parasite and adult mortality in endemic areas is fairly low. The mortality is concentrated among children younger than five years, travelers, migrants from non-malarial into malarial regions, and among populations with repressed immune system, including pregnant women and individuals suffering from HIV.

The human malaria is caused by four different parasite species, belonging to the genus *Plasmodium*. These are: *P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale*. *Plasmodium falciparum* is by far the most prevalent species and causes most problems as a result of its virulence and drug resistance. It has a rapid rate of asexual reproduction in the host and an ability to sequester in small blood vessels, with high risk for development of cerebral malaria (118).

3.2. Conventional malaria chemotherapy

Chloroquine is an inexpensive and readily available drug in many endemic areas. The compound is by far the most used antimalarial although it is considered to be ineffective in many parts of the world due to a widespread resistance in *P. falciparum* and also *P. vivax* malaria (113). Chloroquine is a blood schizonticide, affecting the blood stages of the parasites, although the exact mechanism of action is not known. The resistance to chloroquine is associated with reduced drug concentrations within the parasites due to reduced ingress or increased influx (113). Due to an increased treatment failure, the compound is now being replaced by pyrimethamine/

sulphadoxine. The antifolates proguanil and pyrimethamine are usually used in combination with sulphonamides. Resistance to the combinations is, however, widespread in South America and East Asia and is emerging in West Africa. Mefloquine has been used widely in Southeast Asia and South America. However, resistance to the compound has been observed in treatment of infections caused by *P. falciparum* malaria in Southeast Asia (80, 120). The Cinchona alkaloid, quinine is still effective in treatment of all human malaria species despite over 350 years of use. Parenteral formulations of quinine are used for treatment of severe cases of *P. falciparum* malaria. Primaquine is active against exoerythrocytic as well as latent exoerythrocytic stages of all malaria parasites and is therefore used for treatment of relapsing malaria. Due to lack of recent clinical trials, the actual clinical efficacy of the compound is uncertain in most parts of the world (1).

4. Artemisinin and its derivatives

4.1. A modern antimalarial from an ancient China

The Chinese medical plant qinghao (*Artemisia annua* L.) has been used in traditional Chinese medicine for more than 2000 years. The earliest reference to the plant goes back to "52 Prescriptions", found in the Mawangudi Tomb in an era dating back to 206 BC-AD23. The first description of qinghao for treatment of malaria-related symptoms is found in "The Handbook of Prescriptions for Emergencies" by Ge Hong, who lived during AD 281-340 (121). Isolation of the active moiety qinghaosu, however, took place at a considerably later time-point. Qinghaosu, meaning the extract of qinghao, was isolated in early 1970s from the leaves and flowering tops of the plant by Chinese scientists in their search for new antimalarial compounds. The compound showed good in vitro antimalarial activity and subsequent studies in animal models proved encouraging. By the end of the 1970s, several clinical studies conducted in China found qinghaosu to be an exceptional antimalarial agent with negligible toxicity and high efficacy against human malaria parasites, including those resistant to conventional malaria treatment (59).

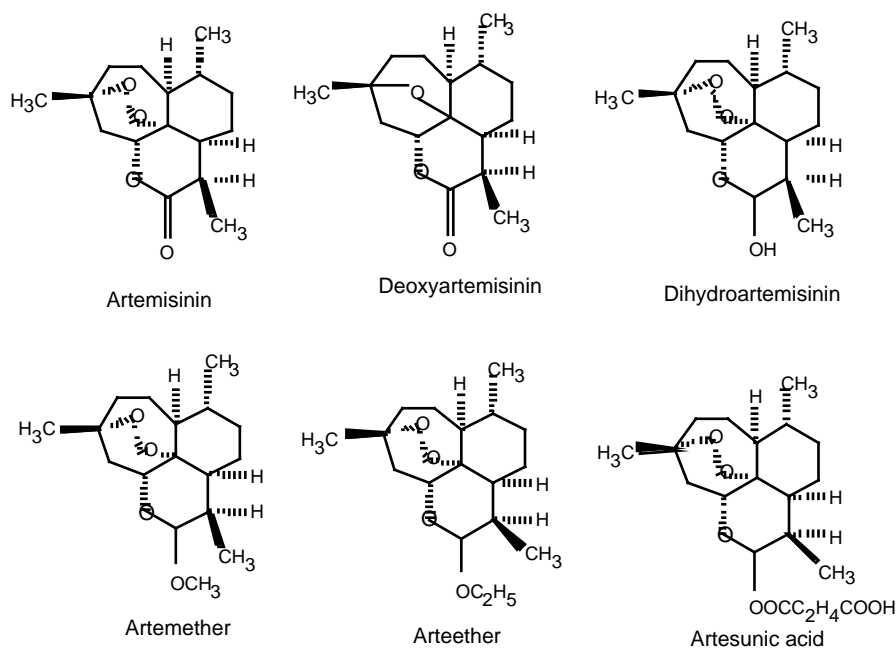


Figure 1: Molecular structures of artemisinin and its related compounds.

Since then, qinghaosu, now known as artemisinin in other parts of the world, has been used in treatment of predominantly falciparum malaria cases around the world.

4.2. Chemistry, general properties, and mechanism of action

The chemical structure of artemisinin is quite different from all previously known antimalarials. The compound is an unusually stable sesquiterpene lactone bearing a peroxy group (Fig. 1). The presence of the peroxide bridge is essential for artemisinin's antimalarial activity as a reduced form of the compound, deoxyartemisinin, lacks the antimalarial activity (53, 119). The white needle crystals of artemisinin are hardly soluble in water or oil and therefore formulations other than oral and rectal are not in clinical use. However, since the peroxide bridge of the compound is stable under certain chemical reactions, several oil- and water-soluble derivatives of artemisinin have been synthesized (Fig. 1). These include dihydroartemisinin, artemether, and artesunate, originally developed by the Chinese scientists, and arteether and artelinic acid. Overviews on synthesis of artemisinin and other derivatives are given elsewhere (109, 121).

Artemisinin is hydrophobic and passes biological membranes easily (13). In vitro studies have suggested an uptake of artemisinin by both healthy and malaria infected red blood cells (8, 82). It is known that artemisinin binds to hem, either in hemoglobin (inside red blood cells) or hemozoin (stored hem within the malaria parasites). Through an iron-mediated cleavage of the peroxide bridge artemisinin free radicals are formed. These free radicals are destructive to different parasite membranes, including mitochondria, rough endoplasmic reticulum, and plasma membranes, thereby killing them (8, 23, 62). It is believed that the hemin-rich internal environment of the parasites is one of the reasons for the selective toxicity of artemisinin toward the malaria parasites as hemin has been shown to interact with the compound (39). Several reviews on the mechanism of action of artemisinin are available elsewhere (24, 69, 103).

4.3. Analytical methods

Due to the lack of chromophore moieties in the structure of artemisinin development of analytical systems for detection of the substance has been a slow process. Although several analytical methods are now available for detection of artemisinin in biological fluids, only two have gained widespread use in pharmacokinetic studies. The first one is based on potentiometric detection, first described by Zhao (123) and later validated in a number of laboratories for various derivatives (50, 68). All these systems use the presence of the peroxide bridge of artemisinin and its derivatives for detection purposes. The method has proven to be sensitive and reliable for detection of both artemisinin and its derivatives possessing the peroxide bridge. However, noise due to the high potentials used and baseline drift caused by the presence of oxygen in the chromatographic system have been the two major limiting factors for these systems resulting in low number of samples being analyzed per day.

The second approach is based on a post-column, on-line derivatization step first described by Edlund *et al* (29), and later modified by others (12, 16). The system has been shown to be robust and sensitive in detection of artemisinin, artesunate, and dihydroartemisinin but not artemether and arteether. It has a relatively high sample turnover capacity with a higher sample turnover capacity. Other reported methods have yet to be applied to clinical studies (15, 37, 60, 72, 93, 97).

4.4. In-vitro interactions with blood components

Artemisinin and artemether showed significant losses when stored in whole blood at room temperature (74). Artemisinin was shown to concentrate in malaria hemozoin where it is covalently bound to hemozoin (39). Concentrations of artemether decreased with time during incubation with whole blood. By using radiolabelled artemether and assessing the recovery of radioactivity from incubations with plasma, hemoglobin, and erythrocyte ghosts, it was suggested that the interaction between the compound and blood components involved the red cell membrane (31). Also radiolabelled artemisinin and dihydroartemisinin were shown to be taken up, and concentrated in isolated red cell membranes (8). However, there was no uptake by intact erythrocytes. Dihydroartemisinin was shown to bind to peripheral membrane proteins located on the cytoplasmic face of red blood cells.

4.5. Human pharmacokinetics

Artemisinin is primarily eliminated by enzymatic metabolism to presumably inactive metabolites, lacking the peroxide bridge (56). Only trace amounts of the compound are detectable in urine in both healthy volunteers and malaria patients after oral administrations (76). The ether and ester derivatives are metabolized to dihydroartemisinin, which accounts for most of the clinical effect of these derivatives after intake. Despite a presumed high absorption the oral preparations are believed to have a low bioavailability due to a significant first-pass extraction (13). Since intravenous administration of artemisinin is not possible, no information on its absolute bioavailability is available. However, an oral formulation of artemisinin showed a 32% relative availability compared to an intramuscular suspension in oils (98). The same extent of relative availability was found for suppositories compared to capsules (11).

