The effects of pharmacogenetics on pharmacokinetics of artemisinin-based combinations in malaria patients

Eva Maria Hodel

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The effects of pharmacogenetics on pharmacokinetics of artemisinin-based combinations in malaria patients

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Summary

Malaria is a vector-borne infectious disease caused by protozoan parasites of the genus *Plasmodium*. If not treated appropriately, human *P. falciparum* malaria can quickly become life-threatening, leading to an estimated 900'000 annual deaths globally. Key interventions to control malaria include prompt diagnosis and effective treatment with artemisinin-based combination therapies (ACTs), use of insecticide treated nets by people at risk, indoor residual spraying with insecticide to control the vector mosquitoes and intermittent preventive treatment for pregnant women (IPTp) and infants (IPTi).

Whether antimalarial treatments are effective or not, depends on parasite and host factors. The ability to define resistance leading to treatment failure has been greatly enhanced by our understanding of the underlying molecular mechanisms causing resistance in *P. falciparum*. However, the potential contribution of host genetic factors, particularly those associated with antimalarial drug metabolism, remains largely unexplored. The same applies for the basic mechanisms involved in the pharmacokinetics of antimalarial drugs and the link between antimalarial drug pharmacokinetics and treatment outcomes. Thus, the purpose of this thesis was to quantify the effects of pharmacogenetics on pharmacokinetics of ACTs.

Between 2007 and 2008, three *in vivo* studies were performed in Cambodia and Tanzania. Patients reporting with fever associated with an infection with *Plasmodium falciparum* were recruited and treated with ACTs according to the national guidelines in the respective country. In Cambodia, 64 patients were recruited for the treatment with artesunate–mefloquine and 61 for the treatment with dihydroartemisinine–piperaquine. In Tanzania, 150 were treated with artemether–lumefantrine. Blood samples for the pharmacokinetic analysis were taken before treatment and at several time points during and after treatment, e.g. on Days 1, 2 and 7 in all studies and in Cambodia also 1 hour after the first dose and on Day 14.

For the analysis of plasma samples collected during our studies, we developed a broad-range liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) assay covering 14 of the currently in-use antimalarial drugs and their metabolites. The assay requires only as little as 200 µl of plasma and is a major improvement over previous methods in terms of convenience, sensitivity, selectivity and throughput. The method was validated according to well-established recommendations. The assay was first used for the analysis of the baseline samples collected in our *in vivo* studies. In all studies more than half of the patients recruited had still antimalarials in their blood. Theses findings enabled us to get a better assessment of

the antimalarials circulating in the local population, and hence of the drug pressure on the parasites in both countries.

Single nucleotide polymorphisms (SNPs) in genes encoding enzymes associated with antimalarial drug metabolism, i.e. cytochrome P450 isoenzymes (*CYP*) and *N*-acetyltransferase 2 (*NAT2*), were analyzed. Based on our previous experience, we developed a DNA microarray to affordably generate SNP data. However, after comparison of microarray data and sequencing data, we concluded that the major limit of the microarray technology was lack of robustness which could not be compensated by superior cost-effectiveness. Consequently, the pharmacogenetic profiles of the patients from the three *in vivo* studies were assessed by direct sequencing of genomic DNA. Whereas for most SNPs allele frequencies were similar in both populations, we found significant inter-ethnic differences in the distribution of genotypes of certain enzymes, namely *CYP2D6*, *CYP3A4/5* and *NAT2*. Is has been shown that the human *CYP3A* subfamily plays a dominant role in the metabolic elimination of more drugs than any other biotransformation enzyme. Therefore, our findings might have implications for treatment policies of not only antimalarials and the widely introduced ACTs in particular, but any other drugs metabolized by these enzymes.

To quantify the effect of pharmacogenetics on pharmacokinetics of ACTs we developed population pharmacokinetic models. The pharmacokinetic parameters we estimated in our models were in agreement with those from previous studies. In order to account for parts of the inter-individual variability in drug-metabolizing capacity of the liver we included pharmacogenetic data as covariate. For artemether, we found that 9% of the inter-individual variability in clearance could be explained by the genotype of *CYP3A5* (reference allele *versus* variant allele *CYP3A5*3*). Heterozygous carriers showed a reduction in clearance of 34%. The alterations in clearance were less pronounced for lumefantrine (increase in clearance of 12% in homozygous carriers of variant allele *CYP3A4*1B*, explaining 2% of the inter-individual variability in clearance) and mefloquine (decrease in clearance of 14% in carriers of homozygous variant allele *CYP3A5*5*, explaining 1% of the inter-individual variability in clearance). These data might partially provide an explanation for the differences in drug efficacy observed with artemether–lumefantrine combination treatment.

In conclusion, we were able to show that there is a correlation between the pharmacogenetic profile of the host and the pharmacokinetics of antimalarial drugs administered in malaria

patients. These results suggest that pharmacogenetics could be one of the basic mechanisms involved in the pharmacokinetics of antimalarial drugs. The knowledge gained from this study could facilitate the selection process of first-line treatment for malaria and would allow dosing adaptation based on the pharmacogenetic profile of the population. Such adaptations are needed especially in the most vulnerable groups, including infants, pregnant women, and those with prevalent co-morbidities, where often therapeutic antimalarial drug concentrations over time are not achieved.

Zusammenfassung

Malaria ist eine von Vektoren übertragbare Infektionskrankheit, die durch Protozoen der Gattung *Plasmodium* verursacht wird. Wird die Krankheit nicht richtig behandelt, kann die humane *P. falciparum* Malaria schnell lebensbedrohlich werden. Dies führt weltweit jährlich zu schätzungsweise 900'000 Todesfällen. Zu den Schlüsselinterventionen gegen Malaria zählen die frühzeitige Diagnose und wirksame Behandlung mit einer Kombinationstherapie Artemisininderivaten (ACT), mit Insektiziden imprägnierte Bettnetze für Risikopersonen, das Sprayen von Insektiziden zur Vektorbekämpfung in Häusern und Hütten sowie die intermittierende vorbeugende Behandlung schwangerer Frauen (IPTp) und Kleinkindern (IPTi).

Ob eine Malariatherapie wirksam ist oder nicht, hängt sowohl von Parasiten- als auch von Wirtsfaktoren ab. Neuste Fortschritte in Genetik und Genomik von Plasmodien haben das Verständnis jener Mechanismen enorm verbessert, welche der Resistenzentwicklung in *P. falciparum* zu Grunde liegen. Jedoch ist der potenzielle Einfluss genetischer Faktoren des Wirtes, die insbesondere den Metabolismus der Malariamedikamente betreffen, noch weitgehend unerforscht. Dasselbe gilt auch für die grundlegenden Mechanismen, die an der Pharmakokinetik der Malariamedikamente beteiligt sind, sowie für den Zusammenhang zwischen Pharmakokinetik und Behandlungsresultat der Malariamedikamente. Daher besteht das Ziel dieser Arbeit darin, die Auswirkungen der Pharmakogenetik auf die Pharmakokinetik der ACT zu quantifizieren.

Zwischen 2007 und 2008 führten wir drei *in vivo* Studien in Kambodscha und Tansania durch. Patienten mit Fieber aufgrund einer Infektion mit *P. falciparum* wurden in die Studie eingeschlossen und mit ACT entsprechend der nationalen Therapierichtlinien behandelt. In Kambodscha wurden 64 Patienten mit Artesunat–Mefloquin und 61 Patienten mit Dihydroartemisinin–Piperaquin behandelt. In Tansania wurden 150 Patienten mit Artemether–Lumefantrin therapiert. Für die pharmakokinetische Analyse wurden Blutproben vor und zu mehreren Zeitpunkten während sowie nach der Behandlung entnommen, d.h. an den Tagen 1, 2 und 7. In den Studien in Kambodscha wurde zudem je eine Probe 1 h nach der ersten Dosis und am Tag 14 entnommen.

Für die Analyse der Plasmaproben entwickelten wir ein Verfahren basierend auf Flüssigkeitschromatographie und Tandem-Massenspektroskopie (LC–MS/MS) zum Nachweis der 14 gebräuchlichsten Malariamedikamente und ihren Metaboliten. Das Verfahren benötigt

lediglich eine Menge von 200 µL Plasma und stellt in Hinblick auf Einfachheit, Sensitivität, Selektivität und Durchsatz eine deutliche Verbesserung gegenüber älteren Methoden dar. Die Methode wurde gemäss gängigen Empfehlungen validiert. Das Verfahren wurde erstmals für die Analyse der Proben verwendet, die vor Therapiebeginn in unseren *in vivo* Studien entnommen wurden. In allen drei Studien wiesen mehr als die Hälfte der Patienten noch Spuren von Malariamedikamenten im Blut auf. Dies ermöglichte zu erkennen, welche Medikamente von der Lokalbevölkerung tatsächlich eingenommen worden waren und welcher medikamentöse Selektionsdrucks auf die Parasitenpopulation daraus resultierte.

Zudem wurden Einzelnukleotid-Polymorphismen (SNPs) in Genen analysiert, die Enzyme kodieren, welche im Zusammenhang mit dem Metabolismus von Malariamedikamenten stehen, d.h. Cytochrom-P450-Isoenzyme (*CYP*) und *N*-Acetyltransferase 2 *(NAT2*). Ausgehend von unserer bisherigen Erfahrung entwickelten wir einen DNA-Microarray, um SNP-Daten kostengünstig zu generieren. Nach dem Vergleich der Microarraydaten mit den Sequenzierdaten mussten wir jedoch feststellen, dass es der Microarraytechnologie an Robustheit fehlte. Diesen Mangel konnte selbst eine höhere Kosteneffizienz des Microarrays nicht rechtfertigen. Entsprechend wurden die pharmakogenetischen Profile der Patienten aus den drei *in vivo* Studien durch direkte Sequenzierung genomischer DNA bestimmt. Während für die meisten SNPs die Allelfrequenzen in beiden Populationen ähnlich waren, fanden wir hingegen signifikante inter-ethnische Unterschiede in der Verteilung der Genotypen einzelner Enzyme, namentlich *CYP2D6*, *CYP3A4/5* und *NAT2*. Es wurde gezeigt, dass unter allen biotranformierenden Enzymen die Familie der humanen *CYP3A* eine besonders wichtige Rolle bei der metabolischen Elimination der Mehrheit von Medikamenten spielt. Daraus folgt, dass unsere Ergebnisse nicht nur für Therapierichtlinien mit Malariamedikamenten und speziell für die weit verbreiteten ACTs, sondern auch für zahlreiche andere Medikamente wichtige Implikationen haben könnten.

Um den Einfluss der Pharmakogenetik auf die Pharmakokinetik von ACTs zu quantifizieren, entwickelten wir populationspharmakokinetische Modelle. Die durch unsere Modelle geschätzten pharmakokinetischen Parameter stimmten mit den Literaturwerten überein. Das pharmakogenetische Profil wurde als Kovariable in die Modelle aufgenommen, um Teile der interindividuellen Variabilität in der metabolischen Kapazität der Leber zu erklären. Für Artemether konnten wir 9% der interindividuellen Variabilität der Clearance durch den Genotypen von *CYP3A5* (Referenzallel *versus* Variante *CYP3A5*3*) erklären. Heterozygote

wiesen eine um 34% reduzierte Clearance auf. Die Änderungen waren weniger ausgeprägt für Lumefantrin (Clearancezunahme um 12% bei homozygoten Trägern des Allels *CYP3A4*1B* und 2% Anteil an der interindividuellen Variabilität der Clearance) und Mefloquin (Clearanceabnahme um 14% bei homozygoten Trägern des Allels *CYP3A5*3* und 1% Anteil an der interindividuellen Variabilität der Clearance). Diese Daten könnten eine Erklärung für die beobachteten geographischen Unterschiede in der Wirksamkeit von Artemether–Lumefantrin liefern.

Zusammenfassend können wir festhalten, dass eine Korrelation zwischen dem pharmakogenetischen Profil des Wirtes und der Pharmakokinetik des verabreichten Medikamentes in Malariapatienten besteht. Diese Ergebnisse lassen vermuten, dass Pharmakogenetik zu den zentralen Mechanismen gehört, welche die Pharmakokinetik von Malariamedikamenten beeinflussen. Die Erkenntnisse aus dieser Studie könnten zukünftig beim Entscheidungsprozess helfen, die Mittel der ersten Wahl in der Malariatherapie festzulegen. Des Weiteren würden unsere Erkentnisse eine Dosisanpassung aufgrund des pharmakogenetischen Profils erlauben. Solche Anpassungen sind insbesondere für gefährdete Patientengruppen nötig, wie etwa Kleinkinder, Schwangere und polymorbide Patienten, da bei diesen Patienten therapeutische Konzentrationen von Malariamedikamenten über die notwendige Zeitspanne oftmals nicht erreicht werden.

Abbreviations

ACT	Artemisinin-based combination therapy
ADDO	Accredited drug dispensing outlet
AIDS	Acquired immunodeficiency syndrome
AL	Artemether–lumefantrine
AM	Artemether
AM	Artemether
AQ	Amodiaquine
ART	Artemisinin
ARV	Antiretroviral
AS	Artesunate
AS	Artesunate
AUC	Area under the plasma concentration time curve
A_X	Amount of drug in compartment X
BW	Body weight
CHUV	Centre Hospitalier Universitaire Vaudois, Switzerland
CID	Collision induced dissociation
CL	Clearance
CNM	National Center for Parasitology Entomology and Malaria Control, Cambodia
CPD	Cambodia, Phnom Dék
CQ	Chloroquine
C_t	Concentration at time t
CV	Coefficient of variation
CVV	Cambodia, Veal Veng
CYP	Cytochrome P450 gene
DAQ	Desethyl-amodiaquine
ddNTP	Dideoxynucleotide
dH_2O	Deionized water
DHA	Dihydroartemisinin
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
DLF	Desbutyl-lumefantrine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DSS	Demographic surveillance system