Artemisinin has an absorption lag-time of 0.5-2 hrs after oral intake, with peak plasma concentrations at 1-3 hours post-administration. It has a relatively short half-life of 1-3 hours (3, 10, 98). Cytochrome P-450 enzyme 2B6 with some possible contribution of CYP3A4 and CYP2A6 have been suggested to metabolize the compound (95, 96). Rectal administration of artemisinin resulted in lower plasma concentrations of the drug compared to oral doses, although no significant difference was found in the

elimination half-lives between the two administration routes (11). Fraction bound artemisinin to plasma proteins averages around 80-85% (10). Artemisinin exhibits time- and dose-dependent kinetics in both healthy volunteers and malaria patients. These include trends for a possible saturable first-pass metabolism (9) and decreased plasma concentrations upon repeated administration of the substance (3, 11, 12). There are also reports indicating an autoinduction effect caused by the derivatives artemether (33, 57, 102) and, although less convincingly, artesunate (52). The lower plasma concentrations toward the end of the treatment period are believed to be due to an increase in the first-pass extraction of the drug, affecting its bioavailability (10, 11). Unchanged artemisinin elimination half-lives during pre- and post-induction states imply the compound to be a high extraction drug with little effect of the induction on its systematic clearance (10, 11).

The capillary and saliva sampling have been suggested as promising replacements for venous sampling in pharmacokinetic studies of artemisinin (90). Indications of a putative arterio-venous concentration difference were reported in the same study.

A general review on the pharmacokinetics of artemisinin and its derivatives is given by Navaratnam *et al* (76).

4.6. Antimalarial efficacy

Artemisinin and its derivatives have successfully been used in treatment of falciparum malaria infections in various parts of the world. More importantly, they have proved effective against strains resistant to conventional antimalarials such as chloroquine and mefloquine (4, 85, 118). No clinically relevant artemisinin-resistant human malaria has yet been reported, although there are reports published on development of the rodent malaria parasite strains resistant to the drug (22, 58). The genetic basis of the resistance in the rodent malaria *P. yoelii* has been elucidated in a recent study (108), which might give information on the mechanism of resistance in human malaria, when it appears.

Artemisinin and its derivatives have a rapid antimalarial effect, decreasing the number of parasite faster than any other known drug (112). Also lower gametocyte carriage

rates have been observed after treatment with artemisinin and its derivatives (87). The fast decline in the number of parasites has been suggested beneficial in combination therapies (115). Another advantage of the rapid clearance of the parasite is the earlier return of the patients to the normal life. However, since the clinical symptoms of malaria disappear within a day or two after treatment initiation, compliance could become a problem. It is also to be noted, that the rapid decline in the number of parasites has not proved to be of clinical benefit compared to quinine in severe malaria cases (18, 21, 84), although there seems to be a lower frequency of side effects in patients treated with the artemisinin compounds (100).

General reviews of the use of artemisinin and its derivatives in treatment of malaria are given elsewhere (66, 67, 86, 103).

4.7. Clinical safety / Toxicity

Despite several reports on neurotoxic effects of arteether and artemether (19, 35, 48, 78), this family of compounds have proven to be extremely well tolerated in clinical practice (25, 105, 116). The neurotoxicity seen in animals after high doses of these compounds has not been reported in humans (7). However, a longer time for recovery from coma in cerebral malaria patients has been observed in those treated with intramuscular artemether compared to intramuscular quinine (100).

Artemisinin derivatives have successfully been used in treatment of malaria in pregnant women with no observed effects on the fetus or newborn children, who were followed up for one year (65). However, randomized studies with these drugs are required to define their true safety and efficacy in pregnant women. In general, the artemisinin compounds are considered to be well-tolerated, with few or none side effects in clinical use (103).

4.8. Combination therapy

One of the major drawbacks in monotherapy with artemisinin or its derivatives is the high rate of recrudescence infections observed in several clinical studies (2, 5, 11, 27, 77). It has been suggested that the short half-life of artemisinin family of antimalarials and the parasite life-cycle, including generation of artemisinin insensitive stages, are

among the major reasons for the inefficiency of these drugs in clearing the parasites totally (111).

Nowadays, the general recommendation in treatment of falciparum malaria with artemisinin or one of its derivatives is a combination with another antimalarial with relatively long half-life (26, 67, 79, 101, 114). The risk of having a patient being infected with a parasite strain resistant to two antimalarials simultaneously is very small (114). Moreover, by reducing the number of parasites rapidly by artemisinin or its derivatives, the risk for resistance development against the second drug of the combination therapy will be reduced. Trends for such effects have been reported for combinations of artesunate and mefloquine, in which halted progression of mefloquine resistance has been observed (81). A combination therapy with an artemisinin compound and a second antimalarial with longer half-life would also decrease the risk for recrudescence due to the long protective action of the second drug. Furthermore, such a combination results in shorter treatment period, which is beneficial from a compliance point of view.

Although different combinations of an artemisinin derivative with mefloquine have been the most used ones, other compounds have also been used in combination with an artemisinin compound. One of these combinations is lumefantrine, a new Chinese drug previously known as benflumetol. A fixed dose combination of artemether and lumefantrine has been studied and is now available for the treatment of uncomplicated falciparum malaria (106, 107). More clinical studies with different combinations of artemisinin compounds and other antimalarials are undertaken. For more detail see Price (86).

5. CYP-450 enzymes

The cytochrome P450 (CYP-P450) enzyme system was discovered in the 1950s as pigments in liver microsomes, binding carbon monoxide at reduced form, and having a characteristic absorbance peak at 450 nm (54). Since then the system has been shown to be a large group of hem-containing proteins, catalyzing monooxygenase reactions (32). Depending on their sequence similarities, CYP-450 enzymes are classified into families (more than 40% identical sequences) or subfamilies (more than

55% identical sequences). These enzymes are found in virtually all living organisms with their major task being conversion of different exogenous and endogenous molecules into more polar compounds, facilitating their removal from the body. However, there are many examples where the metabolites are more toxic and have longer elimination half-lives than the parent compound. Although a major part of CYP-450 enzymes are located in the liver, these enzymes are found in all tissues in mammals, predominantly in the mitochondria and endoplasmic reticulum. Several CYP-450 enzymes are known to be inducible by different compounds, such as phenobarbitals (CYP1A and CYP2B6) and ethanol (CYP2E1).

Sex-dependent difference in the activity of cytochrome P-450 enzymes has been shown in several species, especially rats (73). Most of the information on drug metabolism in humans has been gathered from clinical studies aiming at the pharmacokinetic investigations of different compounds (14, 34, 40). The sex-dependent metabolism does not appear to be very common and thus the drug development process usually does not include examining the potential for such a difference.

Overviews of the CYP-450 enzymes are given elsewhere (54, 70).

6. Saliva and its use in drug monitoring

Saliva is secreted by 3 major pair salivary glands (parotid, sublingual, and submandibular), with the parotid and submandibular glands accounting for 90% of the volume of secreted saliva (89). Sympathetic and parasympathetic stimulations control blood flow as well as glandular activity. Approximately 1200 ml saliva is secreted each day. Saliva pH range from 6.2 to 7.4, with higher pH values observed during increased secretion. The total protein content of saliva is approximately one-tenth that of plasma, although this value may vary (45). Drugs enter saliva predominantly via passive diffusion, a process that is limited to un-ionised drugs (38). Moreover, the diffusion of drug from plasma to saliva is limited to the unbound fraction of the drug (28). Thus saliva levels may be more reflective of drug concentrations at the site of action than are total drug concentrations in plasma.

Saliva offers an easily collected and sometimes useful body fluid for concentration monitoring and PK/PD investigation of various drugs. The ideal drug to be studied using saliva should be secreted to saliva in a manner independent of drug concentration, salivary pH and salivary flow. Factors affecting the transport of drug from plasma to saliva can be divided into two: physiochemical properties of the drug, including molecular size, lipid solubility, pK_a , and the degree of protein binding of the compound, and physiological factors such as salivary pH, flow rate, and possible existing pathophysiology of the oral cavity (47).

Distribution of several drugs into saliva has been investigated. Saliva phenytoin levels were shown to be 1.4 times greater than the free plasma phenytoin concentrations (61). In general, such an observation could be due to binding of the compound to salivary components. For phenytoin, however, this was found to be an artifact due to use of citric acid, which altered the salivary pH. Saliva concentrations of carbamazepine were found to be highly correlated to its unbound plasma concentrations (83). The same was true for phenobarbital (71). A pH-dependent secretion into saliva was reported for procainamide (55) and quinidine (122). A higher saliva/plasma ratio in the absorption phase compared to post absorptive and elimination phase has been observed for several drugs, including digoxin and caffeine (92). These differences have been attributed to an arterial/venous concentration difference in the absorption phase.

Overviews on salivary drug monitoring are given elsewhere (36, 124, 125).

7. Aims of the thesis

The general aim of the thesis was to investigate the phenomenon of artemisinin autoinduction and its implications in clinical settings.

The specific aims were

- To investigate the in-vitro interaction of artemisinin with blood components.
- To study the suitability of saliva as sampling matrix in pharmacokinetic studies of artemisinin.
- To develop a fully automated HPLC system for detection of artemisinin in saliva and plasma samples.
- To investigate the kinetics of artemisinin autoinduction and its effects on the antimalarial efficacy of the compound.

8. General methods

Written, informed consent was obtained from each subject/patient prior to inclusion in any of the clinical studies (papers III-V). Study permissions were obtained from the Ministry of Health, Hanoi, Vietnam, the ethics committee of the Medical Faculty of Uppsala University, Sweden, and the Medical Products Agency, Uppsala, Sweden.

8.1. Study location

The in-vitro study (paper I) and development of the analytical system (paper II) took place at the division of Pharmacokinetics and Drug Therapy at the Faculty of Pharmacy of Uppsala University. Studies involving patients (papers III and IV) took place at health care facilities of the Phú Rieng rubber plantation, Binh Phuoc province, 130 km north of Ho Chi Minh City, Vietnam. The study on the autoinduction of artemisinin, involving healthy volunteers (paper V) was carried out in the Clinical Study Unit of the National Institute of Malariology, Entomology and Parasitology, Hanoi, Vietnam.