EDTA	Ethylenediaminetetraacetic acid
EKBB	Ethikkommission beider Basel
EMIC	Explanatory model interview catalogue
ESI	Electrospray ionization
Ext RE	Mean extraction yield
FA	Formic acide
FDA	Food and Drug Administration, United States
F_{ST}	Fixation index
H	High
Hb	Hemoglobine
Het	Heterozygous carrier
HIV	Human immunodeficiency virus
Hom-REF	Homozygous carrier of the reference allele
Hom-VAR	Homozygous carrier of the variant allele
HPLC	High performance liquid chromatography
I	Intermediate
I.S.	Internal standard
IC50	Half maximal inhibitory concentration
IHI	Ifakara Health Institute, Tanzania
IIV	Inter-individual variability
IPC	Institute Pasteur du Cambodge, Cambodia
IPTp	Intermittent preventive treatment for pregnant women
IQR	Inter-quartile range
k_a	Absorption rate constant
k_e	Elimination rate constant
k_{xy}	Rate constant of transfer from compartment x to y
L	Low
LC	Liquid chromatography
LF	Lumefantrine
LLC	Lower limit of calibration
LLOQ	Lower limit of quantification
LOD	Limit of detection
M	Medium
ME	Mean matrix-mediated ionization

MeCN	Acetonitrile
MeOH	Methanol
MF	Mefloquine
MIC	Minimal inhibitory concentration
MPC	Minimal parasiticidal concentration
MQ	Mefloquine
MS	Mass spectrometry
N.A.	Not applicable
NAT2	*N*-acetyletransferase 2 gene
NCBI	National Center for Biotechnology Information
OFV	Objective function value
PCL	Division of Clinical Pharmacology
PCR	Polymerase chain reaction
PD	Pyrimethamine
PE	Process efficiency
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
PPQ	Piperaquine
PQ	Piperaquine
PY	Pyronaridine
Q	Intercompartmental clearance
Q	Quinine
Q1	First quadrupole
Q2	Second quadrupole
Q3	Third quadrupole
QC	Quality control
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
ROC	Receiver operating characteristic
RT	Room temeratur
S.E.	Standard error
SAP	Shrimp alkaline phosphatase
SD	Sulfadoxine
SD	Standard deviation
SDS	Sodium dodecyl citrate

SNP	Single nucleotide polymorphism
SP	Sulfadoxine–pyrimethamine
SRM	Selected reaction monitoring
SSC	Standard saline citrate
$t_{½}$	Terminal half-life
TB	Tuberculosis
TE	Tris-EDTA
TK	Tanzania, Kibaoni
TPR	Trimipramine-D_3
TSQ	Triple stage quadrupole
UGT	Uridine diphosphate glucuronosyl transferase
V_X	Volume of distribution of compartment X
WARN	Worldwide Antimalarial Resistance Network
WHO	World Health Organization
X	Covariate
ε	Intra-individual (= residual) variability
η	Inter-individual variability
θ	Pharmacokinetic parameter

Introduction

Worldwide malaria burden and global control and elimination strategies

Malaria is a vector-borne infectious disease caused by protozoan parasites of the genus *Plasmodium*. Human *P. falciparum* malaria is a deadly disease that puts at risk half of the world population, i.e. approximately 3.3 billion people [1]. Of the approximately 900'000 annual deaths globally from malaria, 98% occur in 30 countries in Sub-Saharan Africa and 5 countries in Asia [2]. The global strategy to eliminate malaria on long-term bases relies on control strategies at the local level trough effective prevention and case management. Prevention can be achieved with vector control interventions and intermittent preventive treatment for pregnant women (IPTp) and infants (IPTi). Whereas vector control strategies aim at reducing transmission and hence the incidence and prevalence of parasite infection and clinical malaria, IPTp reduces the impact of placental malaria infection and maternal malaria-associated anaemia. IPTi relies on reduction of number of infections with simultaneous build up of immunity. Early and effective case management of malaria will shorten its duration and prevents complications and most deaths from malaria. The main components of effective case management are prompt diagnosis and treatment with an appropriate antimalarial [3].

Antimalarial drugs and treatment outcome

Treatment with an appropriate antimalarial means that the drug (combination) (i) cures the infection by eradicating the infection that caused the illness from the body, (ii) prevents progression to severe disease, (iii) prevents additional morbidity associated with treatment failure, (iv) reduces transmission of the infection to others by reducing the infectious reservoir, (v) prevents the emergence and spread of resistance to antimalarials, (vi) is well tolerated, and (iiv) and shows a fast therapeutic response [4].

Currently used antimalarials stem from seven drug classes [5,6]. Table 1 provides a list of these drugs, their proposed mode of action and the targeted parasite stages. However, the list is not exhaustive, as many more drugs are in clinical development [7].

Table 1. Antimalarials and their mode of action

Drug class	Example(s)	Mode of action	Active against
4-Aminoquinolines	Chloroquine, amodiaquine	Prevention of hem polymerisation into non-toxic hemazoin [8-10]	Trophozoytes [8]
8-Aminoquinolines	Primaquine	Destruction of internal structure of mitochondria [11,12]	Different developmental stages including sexual stages [13-15]
Aminoacridines	Pyronaridine	Inhibit glutathione-dependent degradation of hematin [16]	Schizonts [16]
Antibiotics	Doxycyclin, clindamycin	Interaction with prokaryote-like protein biosynthesis machinery of the mitochondrion and/or the apicoplast [17]	Second intracellular cycle (“delayed kill effect”) [17]
Antifolates	Pyrimethamine, proguanil, cycloguanil, sulfadoxine	Inhibition of tetrahydrofolate biosynthesis by inhibition of either dihydro-floate reductase (DHFR) or dihydropteroate synthetase (DHPS) [18]	Asexual erythrocytic stages [19]
Artemisinines	Artemether, artesunate, dihydroartemisinin	Inhibition of PfATPase6 [20]	Small and late ring stages [21]
Arylaminoalcohols	Quinine, mefloquine, lumefantrine, halofantrine	Seem to interfere with the hem digestion suggested [5,22]	Different developmental stages [4]
Inhibitors of the respiratory chain	Atovaquone	Inhibition of mitochondrial electron transport chain by blocking the ubiquinone binding side of the cytochrome bc_1 complex [23,24]	Hepatic and erythrocytic stages [23,25]

Increasing levels of resistance to conventional antimalarial drugs, such as chloroquine, sulfadoxine–pyrimethamine, and amodiaquine, resulted in increasing malaria mortality and morbidity. Thus, these monotherapies cannot be considered appropriate antimalarial treatment anymore. Consequently, the World Health Organziation (WHO) now recommends that all uncomplicated *P. falciparum* infections should be treated with an artemisinin-based combination therapy (ACT) [26]. However, as in all parasitic diseases, treatment outcome in malaria depends on both, host and parasite factors, and parasite resistance is not regarded to be the only cause of treatment failure. Four basic methods have been routinely used to define or monitor response to antimalarial drugs [27,28]:

(1) Assessment of *in vivo* clinical response [29]. Drug response *in vivo* generally depends on many factors including age [30], disease status [31], pre-existing host semi-immunity [32], co-morbidity [33-35], concomitant treatment [36,37], environmental factors (e.g. food intake [38]), pregnancy [30], and adherence.

(2) Assessment of susceptibility of the parasite *in vitro* [39]. *In vitro* sensitivity assessment of *P. falciparum* strains to antimalarials allows the response of clinical isolates to individual drugs to be assessed, unmodified by host factors that could influence drug efficacy *in vivo*.

(3) Determination of blood/plasma drug concentrations [40]. The achievement of therapeutic drug levels in malaria patients is pivotal to curing malaria [40]. Insufficient exposure to the administered drug is associated with a risk of failure and resistance emergence, and too high levels with a risk of toxicity.

(4) Characterization of molecular markers of resistance [41]. Described resistance mechanisms are mutations in genes and changes in copy number of genes relating to the drug's target or efflux pumps affecting intra parasitic drug concentrations [4]. A recently published systematic review and meta-analysis concluded that there is evidence that genetic molecular markers of the malaria parasites are related to an increased risk of therapeutic failure [42].

These four strategies used in clinical routine (item 1) and clinical studies (items 1–4) allow to account for both, the host and parasite factors responsible for drug response. On the one hand, the ability to define resistance has been greatly enhanced by recent advances in *Plasmodium* genetics and genomics [43]. On the other hand, no data on human pharmacokinetics of

antimalarials had been available until recently, although these drugs have been used for almost 80 years [44].

Pharmacogenetics and population pharmacokinetics

The achievement of therapeutic drug levels is of particular interest for antimalarial drugs for which a rapid onset of the antiparasitic effect (sufficient C_{max} and short t_{max}) and a slow elimination (long $t_{½}$) to protect against recrudescence are required for successful treatment [45]. The analysis of drug absorption, distribution, metabolism, elimination and action is a step towards a broader understanding of inter-individual differences in pharmacokinetic and pharmacodynamic profiles and consequential treatment failures and adverse drug reactions.

Most therapeutic agents are lipophilic and need to be biotransformed before they can be eliminated from the body. Without biotransformation drugs would be cleared more slowly, leading to their accumulation and toxicity. Biotransformation can be divided into two steps, i.e. metabolic oxidation (phase I) and conjugation (phase II). The isoenzymes of the cytochromes P450 (*CYP*) superfamily have a pivotal role in the oxidative conversion of drugs to polar products before elimination [46]. Phase II reaction include methylation, sulphation, acetylation, and glucuronidation.

It has been stated that polymorphism of drug-metabolizing enzymes have by far the highest impact on inter-individual differences in drug response [47,48]. Mutations in a gene coding for a drug metabolizing enzyme can give rise to enzyme variants. If the mutant allele occurs with a frequency of at least 1% in the normal population and causes a different drug response or phenotype, this phenomenon is termed a pharmacogenetic polymorphism [49]. Depending on the alleles an individual is carrying, the metabolism can be altered. Certain enzyme polymorphisms can enhance drug metabolism, whereas others abolish or decrease drug metabolism, and frequencies of such polymorphisms vary among different ethnic groups [50,51]. As a consequence, ethnicity may have a major impact on drug metabolism and hence drug efficacy and safety.

Table 2. Polymorphic enzymes responsible for the metabolism of various antimalarial drugs.

Drug	**Cytochrome P450 oxidase superfamily**								***NAT2***
	2A6	***2B6***	***2C8***	***2C9***	***2C19***	***2D6***	***3A4***	***3A5***	
Amodiaquine			[44,59,60]						
Artemether							[37,44,61]	[61]	
Artemisinin	[44,62]	[44,62,63]					[44,62,63]		
Artesunate	[44,64]	[44]							
β-arteether		[44,65]					[44,65]	[44,65]	
Artelinic acid							[44]	[44]	
Chloroquine			[44,64,66,67]			[44,64,67]	[44,64,66,67]	[44,66]	
Dapsone			[64]	[44,64]	[64]		[44,64]		[64]
Halofantrine						[68]	[37,44,68]	[44]	
Lumefantrine							[37,61]	[44]	
Mefloquine							[44,69-71]	[69]	
Primaquine							[44,64]		
Proguanil					[37,44,72,73]				
Quinine							[37,44,70,71,74-76]	[76]	
Sulfadoxine								[77]	
Sulfamethoxazole				[78]					[79]

Both, *CYP* and the phase II enzyme *N*-acetyltransferase-2 (*NAT2*) are involved in the metabolism of various antimalarial drugs. Table 2 lists some of the currently used antimalarials and the metabolizing enzymes for which phenotypic and/or genotypic polymorphisms have been described [46,52]. However, for some antimalarial drugs the metabolic pathway is still not very well known (i.e. piperaquine, pyrimethamine and pyronaridine), or they are barely metabolized at all (i.e. atovaquone and doxycycline) [53-58].

Understanding the causes of ethnic differences in metabolism of antimalarials may promote improved understanding of inter-individual differences in the pharmacokinetics and tolerance of these antiparasitic drugs. In population pharmacokinetic studies the variability in plasma drug concentrations between individuals can be assessed when standard dosage regimens are administered. Such studies may lead to a better knowledge on the pharmacokinetic properties of antimalarials. They would allow the more precise use of the term "antimalarial drug resistance", as it would indicate when treatment failure is not caused by intrinsic parasite resistance but is instead the result of inadequate drug levels due to the pharmacogenetic profile of the host or other non-genetic modifiers of the pharmacokinetic parameters. We know from other studies on infectious diseases such as HIV, tuberculosis and mycoses, that genetic variants might predict plasma exposure and failure and/or emergence of drug resistant pathogens [80-85].

Objectives

As stated in the World Malaria Report [26], an essential component of malaria control is surveillance of therapeutic efficacy over time in order to revise national drug policies and to ensure effective and safe treatment of malaria. Both, drug efficacy and safety, are strongly dependent on the achievement of appropriate circulating drug concentration, and insufficient exposure is associated with a risk of failure and emergence of resistance (Darwinian selection of parasites), whilst too high levels of drug are associated with risk of toxicity. The latter can affect adherence and hence also contribute to the emergence of resistance. Since differences on ethnicity may have a major impact on antimalarial drug metabolism and hence drug efficacy and safety, public health policies for drug use should incorporate pharmacogenetic data collected at the population level in the decision process. A deeper insight in the inter-population distribution of polymorphisms of genes encoding enzymes responsible for antimalarial drug metabolism could facilitate the selection of appropriate first-line treatment for uncomplicated malaria in a specific population. The study of the pharmacogenetic and

pharmacokinetic data in two genetically different populations in South East Asia and African (i.e. Cambodia and Tanzania) might lead to a better understanding of the different factors influencing treatment outcome in malaria patients.

One way to capture numerous non-synonymous point mutations in the genes which are known to be involved in drug metabolism and parasite resistance, and hence response to drugs, is to use high throughput systems such as microarrays, especially when a large sample size are required.

For the analysis of pharmacokinetic data in a large sample size liquid chromatography–tandem mass spectrometry method (LC–MS/MS) assays has played an important role in pharmacokinetics and metabolism studies at various drug development stages since its introduction to the pharmaceutical industry [86]. Thus, LC–MS/MS occurred to be the method of choice for this study as analysis can be done rapidly with a minimal effort for sample preparation. There is still a lack of a validated LC-MS/MS assay that can be routinely used for the simultaneous determination of plasma concentrations of all different kind of antimalarial drug combinations. The assay presented here can facilitate the analysis of large numbers of plasma samples from pharmacokinetic studies as well as individual clinical samples from patients where therapeutic drug monitoring is indicated.