8.2. Clinical monitoring

All healthy volunteers underwent a routine check-up prior to inclusion. A parasite count in finger capillary blood was performed in all patients immediately before, and every four hours after the start of treatment until three consecutive negative smears. The observed number of parasites was counted against 300 white blood cells. The time until the first (out of three) negative smear was defined as parasite clearance time (PCT). Body temperature was simultaneously recorded sublingually until normalization, i.e. a body temperature below 37°C. Fever subsidence time (FST) was defined as the time from the intake of the first dose until normalization. All subjects were interviewed about side-effects after the first and last intake of the drug.

8.3. Artemisinin administration

All capsules were administered under the supervision of study team members, with each ingestion followed by a protocol stipulating intake of 100 ml water and rinsing of the mouth with another 100 ml water. Solid food was avoided 2 hours before and 2 hours after drug intake on the sampling days.

8.4. Sample collection and handling

Samples collected during the in-vitro experiments (paper I) were kept at 4°C for 10 minutes and then centrifuged at 3500 g for 5 min. The plasma/buffer was removed and stored at -20°C until analysis.

Venous blood samples (2.0 -2.5 ml each) were collected from each patient into 5-ml Li-heparin Vacutainer[®] tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) using an indwelling catheter placed in a forearm vein (paper III). Capillary samples (0.5 ml each) were collected in Li-heparin Microtainer[®] tubes with gel (Becton-Dickinson) by lancing fingertips (papers III-V). All samples were kept at room temperature for five minutes before centrifugation for five minutes followed by immediate separation of plasma. Unstimulated saliva samples (2 ml each) were collected into 3-ml sampling tubes (Nunc, Hereford, England). Plasma and saliva samples were immediately frozen and kept at -20°C prior to and after arrival to Sweden. The samples were, within two months, placed and kept at -80°C before artemisinin quantification.

8.5. Chemical analysis

All samples were analyzed at the Division of Pharmacokinetics and Drug Therapy of the Faculty of Pharmacy, Uppsala University. Samples collected during the in-vitro study (paper I) and all plasma samples were analyzed according to a post-column alkali-derivatization method (12). All plasma samples from the same patient/subject were analyzed within the same run. Saliva samples were analyzed by the coupled column system described in paper II. All saliva samples from the same patient/subject were analyzed in the same experimental occasion.

8.6. Statistical methods

The following statistical methods were utilized where appropriate: Chi-squared test, multiple t-test with sequentially rejective Bonferroni test, MANCOVA with Fisher's PLSD post-hoc test, canonical correlation analysis, linear regression, and comparisons based on construction of confidence intervals.

8.7. Pharmacokinetics

8.7.1. Parameter estimation

Maximum artemisinin concentration (C_{\max}) and the time to reach this concentration (t_{\max}) were taken directly from the observed concentration-time data for both saliva (paper III-V) and plasma (paper III) for each individual. Artemisinin elimination rate constant (k_e) was estimated in each individual by log linear regression of 3-5 terminal saliva concentration-time data. Terminal half-life ($t_{1/2}$) was calculated as $\ln 2 / k_e$. Area under the concentration- time curves from the time of drug administration until the last quantified sample (AUC_{0-t}) were calculated using linear trapezoidal method for the ascending and log-linear trapezoidal rule for the descending phase of the curves. $AUC_{t-\infty}$ was estimated by dividing the last predicted concentration data by the elimination rate constant. $AUC_{0-\infty}$ was calculated as the sum of AUC_{0-t} and $AUC_{t-\infty}$. Oral clearance (CL/F) for each subject was derived as dose divided by $AUC_{0-\infty}$. Mean residence time (MRT) for each subject was calculated as the $AUMC/AUC_{0-\infty}$, where AUMC was calculated by the linear trapezoidal rule with the residual area from the last sampling time point extrapolated by C_{calc}/k_e^2 . The same procedures were applied using the number of parasites vs. time in order to estimate parasite AUC (PAUC) and parasite mean residence time (PMRT).

8.7.2. Modeling of the autoinduction

A population modeling approach, using first-order method (FO) without centering in the nonlinear mixed effects modeling program NONMEM, version VI (17), was utilized in modeling the autoinduction phenomenon of artemisinin. Different structural models were fitted to the available saliva concentration-time data from all individuals simultaneously. Discriminations between hierarchical models were based on the objective function value (OFV) provided by NONMEM, at a significant level of 0.05, equal to a drop of 3.86 in the OFV. Xpose, version III (46) was used for graphical analysis of residuals and predictions in model diagnostics. Confidence intervals for parameters of the final model were obtained by the likelihood profile method (17). Effects of the covariates age, body weight, and smoking habits on different parameters were tested using a GAM analysis (63), implemented in Xpose.

9. In-vitro interaction of artemisinin with red blood cells; effects of hem and presence / absence of oxygen (paper I)

9.1. Study aims

The present study investigated the kinetics of the *in vitro* interaction of artemisinin with intact human erythrocytes, erythrocyte ghosts, hemoglobin, and carbonic anhydrase, using a specific method for drug quantitation. Moreover, the influence of oxygen tension on the interactions was evaluated. Such information was considered valuable when specifying handling procedures for blood samples collected for pharmacokinetic analysis.

9.2. Study design

Erythrocyte suspensions in plasma at three different concentrations (5, 18 and 33% v/v respectively) were used in the experiments. Also erythrocyte ghost suspensions in plasma were prepared in three different concentrations (1, 5 or 9% v/v). Incubation took place under an argon-carbon dioxide (95%- 5%) or oxygen-carbon dioxide (95%- 5%) atmosphere, with three different initial artemisinin concentrations of 400, 800, and 1600 ng ml⁻¹.

Human hemoglobin and carbonic anhydrase II were dissolved in phosphate buffer to give concentrations of 0.45 and 90 mg.ml⁻¹ respectively. These were incubated with an initial artemisinin concentration of 1500 ng ml⁻¹. To a third flask, containing artemisinin, was added phosphate buffer only. Carbonic anhydrase and phosphate buffer experiments were performed under air. The incubation with hemoglobin was performed under either oxygen-carbon dioxide (95%- 5%), or an argon-carbon dioxide (95%- 5%) atmosphere.

All experiments were performed under gentle agitation at 37°C with sampling during the first 8 hours.

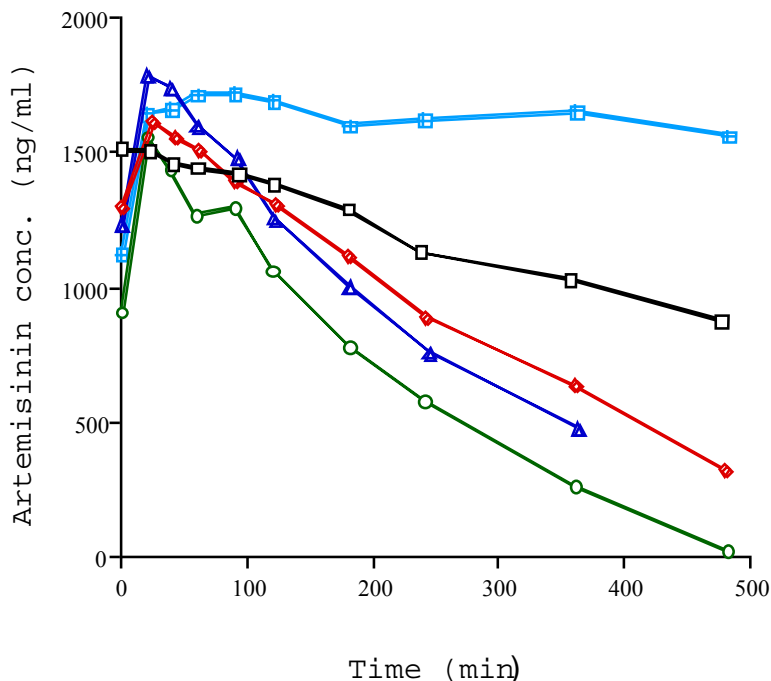


Figure 2: Artemisinin plasma concentrations in vitro during incubation with human whole blood (▲), plasma (■), and erythrocyte-plasma suspensions of 5% (□), 18% (◆), and 33% (○) hematocrits under an argon: carbon dioxide atmosphere (95:5) in flasks pre-dosed to yield initial drug concentrations of 1600 ng/ml.

9.3. Results and discussions

In agreement with previous studies (30, 74) a disappearance of artemisinin during incubation with whole blood or erythrocyte suspensions was observed (Fig. 2). In addition, a marked difference in artemisinin disappearance rates depending on whether the incubations were performed under an atmosphere rich or poor in oxygen was found (Table 1). This finding was consistent in all incubations and might be attributed to a direct competition between oxygen and the peroxide bridge for binding to hemoglobin. As a result of the enhanced disappearance of artemisinin in the absence of oxygen, an increased formation rate of free radicals under conditions of low oxygen tension may be hypothesized. Such a condition is expected within the *Plasmodium falciparum* parasite (6, 99), which may partly explain the selective toxicity of the artemisinin class of antimalarials.

No dose-dependency in the disappearance of artemisinin was found. The rate of artemisinin disappearance increased with higher hematocrit of the erythrocyte plasma suspensions under the argon-carbon dioxide atmosphere. However, the disappearance was more rapid in the 33% erythrocyte suspension compared with whole blood, whereas the opposite was expected. This could not be attributed to different degrees of hemolysis in the incubations. An interaction of the compound with other blood

Table 1: Artemisinin disappearances half-lives and dose-adjusted AUCs measured in plasma, following incubations under either oxygen-carbon dioxide gas or an argon-carbon dioxide atmosphere in whole blood, or erythrocyte suspensions of varying hematocrits.