In an individual pharmacokinetic multi-compartmental model a large number of samples are necessary in order to estimate all pharmacokinetic parameters. However, in some cases an intensive sampling schedule cannot be applied because of logistic reasons, e.g. rural areas in developing countries where infrastructure and human resources of the health centres are often very limited, and repeated blood sampling in children causes ethical concerns. This problem can be overcome by population pharmacokinetic analysis for which sparse data, i.e. 3–4 samples per patient, is sufficient to estimate the mean kinetic parameters in the respective population. Population pharmacokinetics allows both, to measure variability of kinetic parameters within the population and to account for it in terms of patient variables, such as age, sex, weight or disease state [87]. The analysis of sparse data sets needs more sophisticated statistical models than required for classical pharmacokinetic analysis. There is a large variety of methods proposed for population pharmacokinetic modelling [88]. NONMEM® (eponym of nonlinear mixed-effects model) is a computer package developed by Beal and Sheiner and designed to fit general statistical (nonlinear) regression-type models to

data [89]. NONMEM® is the most widely used program for the analysis of population pharmacokinetic data [87] and it is based on mixed-effects models taking into account independent variables like time or dose (so called fixed effects) and kinetic parameters. Furthermore, additional variables (so called covariates) such as for instance demographic characteristics can be included in the model as part of the fixed effects. The random effects include the inter-individual variability and the residual variability.

General goal

To quantify the effects of pharmacogenetics on pharmacokinetics of artemisinin-based combinations in malaria patients.

Research questions

(1) Is it possible to measure accurately and precisely the plasma concentration of several antimalarial drugs in one sample at the same time?

(2) What are the different pharmacogenetic profiles of the populations in Cambodia and Tanzania regarding genes known to govern drug disposition?

(3) Is there a correlation between the pharmacogenetic profile of the host and the pharmacokinetics of antimalarial drugs administered in malaria patients?

Specific aims

(A) To develop a LC-MS/MS assay to determine the plasma concentration of the main antimalarial drugs currently available in different countries.

(B) To compare the population's pharmacogenetic profile of genes encoding for proteins relevant for the metabolism of the main antimalarial drugs currently available in Cambodia and Tanzania, i.e. isoenzymes of the cytochrome P450 oxidase superfamily (*CYP*) and *N*-acetyltransferase-2 (*NAT2*).

(C) To correlate the pharmacogenetic profile of malaria patients with the pharmacokinetics of drugs administered, accounting for variability in usual demographic factors such as age, sex, weight etc.

Methodology

Collaborations (aims A,B and C)

In collaboration with the National Center for Parasitology, Entomology and Malaria Control (CNM) of Cambodia, the Pasteur Institute in Cambodia (IPC) and the Ifakara Health Institute (IHI) in Tanzania three *in vivo* treatment studies in malaria patients were performed in Cambodia and Tanzania. The LC–MS/MS assay and the population pharmacokinetic models were developed at the Division of Clinical Pharmacology of the University Hospital in Lausanne, Switzerland.

Ethical considerations (aims A,B and C)

The *in vivo* studies were performed according to the WHO guidelines for monitoring malaria treatment [90]. All the applied protocols were approved by the ethics committee of the two cantons of Basel (Ethikkommission beider Basel) and the responsible local authorities (Medical Research Coordination Committee of the National Institute for Medical Research in Tanzania and National Ethics Committee for Health Research in Cambodia). Blood samples were obtained after written informed consent in the local language (Khmer or Swahili) from the participants or their responsible guardians.

Study design (aims A,B and C)

Between 2007 and 2008, three *in vivo* studies were performed in Cambodia and Tanzania. Patients reporting with fever (axillary temperature above 37.5 °C at admission or history of fever in the last 48 hours) associated with an infection with *Plasmodium falciparum* (between 1000 and 100’000 asexual parasites per µl blood) were asked to participate in the studies. Exclusion criteria were age below the age for which the studied antimalarials were approved (1 year for artemether–lumefantrine, 2 years for artesunate–mefloquine and 6 years for dihydroartemisinine–piperaquine), pregnancy (in Cambodia only), severe malnutrition (weight-for-height below –2 standard deviations of the National Center for Health Statistics (NCHS)/WHO reference values in children, body mass index below 16 in adults [91]), hemoglobin less than 5 g/dl, severe co-infection with other diseases requiring hospitalization, ongoing treatment with antibiotics with antimalarial activity, antimalarial treatment in the

previous 28 days, and signs of severe malaria (e.g. cerebral malaria, severe aneamia, renal failure, pulmonary oedema, hypoglyceamia, circulatory collapse/shock [90]). The patients were treated according to the national guidelines in the respective countries. In Cambodia, 64 patients were recruited for the treatment with artesunate–mefloquine and 61 for the treatment with dihydroartemisinine–piperaquine. In Tanzania, 150 were treated with artemether–lumefantrine. Patients willing to participate in the study were seen on several follow-up visits (Days 1, 2, 3, 7, 14, 28, and 42 in Tanzania; Days 1, 2, 3, 7, 14, 21, 28, 35 and 42 in Cambodia). On every visit the patients were asked a few questions about their health condition and the history of the disease, axillary temperature and respiratory rate were measured and 0.2 mL of blood taken by fingerprick to assess parasite species and density. Samples for the pharmacokinetic analysis were taken before treatment (about 5 mL in order to have sufficient material also for the pharmacogenetic analysis) and at several time points during and after treatment (about 1 mL each time), e.g. on Days 1, 2 and 7 in all studies and in Cambodia also 1 hour after the first dose and on Day 14. Age, sex, weight, height, smoking status, presence of known renal or hepatic disease, co-medication, actual times of dose intake and blood sampling, concurrent intake of food with drug administration were recorded.

Laboratory analysis (aimes A and B)

To accommodate aim A, a broad-range LC–MS/MS assay covering 14 of the currently in-use antimalarial and their metabolites was (CHAPTER 1). The performance of the assay was investigated in three *in vivo* studies in Tanzania and Cambodia where baseline samples from patients with *Plasmodium falciparum* malaria recruited in the study were analyzed (CHAPTER 4 and CHAPTER 5) and the pharmacokinetic profile under treatment was assessed (CHAPTER 6).

To address aim B, the pharmacogenetic profile of the patients from the three *in vivo* studies was analyzed by direct sequencing of genomic DNA (CHAPTER 2). Furthermore, a DNA microarray was developed and the results obtained were compared with those from the sequencing (CHAPTER 3).

Data analysis (aim C)

To achieve aim C, the data from the above mentioned studies were included in population pharmacokinetic models for the drugs used in the *in vivo* studies, assessing the pharmacogenetic profile as the main covariate (CHAPTER 6).