Atmosphere Initial artemisinin conc. (ng/ml)	95% O ₂ , 5% CO ₂			95% AR, 5% CO ₂		
	400	800	1600	400	800	1600
Whole blood						
t _{1/2} (hrs)	9.5	10.5	10.7	1.5	1.7	2.8
AUC/D (min/ml)	4.2	4.2	4.3	1.8	2.3	2.4
RBC 5%						
t _{1/2} (hrs)	20.3	40.6	17.8	7.1	8.5	9.1
AUC/D (min/ml)	4.0	4.1	4.0	3.3	3.5	3.5
RBC 18%						
t _{1/2} (hrs)	4.2	16.0	21.3	1.9	2.0	3.0
AUC/D (min/ml)	3.9	4.2	4.2	2.4	2.7	2.8
RBC 33%						
t _{1/2} (hrs)	7.7	8.3	7.7	1.5	1.4	0.9
AUC/D (min/ml)	3.5	3.7	3.8	1.7	1.8	2.0

cells could not be ruled out. Artemisinin exhibited a rapid disappearance with a half-life of approximately 14 minutes in the 90 mg ml⁻¹ hemoglobin solution under an oxygen-rich atmosphere. Under the argon-carbon dioxide atmosphere, all artemisinin concentrations except one single determination at zero time were below the limit of quantitation.

A previous observation found dihydroartemisinin to bind to erythrocyte membranes but not to intact red blood cells (Asawamahasakda et al 1994). The present study clearly demonstrates an interaction between artemisinin and intact red blood cells and does not confirm any quantitative binding to erythrocyte membranes (Fig. 3). This apparent discrepancy may be explained by the use of a selective quantitation method in the present study, unlike previous studies using radiolabeled compound.

It has been proposed that the putative binding of artemisinin compounds to erythrocyte membranes invalidates pharmacokinetic investigations based on plasma concentrations and their relevance to pharmacological response (31). However, should there be significant binding to blood cell membranes (for other derivatives aside from artemisinin), one may expect such binding to be non-specific and of no relevance for

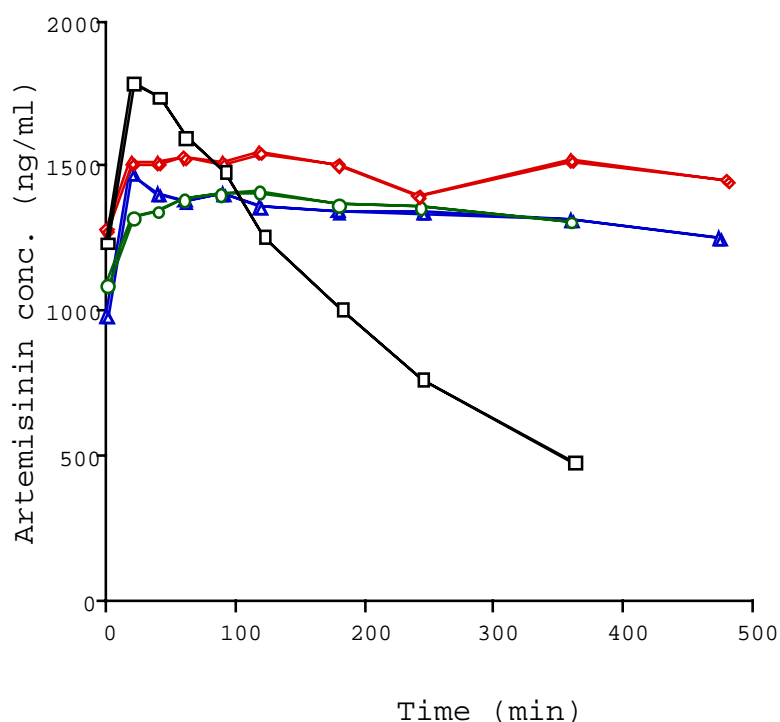


Figure 3: Artemisinin plasma concentrations in vitro during incubation with human whole blood (□) and with erythrocyte ghosts of 1% (◇), 5.0% (○), and 9% (△) v/v suspended in plasma under an argon: carbon dioxide (95:5) atmosphere in flasks pre-dosed to yield initial drug levels of 1600 ng/ml.

the therapeutic effects of the drug. Such interactions would be of little concern since they would not influence intraparasitic concentrations of unbound drug, for which the driving forces are unbound drug plasma levels. It was therefore concluded that pharmacokinetic studies based on plasma drug levels are appropriate and that their interpretation carries clinical relevance. Further investigations may demonstrate whether it is necessary to include drug disappearance in blood as an irreversible clearance in a pharmacokinetic model. These findings also illustrate the precariousness of relating clinically observed drug concentrations with potency parameters such as IC_{50} determined by in-vitro susceptibility tests. The Candle Jar method (44) employs conditions quite similar to the experiment with 5% hematocrit under an argon-carbon dioxide atmosphere.

10. Development of the analytical system (paper II)

10.1. Study aims

The post-column alkali derivatization method developed by Edlund *et al.* (29) has been successfully used for quantitation of artemisinin in plasma in several pharmacokinetic studies of the compound (3, 9-12, 96). Although robust and reliable, this method requires a time and labor-consuming step of liquid-liquid extraction. The extraction procedure consists of several steps, each introducing a source of error to the

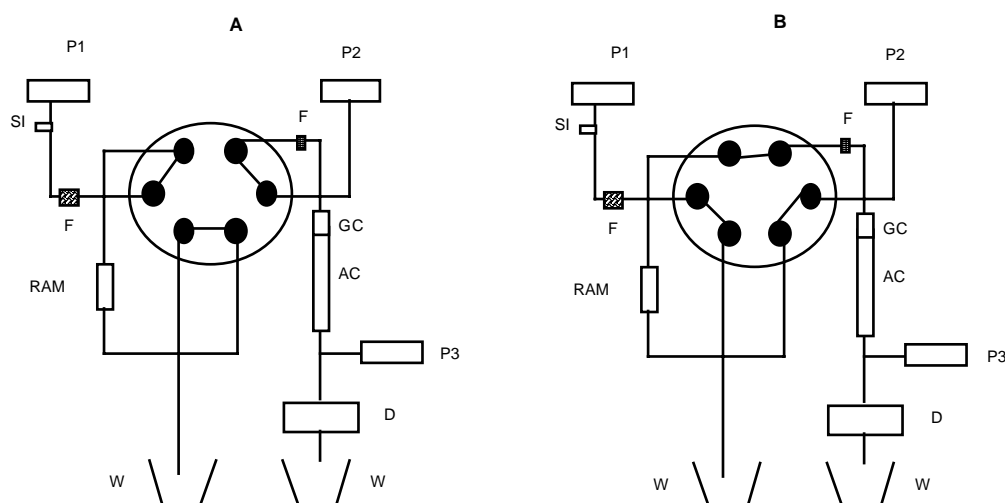


Figure 4: Schematic presentation of the column switching system: A, inject position and B, load position. Abbreviations: AC: analytical column, D: detector, F: filter, GC: guard column, P1 and P2: pumps 1 and 2 respectively, P3: Post-column derivatization pump (KOH), RAM: restricted access material column, SI: sample injection and W: waste.

final analysis.

Due to intensive sampling during the clinical studies (papers III-V), more than 5500 samples were collected. Analysis of these samples with a system requiring the extraction step would take an unreasonable period of time. Thus, a system with direct and automated injection of samples was developed.

10.2. Study design

A restricted access material (RAM) column (LiChrospher[®] ADS C-18, Merck, Darmstadt, Germany) was used to replace the liquid-liquid extraction step. Plasma or saliva was washed through the column with a mobile phase with 2% v/v acetonitrile in water (Fig. 4). After removal of proteins, a second mobile phase (acetonitrile: water, 50% v/v) passed through the column in a back-flush mode removing artemisinin from the RAM-column, introducing it to the separation column (reverse-phase, C18). After passage through the column, the eluent was mixed with a 0.3M KOH in methanol and allowed to react at 70°C before passage through a UV-detector.

Table 2: Within-day accuracy and precision for quantification of artemisinin in plasma and saliva samples at different concentrations.

Matrix	Conc. (ng/ml)	Mean (\pm SD) found conc. (ng/ml)	CV (%)	Accuracy (%)
Plasma	10.1 (n = 8)	9.96 \pm 1.05	10.5	98.6
	20.1 (n = 8)	20.8 \pm 0.83	4.0	103.4
	2014 (n = 6)	2014 \pm 20.6	1.0	100.0
Saliva	2.0 (n = 8)	1.97 \pm 0.22	11.1	98.6
	231.4 (n = 8)	231.4 \pm 7.0	3.0	100.0

10.3. Results and discussions

The major challenge in developing the system was the low artemisinin levels in saliva samples, which necessitated the injection of high volumes (1.00 ml) into the RAM-column. The problem was solved through a combination of gradient increase in the flow rate of the first mobile phase, and longer wash time. In the final system a total volume of 25 ml of the first mobile phase washed each saliva sample through the RAM-column. It was also decided to place an in-line filter in front of both columns, which resulted in longer lifetimes for both columns. Different properties of the final system are depicted in tables 2 and 3.

Use of buffers is usually recommended when RAM-columns are utilized. It was found, however, that a mix of water and acetonitrile resulted in the lowest baseline noise and was therefore used in further work. Also lower KOH concentration of 0.3M (compared to 1.0M in the original method) in the post-column derivatization step was found to be the most optimal one for detection of artemisinin.

Since simultaneous use of other medications together with artemisinin is a common practice, the chromatographic interference between artemisinin and several

Table 3: Between-day precision for artemisinin quantified in plasma (n = 6) and saliva samples (n = 6), respectively.