CHAPTER 1

A single LC–tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma

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Abstract

Among the various determinants of treatment response, the achievement of sufficient blood levels is essential for curing malaria. For helping us at improving our current understanding of antimalarial drugs pharmacokinetics, efficacy and toxicity, we have developed a liquid chromatography–tandem mass spectrometry method (LC–MS/MS) requiring 200 µl of plasma for the simultaneous determination of 14 antimalarial drugs and their metabolites which are the components of the current first-line combination treatments for malaria (artemether, artesunate, dihydroartemisinin, amodiaquine, N-desethyl-amodiaquine, lumefantrine, desbutyl-lumefantrine, piperaquine, pyronaridine, mefloquine, chloroquine, quinine, pyrimethamine and sulfadoxine). Plasma is purified by a combination of protein precipitation, evaporation and reconstitution in methanol/ammonium formate 20 mM (pH 4.0) 1:1. Reverse-phase chromatographic separation of antimalarial drugs is obtained using a gradient elution of 20 mM ammonium formate and acetonitrile both containing 0.5% formic acid, followed by rinsing and re-equilibration to the initial solvent composition up to 21 min. Analyte quantification, using matrix-matched calibration samples, is performed by electro-spray ionization–triple quadrupole mass spectrometry by selected reaction monitoring detection in the positive mode. The method was validated according to FDA recommendations, including assessment of extraction yield, matrix effect variability, overall process efficiency, standard addition experiments as well as antimalarials short- and long-term stability in plasma. The reactivity of endoperoxide-containing antimalarials in the presence of hemolysis was tested both *in vitro* and on malaria patients samples. With this method, signal intensity of artemisinin decreased by about 20% in the presence of 0.2% hemolysed red-blood cells in plasma, whereas its derivatives were essentially not affected. The method is precise (inter-day CV%: 3.1–12.6%) and sensitive (lower limits of quantification 0.15–3.0 and 0.75–5 ng/ml for basic/neutral antimalarials and artemisinin derivatives, respectively). This is the first broad-range LC–MS/MS assay covering the currently in-use antimalarials. It is an improvement over previous methods in terms of convenience (a single extraction procedure for 14 major antimalarials and metabolites reducing significantly the analytical time), sensitivity, selectivity and throughput. While its main limitation is investment costs for the equipment, plasma samples can be collected in the field and kept at 4 °C for up to 48 h before storage at −80 °C. It is suited to detecting the presence of drug in subjects for screening purposes and quantifying drug exposure after treatment. It may contribute to filling the current knowledge gaps in the pharmacokinetics/pharmacodynamics relationships of antimalarials and better define the therapeutic dose ranges in different patient populations.

1. Introduction

In the past few years, the therapeutic armoury against malaria has changed dramatically from the traditional few, failing single-agent treatments to an unprecedented wealth of antimalarial products, basically combinations of artemisinin derivatives with older and newer quinolines available either as fixed-dose (artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloquine, dihydroartemisinin/piperaquine), co-blistered or individually formulated products (artemisinin-based combination therapies, ACTs) [4,29,92,93]. More drugs are in the Research & Development pipeline.

However, essential data on the disposition of the products in use are lacking especially in children with malaria [40]. In addition, the majority of these drugs have not been developed with respect to the selection of doses and dosing regimens based on stringent pharmacokinetics–pharmacodynamics relationships. This means that we have inadequate information on the appropriate dosing and levels they generate in patients for the currently recommended regimens. As a consequence, some patients or patient categories may be underdosed (resulting in treatment failures and promoting parasite resistance) or overdosed (a cause of toxicity).

One of the obstacles to obtaining this information has been the lack of sensitive, reliable, robust analytical methodologies. Ideally, the assay should be able to extract and detect several drugs and their main metabolites simultaneously with no limitation for drug classes. The availability of such techniques would result in an overall reduction in analytical time and costs while allowing screening and monitoring drug intake in clinical and epidemiological studies.

High performance liquid chromatography (HPLC) is widely used and relatively economical but has limitations—mostly related to the choice of the detector relative to the chemical class of the drug, sensitivity and throughput, as frequently encountered with the unspecific UV or spectrofluorimetic detection [94-98]. Few of the methods developed so far aim at detecting a range of antimalarials, including an HPLC-UV method for sulfadoxine, pyrimethamine, chloroquine, amodiaquine and desethylamodiaquine from whole blood using liquid–liquid extraction, reversed-phase chromatography and UV detection [98], and a HPLC-ECD method with simultaneous extraction and quantification with an electrochemical detector operating in the reductive mode for artesunate/dihydroartemisinin and mefloquine [99] or oxidative mode

for amodiaquine [100]. Equally limited is the experience with liquid chromatography coupled to mass (LC–MS) or triple stage tandem mass spectrometry (LC–MS/MS), mostly aimed at detecting a single or few antimalarials generally belonging to a single chemical class [58,101-130].

Triple stage mass detection qualifies for the measurement of arrays of structurally unrelated antimalarial agents as well as their metabolites in a single analytical run.
Here, we describe a sensitive LC–MS/MS method for the simultaneous analysis in a small volume of plasma of the major antimalarial agents currently used as drug combinations (artemether, artesunate, lumefantrine, piperaquine, pyronaridine, amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine and pyrimethamine) as well as some of their active metabolites (dihydroartemisinin, desbutyl-lumefantrine, desethyl-amodiaquine).

2. Experimental

2.1. Chemicals and reagents

Dihydroartemisinin (DHA) and artesunate (AS), arthemether (AM), lumefantrine (LF) and desbutyl-lumefantrine (DLF), mefloquine hydrochloride (MF), sulfadoxine (SD), pyrimethamine (PM), piperaquine phosphate (PQ) were kindly provided by Abbott AG (Liestal, Switzerland), Novartis Pharma SAS (Rueil-Malmaison, France), Novartis Pharma AG (Basel Switzerland), Roche (Hoffmann-la Roche Pharma Research, Basel, Switzerland), and Sigma-tau (Pomezia, Roma, Italy), respectively. Pyronaridine (PY) was offered by Dr. Sergio Wittlin (Swiss Tropical Institute, Basel) and desethyl-amodiaquine (DAQ) standard was a gift from Prof. Giovanni Di Perri (Sezione di Malattie Infettive, Università Degli Studi di Torino, Italy) and RCC Ltd. (Füllinsdorf, Switzerland) *via* the Antimalarial Drug Resistance, Global Malaria Program (Dr. Pascal Ringwald, WHO, Geneva). Chloroquine diphosphate (CQ), amodiaquine dihydrochloride dihydrate (AQ), quinine hydrochloride dihydrate (Q) were purchased from Sigma–Aldrich (Schnelldorf, Germany). The internal standards (I.S.) artemisinin (ART) and trimipramine-D_3 (TPR) were obtained from Sigma–Aldrich (Schnelldorf, Germany) and Cerilliant Corporation (Round Rock, TX, USA), respectively. Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (MeCN) purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate

was purchased from Fluka (Buchs, Switzerland). Formic acid (98%) and methanol for chromatography Lichrosolv® (MeOH) were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Blank plasma used for the assessment of matrix effect and for the preparation of calibration and control samples were isolated (1850 × *g*, 10 min, +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from blood withdrawn from patients with Vaquez Disease.

2.2. Equipment

The liquid chromatography system consisted of Rheos 2200 quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fischer Scientific Inc., Waltham, MA). Separations were done on a 2.1 mm × 50 mm Atlantis® dC18 3 µm analytical column (Waters, Milford, MA, USA) and placed in a thermostated column heater at 25 °C (Croco-Cil, Cluzeau Info Laboratory, Courbevoie, France). The chromatographic system was coupled to a triple stage quadrupole (TSQ) Quantum Ion Max mass spectrometer (MS) from Thermo Fischer Scientific, Inc., equipped with an electro-spray ionization (ESI) interface and operated with Xcalibur 2.0 software (Thermo Fischer Scientific Inc., Waltham, MA).

2.3. Solutions

2.3.1. Mobile phase and solution for extracts reconstitution

The mobile phase used for chromatography was 20 mM ammonium formate in ultrapure water (buffer A) and acetonitrile (solvent B), both containing 0.5% formic acid (FA). A mixture of MeOH/20 mM ammonium formate 1:1 (volume/volume, v/v), adjusted to pH 4.0 with FA was used for the reconstitution of extracted plasma samples prior to their LC–MS/MS analysis. Solvents were regularly prepared for each series of analysis and stored in the dark at +4 °C prior use.

2.3.2. Internal standard, calibration standards and quality controls (QCs) solutions

A stock solution of trimipramine-D_3 (TPR) 1 μg/ml in MeOH and a stock solution of artemisinin 100 μg/ml were diluted with MeOH to obtain a working I.S. solution at 100 and 2000 ng/ml, respectively.

Standard stock solutions of antimalarial drugs (depicted in Figure 1) were prepared in solvents indicated in Table 1. The stock solutions were stored in polypropylene flasks with caps tightly wrapped and protected from light and stored at +4 °C. Appropriate volumes of stock solutions were serially diluted with H_2O/MeOH 3:1 as indicated in Table 1 to obtain a single working solution of antimalarials at concentrations ranging from 0.006 to 100 μg/ml. Finally this working solution was diluted 1:20 (i.e. 5%) with blank plasma to obtain the calibration samples from 0.3 to 5000.0 ng/ml and the corresponding four quality control (low (L), intermediate (I), medium (M) and high (H) QCs) samples from 0.9 to 3750 ng/ml. All solutions were prepared according to the recommendations on bioanalytical methods validation stating that the total added volume must be ≤10% of the biological sample volume [131]. The calibration standard and control plasma samples were stored as 200 μl-aliquots at −80 °C prior to analysis.

Table 1. Preparation of calibration and QC samples.

Drug	**Stock solution solvent**	**Calibration range (obtained by dilution of working solution with plasma 1/20) (ng/ml)**	**QCs controls (ng/ml)**
Piperaquine (PQ)	H_2O	2–4000	6, 30, 300, 3000
Desethyl-amodiaquine (DAQ)	H_2O	0.3–600	0.9, 4.5, 45, 450
Chloroquine (CQ)	H_2O	2.5–5000	7.5, 37.5, 375, 3750
Amodiaquine (AQ)	H_2O	0.3–600	0.9, 4.5, 45, 450
Pyronaridine (PY)	MeOH + 0.5% FA	1–1000	3, 7.5, 75, 750
Quinine (Q)	H_2O	2.5–5000	7.5, 37.5, 375, 3750
Sulfadoxine (SD)	MeOH	0.5–1000	1.5, 7.5, 75, 750
Pyrimethamine (PM)	MeOH	0.5–1000	1.5, 7.5, 75, 750
Mefloquine (MF)	MeOH	2.5–5000	7.5, 37.5, 375, 3750
Dihydroartemisinin (DHA)	MeOH	1–2000	3, 15, 150, 1500
Artesunate (AS)	MeOH	2–2000	6, 15, 150, 1500
Artemether (AM)	MeOH	5–2000	15, 150, 1500
Lumefantrine (LF)	MeOH + 0.5% FA	4–4000	12, 30, 300, 3000
Desbutyl-lumefantrine (DLF)	MeOH + 0.5% FA	4–4000	12, 30, 300, 3000

All stock solutions were mixed together to give a single working solution.

Table 2. Instrument method for the LC–MS/MS analysis for antimalarials/metabolites.

Drug	Parent (*m/z*)	Product (*m/z*)	CE (eV)	Tube lens (V)	Mean RT (min)	Segment	Polarity mode
Piperaquine (PQ)	535.20	287.90	37	68.33	3.9	1	Positive
Desethyl-amodiaquine (DAQ)	328.30	283.00	26	66.08	4.5	1	Positive
Chloroquine (CQ)	320.20	247.00	26	72.08	4.6	1	Positive
Amodiaquine (AQ)	356.30	283.10	26	73.33	4.7	1	Positive
Pyronaridine (PY)	518.20	447.10	20	75.09	4.8	1	Positive
Quinine (Q)	325.10	307.10	32	88.85	5.0	1	Positive
Sulfadoxine (SD)	311.00	155.90	28	77.34	6.1	1	Positive
Pyrimethamine (PM)	249.10	233.00	38	103.00	6.5	1	Positive
Trimipramine-D_3 (I.S.1)	298.21	103.20	26	71.08	8.8	2	Positive
Mefloquine (MF)	379.00	361.00	31	37.00	9.0	2	Positive
Dihydroartemisinin (DHA)	221.00	163.00	19	61.32	9.3	2	Positive
Artesunate (AS)	221.00	163.00	19	61.32	10.6	2	Positive
Artemisinin (I.S.2)	283.00	247.20	18	41.30	11.1	2	Positive
Artemether (AM)	221.00	163.00	19	61.32	13.0	2	Positive
Desbutyl-lumefantrine (DLF)	472.10	454.00	22	84.10	13.1	2	Positive
Lumefantrine (LF)	530.10	512.10	29	110.37	15.9	2	Positive

CE = Collision energy, RT = retention time, MS acquisition time (min) = 17.0, Segment 1 = 0–7.5 min, Segment 2 = 7.5–17.0 min, Q2 collision gas pressure (mTorr) = 1.00