Matrix	Conc. (ng/ml)	Between-day variation (CV%)
Plasma	24.94	15.8
	402.3	10.2
	1609.2	8.6
Saliva	5.3	10.17
	64.5	11.79
	129.0	8.63

antimalarial drugs and their main metabolites was tested. None of the tested compounds (mefloquine, chloroquine, deacetyl-chloroquine, sulfadoxine, acetylsulfadoxine, pyrimethamine, proguanil, cycloguanil, and 4-chlorophenylbiguanide) showed any interference when co-injected with artemisinin in water solution.

In conclusion, the RAM-system offers an easy to use, specific, and accurate method in determination of artemisinin concentrations in plasma and saliva samples. Moreover, a three-fold increase in the analysis turn-over is achieved by the system.

11. Saliva / plasma correlation (paper III)

11.1. Study aims

Venous blood sampling in field studies is associated with several problems, including logistical limitations and patients' reluctance. Thus, the search for new and preferably non-invasive sampling sites to substitute the venous plasma was considered necessary. An early Chinese study suggested secretion of artemisinin into saliva, reaching concentrations 1/8 of those of total plasma (123). A preliminary investigation involving Swedish healthy volunteers confirmed these findings (90). Based on comparison between the capillary and venous plasma concentrations of artemisinin, the authors also suggested a possible arterio-venous concentration difference for the compound. The aim of the present study was to investigate the suitability of saliva and capillary plasma as substitutes for venous plasma samples.

11.2. Study design

Ten patients were administered a first dose of 500 mg oral artemisinin, while another 8 received a first oral dose of 100 mg of the compound. Eight venous blood, 6 capillary samples, and 8 saliva samples were collected at the same time-points within 8 hours after artemisinin intake. Different pharmacokinetic parameters were calculated based on the concentration-time data from the three different sampling sites.

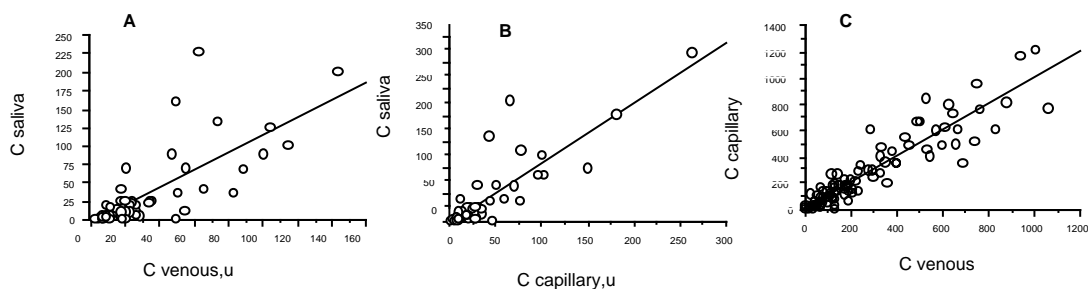


Figure 5: Regression lines for saliva and plasma concentrations (ng/ml) in 18 male Vietnamese patients 1-8 hours after the first oral dose of 100 mg or 500 mg artemisinin. A: Saliva vs. unbound venous plasma. B: Saliva vs. unbound capillary plasma. C: Total capillary vs. total venous plasma.

11.3. Results and discussions

The overall absolute differences in artemisinin concentrations in saliva and unbound venous or capillary plasma were small and non-significant. The same was true for average differences between capillary and venous total plasma concentrations. Saliva and unbound plasma (both venous and capillary) artemisinin concentrations were found to be correlated (Fig. 5). Capillary to venous plasma concentration ratios were significantly higher than unity for the samples taken at 30 and 60 minutes (Fig. 6), indicating an arterio-venous concentration difference. Such differences may be observed for drugs with high organ extraction ratios and large volumes of distribution. Capillary blood is a mix of arterial and venous blood wherefore the actual difference in the case of artemisinin may be greater than here indicated.

The slope of the regression line of saliva versus venous plasma concentrations was similar to the unbound fractions in plasma (slope = 0.18 and $f_u = 0.14$, respectively). Thus saliva sampling offers a simplified means for determination of unbound plasma artemisinin levels. Abruptly high saliva concentrations were detected in samples taken at 30 minutes from several patients, most probably due to residual amounts of artemisinin in the mouth after the intake of the capsules. Samples taken at this time-point were excluded in the final analysis in all patients. The contamination is an obstacle for the use of saliva sampling in large field studies, and may be exacerbated if oral formulations other than capsules are to be used.

The presented findings suggest the use of saliva sampling in pharmacokinetic studies of artemisinin. Capillary sampling is an alternative especially if applying methods for

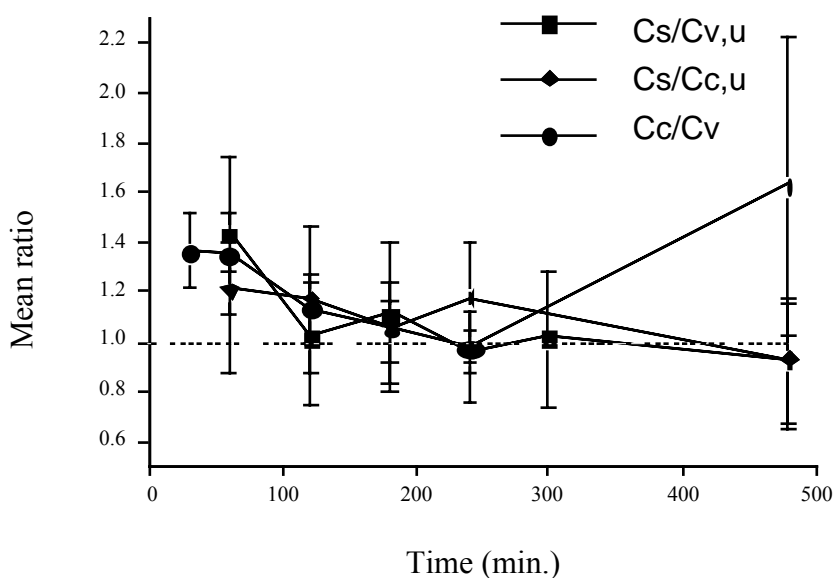


Figure 6: Mean (\pm SEM) ratio of artemisinin concentrations in different sampling sites with time in patients with uncomplicated falciparum malaria.

sparse data analysis. Substituting venous sampling with saliva collection would facilitate the performance of larger field studies, as well as possibly having a greater clinical relevance since saliva concentrations of artemisinin agree well with its unbound plasma concentrations. However, due to low salivary concentrations of the compound more sensitive analytical systems are required. Moreover, saliva seems to be useless in pharmacokinetic studies of artemether and dihydroartemisinin due to low salivary levels (104).

12. Artemisinin PK/PD in uncomplicated malaria patients treated with two different dosage regimens (paper IV)

12.1. Study aims

A major drawback with artemisinin and its derivatives is high recrudescence rates within 2-3 weeks after monotherapy (59, 79, 103). Although these new malaria episodes may be caused by new infections, recrudescence cases in patients kept in vector-free environments for a longer period of time (49, 51, 75), as well as findings supported by PCR technique (42, 85) suggest a true recrudescence to take place in many patients. It has been proposed that occurrence of recrudescence may partly be due to the decreasing artemisinin concentrations toward the end of treatment (11). Thus the time-dependent kinetics of the drug can be of importance in dosing suggestions.

Treatment with artemisinin suppositories was shown to have similar efficacy compared to capsules, despite lower plasma concentrations of the compound after the rectal administration (11). The aim of the present study was to investigate whether a dosing regimen consisting of low initial and then escalating doses of artemisinin was as efficacious as the standard high-dose regimen with respect to the immediate clinical endpoints. It was also of interest to evaluate if lower initial doses of artemisinin would lead to a less pronounced induction of its elimination in patients with uncomplicated malaria. If so being the case, this would result in higher drug concentrations toward the end of treatment period, which in turn could hypothetically reduce the recrudescence rates.

12.2. Study design

Seventy-seven male and female adults with uncomplicated falciparum malaria were included in this double-blind, comparative, parallel group study. Group A patients (n=40) were administered the standard dosage regimen of 500 mg oral artemisinin daily for 5 days, whereas those in group B (n=37) received an escalating dose-schedule of 100+100+250+250+500 mg artemisinin during the five-day treatment. All patients received the same total number of capsules on each occasion. Frequent saliva sampling was applied on the first and last study days. Parasite clearance and fever subsidence were monitored every 4 hours.

12.3. Results and discussions

No significant differences were in evidence between the two groups with respect to initial number of parasites, body weight, age, and gender distribution. Artemisinin was well tolerated by all patients.

Mean PCT and PMRT were found to be significantly longer in the escalating-dose compared to the standard-dose group patients without any evidence for such differences in FST or PAUC between the two groups (Fig. 7, Table 4). The logarithm of the highest parasitemia had a significant effect on PCT and PAUC, and a border-line significant influence on FST when used as covariate. The longer PCT and PMRT are explained by inadequate artemisinin concentrations in the escalating-dose group,

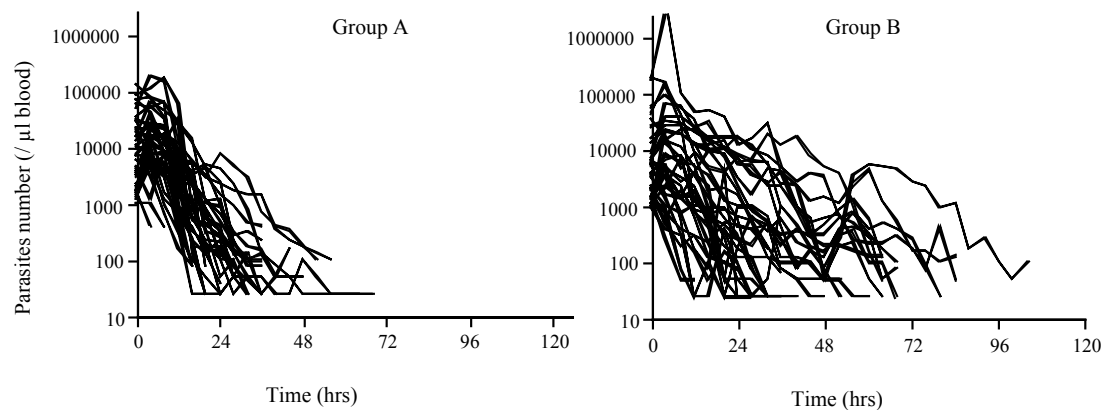


Figure 7: Number of asexual *P. falciparum* parasites per microliter blood in adult Vietnamese patients treated with either a standard oral regimen of 500 mg artemisinin for five days (group A) or an escalating-dosage oral regimen of 100 mg on days 1 and 2, 250 mg on days 3 and 4, and a final dose of 500 mg on day 5 (group B).

as manifested by 10-fold lower saliva AUCs and 7-fold lower saliva C_{max} values. In general, the prolongation of PCT is undesirable, resulting in increased gametocytogenesis and transmission (20, 87). One patient in the escalating-dose group did not clear parasites up to 104 hours after intake of the first dose. In the statistical tests, PCT for this patient was given the arbitrary value of 120 hours. A sensitivity analysis of the outcome of the tests showed no differences with or without the inclusion of this patient.

Table 4: Clinical response parameters (mean \pm SD) in Vietnamese adults with uncomplicated *falciparum* malaria treated with a standard- or escalating-dose regimen of oral artemisinin.

Clinical Response	Standard dosing	Escalating-dose dosing	p-value
PCT (hr)	34.1 \pm 14.1	50*.2 \pm 23.4	<0.01
95% CI	29.6, 38.6	42.3, 58.0	
PMRT (hr)	7.3 \pm 2.7	11.1 \pm 7.0	<0.01
95% CI	6.5, 8.2	8.8, 13.4	
PAUC (parasites.hr/μl blood)	271522 \pm 353430	536359* \pm 1724730	0.09
Geometric mean	138971	100386	
FST (hr)	24.8 \pm 9.6	25.7 \pm 15.9	0.60
95% CI	21.7, 27.9	20.4, 31.0	

* - Including the arbitrary value of 120 hours for one patient. For details see text.

Analysis by canonical correlation indicated no evidence for any correlation between the pharmacodynamic variables and any of the pharmacokinetic variables (p-value = 0.21). No trends for any correlation could be detected upon visual inspection of scattergrams of PD versus PK-variables.

Low salivary artemisinin concentrations in several patients resulted in the pharmacokinetic parameters to be based on data from 43 male (24 and 19 in the standard- and escalating-dose groups, respectively) and 16 female patients (7 and 9 in the standard- and escalating-dose groups, respectively). Artemisinin concentrations in samples taken at 30 minutes after drug intake were abruptly high in several patients. Thus, the 30-min samples from all patients were not included in the final analysis.

There was a significant difference in CL/F between the two groups on the first treatment day with higher values for the escalating-dose group (Table 5). The here observed difference indicates a saturable first-pass extraction such that the higher dose would have higher oral bioavailability. Indications of saturable artemisinin kinetics after oral administration has been found in healthy subjects previously (9). The apparent lower bioavailability on day 1 in patients receiving the 100 mg dose resulted in concentration levels lower than expected, which obviously contributed to the prolonged PCT in this group. The data indicates a border-line significant difference in the MRT between the groups, with higher MRT values for the standard-dose group patients. One possible explanation would be a difference in the mean absorption time (MAT) between the groups, since MRT after extravascular administration is the sum of the MRT in the body and the MAT. However, it should be noted that although significant, the difference in the MRT values between the two groups was rather small (0.7 hours, Table 5).

Artemisinin oral clearance values on day 5 did not differ between the two groups. However, these values were significantly higher compared to day 1 in both groups (Table 5). The increase is in accordance with earlier findings based on plasma concentrations in both malaria patients and healthy volunteers (3, 9, 11, 12).

Table 5: Pharmacokinetic parameters (mean \pm SD) in Vietnamese male and female adults with uncomplicated falciparum malaria treated with a standard- or escalating-dose regimen of oral artemisinin. The overall level of significance was set at 0.05 in all analysis.

	Standard dosing		Escalating dosing		P-value [†]	
	Day 1 (500 mg)	Day 5 (500 mg)	Day 1 (100 mg)	Day 5 (500 mg)		
CL/F (L/hr)	1019 \pm 633	8104 \pm 7837 ^(b)	2019 \pm 1277 ^(b)	7160 \pm 5614 ^(b)	D1	< 0.001
95% CI	783, 1255	5971, 12895	1503, 2535	4983, 9336	D5	0.79
P-value ^{††}	< 0.0001		< 0.0001			
t_{1/2} (hr)	1.3 \pm 1.1 ^(a)	1.4 \pm 2.4 ^(b)	1.1 \pm 0.7 ^(b)	1.1 \pm 0.8 ^(a)	D1	0.40
95% CI	0.9, 1.7	0.8, 1.7	0.8, 1.4	0.8, 1.4	D5	0.79
P-value ^{††}	0.99		0.87			
C_{max}/D (ng/ml)	244 \pm 202 ^(a)	53 \pm 47 ^(b)	185 \pm 194 ^(a)	57 \pm 43 ^(b)	D1	0.36
95% CI	162, 311	35, 71	93, 232	37, 70	D5	0.79
P-value ^{††}	< 0.0001		0.004			
t_{max} (hr)	2.9 \pm 1.9 ^(a)	2.6 \pm 1.2 ^(b)	2.8 \pm 1.5 ^(b)	2.5 \pm 1.0 ^(b)	D1	0.88
95% CI	2.3, 3.8	2.1, 3.0	2.4, 3.7	2.1, 2.9	D5	0.79
P-value ^{††}	0.14		0.19			
MRT (hr)	4.2 \pm 1.4 ^(a)	4.4 \pm 3.9 ^(b)	3.5 \pm 1.1 ^(b)	3.7 \pm 1.3 ^(a)	D1	0.05
95% CI	3.7, 4.8	2.8, 5.8	3.2, 4.0	3.3, 4.2	D5	0.79
P-value ^{††}	0.98		0.48			

†- D1 and D5 refer to between-group differences on the first and last treatment days, respectively.

††- P-values for within-group differences on the first and last treatment days.

a): n = 29-31; b): n = 26-28

Standard-dose group patients in our study exhibited an average increase (measured as CL/F_{day1} divided by CL/F_{day5}) of 5-fold in their oral clearance from day 1 to 5, whereas the increase in the escalating-dose group averaged at 2.5 times.

Both gender and body weight showed a significant influence on the CL/F in the first day of administration between the groups with lower values for female patients and higher values with increased weight. Both covariates also exhibited a border-line significant effect on dose adjusted C_{max} values as well. In a population kinetic study lower oral clearance and distribution volumes for artemisinin were found in female children (91), although the difference was not concluded significant by the authors.

Artemisinin is metabolized by Cytochrome P-450 enzyme 2B6 with some possible contribution of CYP3A4 and/or CYP2A6 (95). It has also been reported to induce CYP2C19 but not CYP3A4 (96). Reports on gender differences with respect to the activity of these enzymes are scarce. Indications of higher activities in female subjects have been reported for CYP2A6 in both Turkish and Finnish healthy volunteers (43, 88), whereas no sex differences were found in the expression of CYP2B6 using specific antibodies (94). Also the reports on the gender-related differences on the activity of CYP3A4 are few and contradicting. The activity of this enzyme was reported to be higher in female subjects (41). However, other investigators found this gender effect to be evident only in combination with age (64). In the present study no effect of gender on the artemisinin oral clearance was in evidence on the last treatment day.

This is the first patient study in which saliva sampling is used for investigations of the pharmacokinetics of artemisinin. Agreement of the study results with previous publications on the non-linearity in the kinetics of the compound and its autoinduction capacity encourages the use of saliva as a suitable sampling matrix. However, due to low salivary concentrations, more sensitive analytical systems than the present are necessary for further investigations based on saliva sampling.

Combination therapies for treatment of malaria using an artemisinin analogue and another antimalarials with longer elimination half-lives have been proposed (79, 110). However, due to the potential for drug-drug interactions caused by the induction of drug metabolizing enzymes, artemisinin is probably the least suitable of its class for combination chemotherapy.

In conclusion, the kinetics of artemisinin was found to exhibit both time- and dose-dependency. The studied escalating-dose regimen with lower initial doses of artemisinin did not result in higher exposure to the drug toward the end of the treatment. The escalating-dose regimen investigated in the present study resulted in prolonged parasite clearance times and its clinical implementation is not advised.

13. Modeling the artemisinin autoinduction (paper V)

13.1. Study aims

The aim of the study was to investigate the kinetics of the phenomenon of artemisinin's autoinduction in healthy volunteers. It was of interest to model the enzyme kinetics based on data derived from the kinetics of artemisinin. Such a model would provide estimates of the time-course of the enzyme induction, including the return of the enzyme activity to pre-induced level.

13.2. Study design

Twenty-four healthy, male, Vietnamese adults were included in this randomized, comparative, parallel group study. Subjects were enrolled in the two groups: group A subjects (n=12) received 500 mg artemisinin per day for 5 days, whereas group B subjects (n=12) were administered artemisinin according to an escalating dose scheme starting with 100 mg of the drug for the first two days, increased to 250 mg on days 3 and 4, and a final dose of 500 mg on the fifth day. All doses were administered as single-doses. Two new subjects from each group were asked to return for an extra administration on one of the following days: 7, 10, 13, 16, 20, or 24 after the study initiation, at which they received a single oral dose of 500 mg artemisinin. Frequent saliva samples were collected during days 1, 3, 5, and the return day of the study.

13.3. The induction model

In the final model (Fig. 8), artemisinin is introduced to the system in a gut compartment, with subsequent absorption into the liver compartment. The compound is distributed further into a saliva compartment, where the concentration observations are made. The transfer rate from the saliva back to the liver compartment was set equal to the elimination rate of the drug from the latter.

The enzyme part of the model consisted of a precursor compartment and an enzyme pool, with the zero-order precursor production rate set equal to the first-order enzyme elimination rate (K_{enz}). The precursor transformation to enzyme was given another rate (K_{pen}). Artemisinin amounts in the liver compartment increased the enzyme

production rate. The intrinsic clearance of artemisinin was directly proportional to the amount of enzymes in the enzyme pool compartment. Thus, elevated enzyme amounts due to induction resulted in a decrease in the bioavailability of artemisinin from the liver to the saliva compartment. Absorption lag-time and absorption rate-constant were fixed to values estimated from the first administration day. Artemisinin unbound fraction was fixed to 0.14, based on previous findings (paper III). An exponential

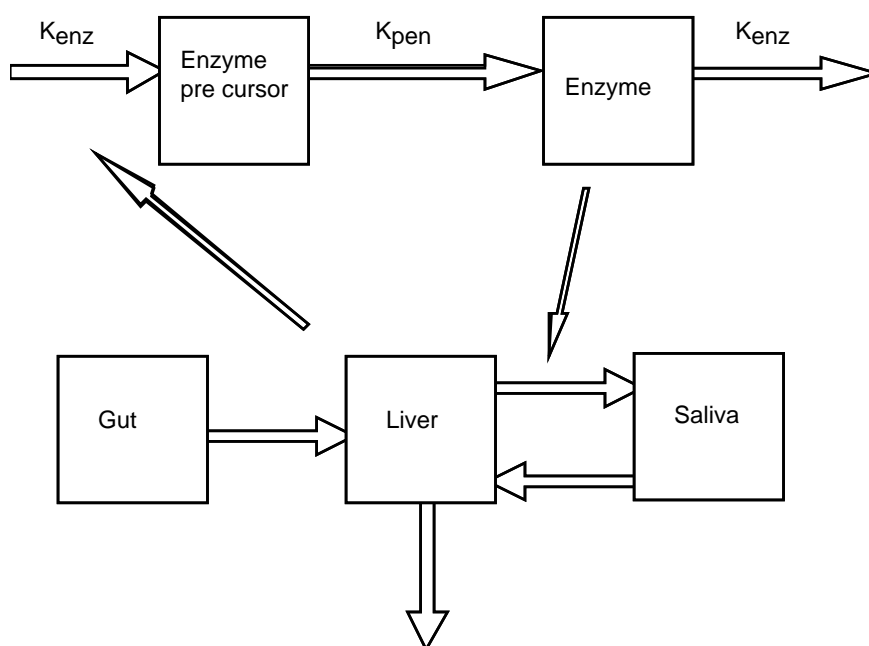


Figure 8: Schematic presentation of a model describing the auto-induction phenomenon of artemisinin. According to this model the amount of artemisinin in liver increases the production rate of enzyme precursors, leading to higher amounts of the enzyme in the enzyme pool. The enzyme amount in this pool has a negative impact on the bioavailability of the compound from liver to saliva, where artemisinin concentrations were measured.

variance model was used to describe the inter-individual variability in the intrinsic clearance and the volume of distribution. Exponential terms, describing the inter-occasional variability in artemisinin absorption rate and absorption lag-time were incorporated in the model. The model was applied to the log-transformed saliva concentration data. A proportional model was applied in the residual error model.

During the early model-building phase several other models were tested. These included a model with drug elimination from both the liver and saliva compartments, a model with only one compartment for the enzyme part, a model with a non-linear

effect of the artemisinin amount in liver on the production rate of the enzymes, a model with a non-linear effect of the enzyme amounts in the enzyme pool, as well as the amounts of artemisinin in the liver compartment, on the bioavailability of artemisinin from the liver to the saliva compartment, and a model with the liver artemisinin amounts inhibiting the elimination rate of the enzymes.

Effects of the covariates age, body weight, and smoking habits on different parameters were tested during the model building procedure.

13.4. Results and discussions

Artemisinin was well tolerated by all subjects with no reported adverse effect. All subjects fulfilled the study according to the protocol. Due to mechanical problems during the HPLC analysis of the samples, data from one subject from group A could not be included in the final analysis. This subject had received the final dose on day 24 after study initiation.

As expected, a decrease in artemisinin AUCs with time was observed during the continuous administration of the compound (Fig. 9). The model assumes an increase in the enzyme precursor synthesis rate of the artemisinin metabolizing enzymes to be the cause of the decreased concentrations of the drug. Such an increase, caused by artemisinin amounts in the liver, results in elevated enzyme amounts in the enzyme pool. The proposed model described the observed decrease in artemisinin's concentrations successfully (Fig. 10).

Our model estimated an enzyme elimination half-life of 32.9 hours (Table 6). Since the information on what enzyme(s) are induced by artemisinin is limited and incomplete, the model-estimated half-life could account for a mix of several enzymes. The estimated half-life implies that after a last dose of artemisinin it would take 5-7 days for the enzyme activity to return to its pre-induction levels. Pharmacokinetics of single oral doses of artemisinin were found to have been affected by another dose administered a week earlier (9). In another report, plasma samples were collected from healthy, male adults after administration of a new dose of artemisinin after a

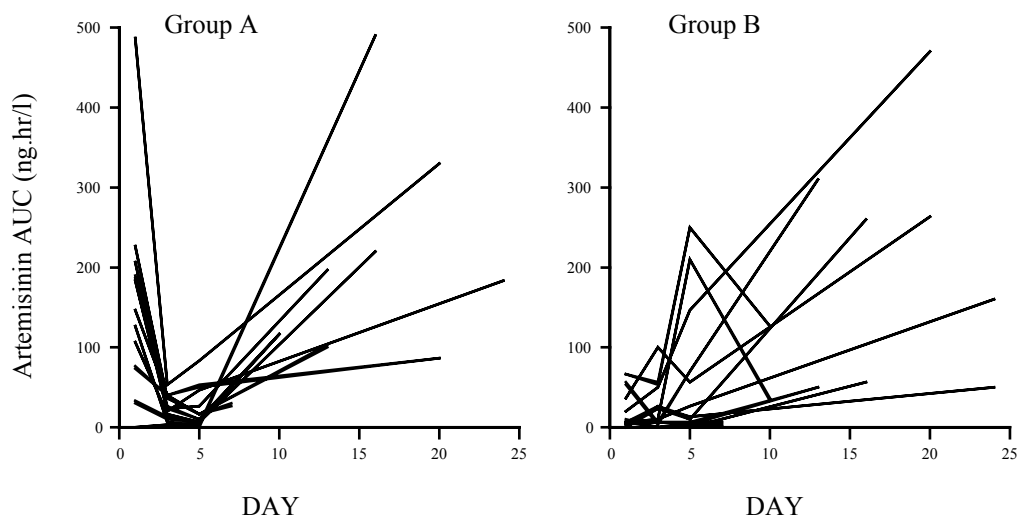


Figure 9. Artemisinin saliva AUCs in healthy Vietnamese volunteers receiving 500 mg of the drug daily for 5 days (group A, n=11), or a dosage regimen of 100+100+ 250+250+500 during a five-days period (group B, n=12). Two subjects from each group received an extra dose of 500 mg on one of the following days: 7, 10, 13, 16, 20, or 24.

Table 6. Artemisinin's typical pharmacokinetic parameter values and associated inter-individual (IOV) and inter-occasional (IIV) variability in 23 healthy Vietnamese subjects.

Parameter	Estimate (RSE%)	IOV (RSE%)	IIV (RSE%)
Enz_{1/2} (hrs)	32.9 (25)	NE	0.31 (58)
Slope_{ind}	0.0305 (34)	NE	NE
CL_{int} (L/h)	2300 (24)	NE	NE
V_d (l)	103 (21)	NE	NE
Lag-time (h)	1.4 (FIXED)	2.8 (4)	NE
k_a (hr ⁻¹)	0.254 (FIXED)	0.95 (29)	NE
F_u	0.14 (FIXED)	NE	NE
Ind_{t1/2} (hrs)	3.15 (71)	NE	NE

Enz_{1/2}: Enzyme elimination half-life, **Slope_{ind}**: Slope of the inducing effect of artemisinin amounts in the compartment on the enzyme production rate, **CL_{int}**: Artemisinin intrinsic clearance, **V_d**: Volume of distribution, **Lag-time**: Absorption lag-time, **k_a**: Absorption constant rate, **F_u**: plasma unbound fraction, **Ind_{t1/2}**: Induction lag-time.

wash-out period of 2 weeks preceded by a 7-days daily administration of the compound (10). AUC values on day 21 were 68% of those of day 1, concluded by the authors to indicate a normalization of drug disposition. In yet another study with a 7-days continuous administration of the compound to healthy volunteers, plasma levels of artemisinin were determined on the first and last administration days (96), as well as after a new administered dose on day 14. The authors suggested the estimated oral clearance values on day 14 to have returned to day 1 levels.

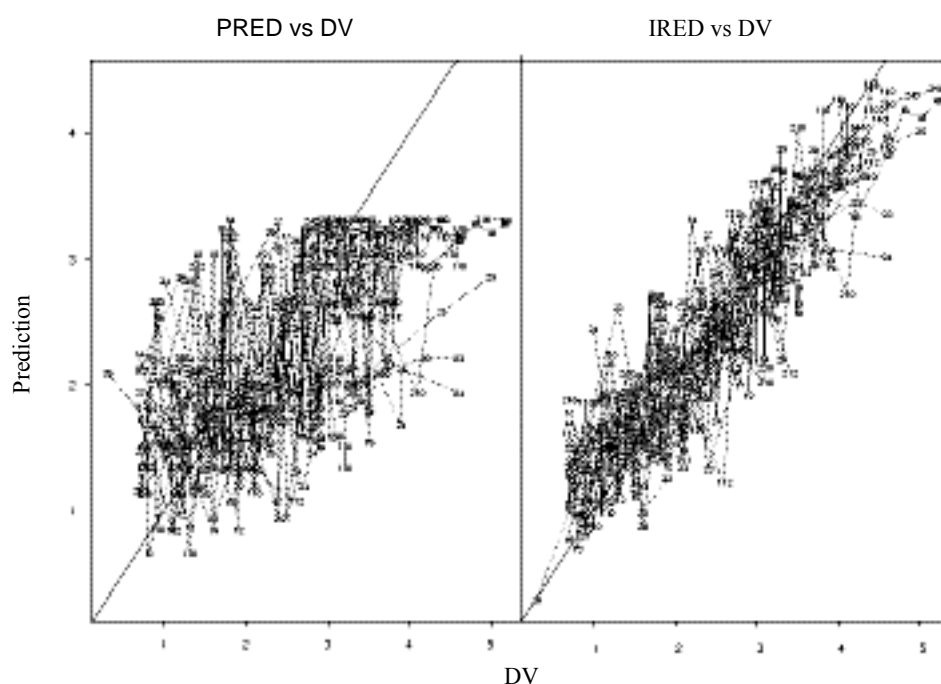


Figure 10. Population and individual predictions v.s. measured saliva concentration in healthy subjects allocated to two different dosage regimens.

An induction lag-time of 3.15 hours was estimated by the model. The enzyme amounts are governed by the induction as well as elimination half-life of the enzymes, with latter being the most influential one in case of artemisinin autoinduction. The short induction lag-time of the enzymes implies that lower artemisinin AUCs would have been observed during the second administration day, had they been determined. In the present study artemisinin saliva concentrations were measured on days 1, 3, 5 and a re-test day (7, 10, 13, 16, 20, or 24). Dose-adjusted artemisinin saliva AUCs were found to have decreased by an average of 80% on day 3. In a previous study the time-course of artemisinin induction in healthy volunteers during a 7-days period was investigated with plasma samples collected on days 1, 4, and 7 (10). Artemisinin

AUCs on day 4 were observed to have decreased to 43% of the first day AUCs. The short induction lag-time also implies that the kinetics of the compound after a single dose is affected by its own inducing effect. Figure 11 represents simulation of artemisinin bioavailability vs. time for the two study arms. As seen in the figure, a decrease in the bioavailability of the compound is observed with time after each

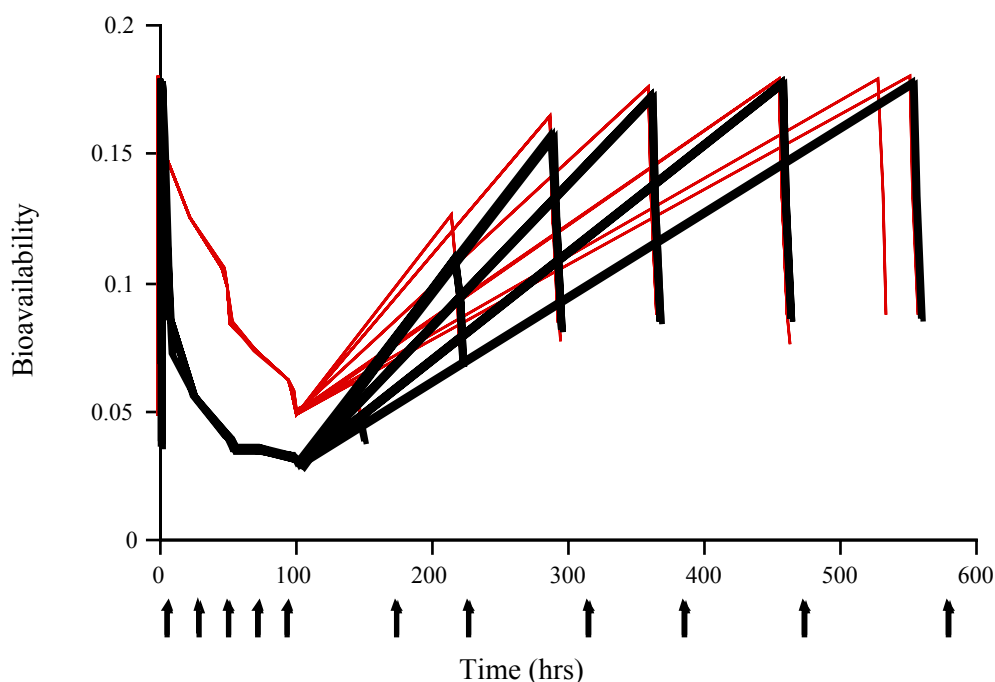


Figure 11. Simulation of artemisinin bioavailability v.s. time in group A (—) or group B subjects (—). Arrows show the administration occasions.

artemisinin administration. The simulation also predicts a dose-dependent induction, which results in a lower extent of induction for those individuals who were administered the lower doses of the compound.

The pre-induced intrinsic clearance of artemisinin was estimated to 2300 L/h. Assuming an average liver blood flow of 70 L/h for a 55-kg Vietnamese subject and an unbound artemisinin fraction in plasma equal to 0.14, the extraction ratio of the compound would be calculated to 82% prior to induction, resulting in a bioavailability of 12%. The high extraction ratio of artemisinin is in accordance with previous suggestions (3, 10, 12). Thus, an increased intrinsic clearance would have little effect on the systemic clearance of artemisinin but decrease its bioavailability significantly.

A large inter-occasional variability was observed for the absorption lag-time and rate constant of the compound. This might be due to artemisinin being hardly soluble in water resulting in erratic and irregular absorption after different administration occasions. It is also probable that lower saliva concentrations of the compound during the induction phase have contributed to the estimation of such high variability. Moreover, with a presumed high extraction ratio of the drug, high variability in its absorption properties would be expected.

This paper presents a semi-physiological model for describing the phenomenon of artemisinin autoinduction. The proposed model was successfully applied to saliva concentration-time data from healthy volunteers, receiving two different dosage regimens of the compound. Our model provides estimations of different parameters describing the time-course of both artemisinin and the induced enzymes, including induction lag-time, enzyme elimination half-life, as well as artemisinin intrinsic clearance.

14. Conclusions

Artemisinin's clinical pharmacokinetics and in-vitro interactions with blood components were investigated in this thesis.

Artemisinin was found to distribute irreversibly into the red blood cells. The rate of the process was faster in the absence of oxygen, indicating a possible competition to the binding sites of hemoglobin. In contrast to previous findings, the compound was shown not to bind to red blood cell membranes.

A coupled column HPLC system was developed for detection of artemisinin in biological samples. Artemisinin concentrations in saliva and plasma samples were quantified using a RAM-column, which allowed direct injections of the samples.

Artemisinin concentrations in saliva were correlated with its unbound venous and capillary plasma levels. The absolute concentration differences for saliva, venous plasma, and capillary plasma were found to be negligible. Higher capillary to venous plasma concentration ratios during the early phases of drug absorption indicate an arterio-venous concentration difference for the compound.

Artemisinin pharmacokinetics in uncomplicated malaria patients treated with two different oral dosage regimens of the compound was found to exhibit dose- and time-dependency. A saturable first-pass metabolism was observed in patients receiving the standard dose of 500 mg. Average oral clearance of artemisinin was found to have increased on the last treatment day (day 5) in both groups. Lower initial doses of artemisinin resulted in the same low concentrations on day 5 as the standard dosing regimen. Sex had a significant effect on artemisinin oral clearances on day 1 with higher values for female patient. The initial low-dose regimen resulted in longer parasite clearance times.

A semi-physiological model was applied to describe artemisinin autoinduction. An induction lag-time of 3.2 hours was estimated for the compound, whereas the enzyme elimination half-life was estimated to 32.9 hours. A large inter-occasional variability was observed for drug absorption lag-time and rate constant.

15. Personal remarks

I came in contact with malaria in my childhood. My father, the chief of field operations, used to take me to his office when he looked at different blood smears. It was not a difficult decision for me to finish my undergraduate studies working on an antimalarial drug.

During my field studies in Vietnam I have come to an understanding of the enormous negative impact the disease has on ordinary people's daily life. The suffering not only affects the individual patient, but also results in extra burden on his/her surrounding in form of the absence of income and costs for treatment.

How to combat the disease? It is my belief that the problem of malaria will not be solved by biological means alone. At the moment we have access to several compounds with good antimalarial efficacy. A less appealing alternative in form of insecticides is also available. More than two million people, however, die every year as a direct consequence of malaria infection. And yet, in places where one expects the disease to be prevalent, it is practically eradicated. I believe that treatment of the acute cases should be regarded as a complementary approach in our combat against malaria. A far more effective cure to the problem would be an increase in the standard of living for the general public in countries hit by the disease. As miraculous as artemisinin and any other antimalarials might be in curing individuals, they are not the answer to the issue of reducing the impact of the disease.

I have had the chance to work with a very interesting drug. It is inexpensive, easy to produce, and highly effective. Its use has resulted in decreased mortality in malaria in several countries. I wonder what else the Chinese scientists in the Artemisinin group should have done to deserve a Nobel Prize for their efforts.

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