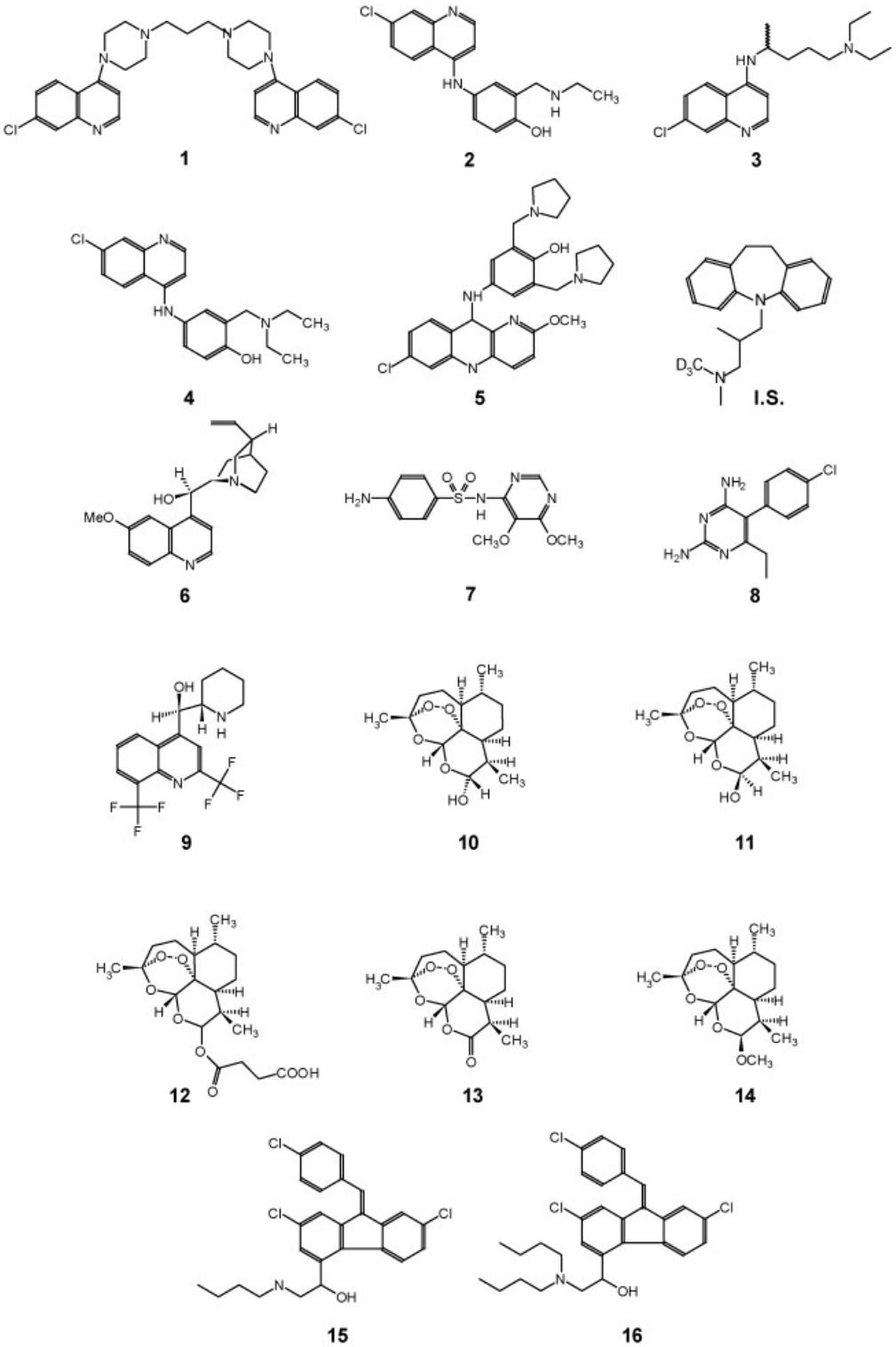

Figure 1. Chemical structures of antimalarials, some of their metabolites, and the I.S. trimipramine (D_3) (1, piperaquine, PQ; 2, N-desethyl-amodiaquine, DAQ; 3, chloroquine, CQ; 4, amodiaquine, AQ; 5, pyronaridine, PY; 6, quinine, Q; 7, sulfadoxine, S; 8, pyrimethamine, PM; 9, mefloquine (only one stereo-isomer is shown), MF; 10, α-dihydroartemisinin, α-DHA; 11, β-dihydroartemisinin, β-DHA; 12, artesunate, AS; 13, artemisinin, AS; 14, artemether, AM; 15, desbutyl-lumefantrine, DLF; 16, lumefantrine, LF). Shown according to order of elution during the chromatographic separation (Figure 2a and b).

2.4. LC–MS/MS conditions

The mobile phase was delivered using a stepwise gradient elution program: 2% of acetonitrile (solvent B) at 0 min, 59% of B at 10 min and 68% of B at 17.0 min with a flow rate of 0.3 ml/min. The second part of the run included 4 min of rinsing (68% B with 0.4 ml/min) and a re-equilibration step to the initial solvent up to 21 min. Moreover, three blank samples were analysed immediately after the high calibration level to eliminate potential memory effect from basic drugs (see below). The thermostated column heater was set at 25 °C and the autosampler was maintained at 10 °C. The injection volume was 10 µl.

The LC–MS/MS conditions were as follows: ESI in positive mode; capillary temperature 350 °C; in source collision induced dissociation (CID) 10 V; tube lens range voltage 37–110.37 V; spray voltage 4 kV and sheath and auxiliary gas (nitrogen); sheath gas pressure 35 psi and auxiliary gas pressure 10 (arbitrary units), respectively. The Q2 collision gas (argon) pressure was 1 mTorr (0.13 Pa). MS acquired in selected reaction monitoring (SRM). The determination of optimal potential settings and MS/MS transitions were chosen by direct infusion of each compound solution separately into the MS/MS detector at a concentration of 1 µg/ml in 1:1 of MeOH/buffer A. The selected *m/z* transitions and the collision energy for each analyte and I.S. are reported in Table 2.

The first (Q1) and third (Q3) quadrupoles were set at 1 amu mass resolution (full-width half-maximum = 0.7). Scan time and scan width were 0.05 s and 0.5 *m/z*, respectively, and each chromatographic peak was the result of at least 15 scans. MS acquisitions were done in centroid mode. Two distinct segments of data acquisition were programmed in the positive mode: the first acquisition segment from 0 to 7.5 min, and a second segment from 7.5 to 17.0 min. Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of Xcalibur software package (version 2.0) (ThermoQuest, Thermo Fischer Scientific Inc., Waltham, MA).

2.4.1. Blood samples collection for antimalarial drugs determination

Blood samples were obtained from patients treated with ACTs for uncomplicated falciparum malaria as part of a multicountry pharmacokinetic study approved by the National Ethics Committee for Health Research in Cambodia and the National Institute for Medical Research in Tanzania. Written informed consent was obtained from all patients. Approximately 1 ml of

venous blood was collected from patient using Vacutainer™ tubes (BD, Franklin Lakes, NJ, USA) just before treatment initiation on Day 0, Days 1 and 2 (i.e. trough levels), and thereafter on Day 7. In Cambodia, two additional samples were taken on Day 0 approximately 1 h after the first dose, and on Day 14. Samples were gently inverted 8–10 times for careful homogenization with the anticoagulant (EDTA). A small volume of whole blood (200 µl) was transferred into a 1.8 ml Nunc or Greiner CryoTube and placed in liquid nitrogen at −190 °C in Cambodia and in a −80 °C freezer in Tanzania. The remaining whole blood was centrifuged at ambient temperature for 15 min with a manual centrifuge in Cambodia, and for 10 min in an electrical centrifuge (approximately 1650 × *g*) in Tanzania. The plasma and remaining red-blood cells were placed in liquid nitrogen at −190 °C or in a −80 °C freezer. In Cambodia, the samples were transferred in a −80 °C freezer within 1 week. Samples from both countries were subsequently shipped on dry ice to the CHUV laboratory in Switzerland and stored at −80 °C prior to analysis.

2.4.2. Selection of the reconstitution solvent

During the initial development of the method, the following solvents were evaluated for the reconstitution of the plasma extract residue: MeOH/ammonium formate 20 mM (pH 4.0) 60:40, 50:50 30:70, 20:80. Among the solvent mixtures tested, the 50:50 provided the best chromatographic behavior and peaks area intensity overall for the 14 antimalarials/ metabolites and the two I.S., and was consequently used thereafter throughout the method validation and subsequent patient samples determination.

2.4.3. Plasma sample extraction procedure

Plasma aliquots (200 µl) were mixed with a 100 µl-volume of I.S. solution (100 ng/ml trimipramine-D_3 and 2000 ng/ml artemisinin) and vortex-mixed. The resulting sample was subjected to protein precipitation with acetonitrile (700 µl) and carefully vortexed-mixed. The mixture was finally centrifuged at 4 °C for 10 min at 20,000 × *g* (14,000 rpm) on a benchtop Hettich® Centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). The supernatant (900 µl) was transferred into a polypropylene tube and evaporated to dryness under nitrogen at room temperature. The solid residue was reconstituted in 150 µl MeOH/ammonium formate 20 mM 1:1 adjusted to pH 4.0 with formic acid and vortex-mixed and centrifuged again under the above-mentioned conditions. The 130 µl supernatent was

introduced into 200 µl glass HPLC microvials maintained at +10 °C in the autosampler rack during the entire LC–MS/MS analysis.

2.5. Quantification

2.5.1. Calibration curves

Quantitative analysis of the 14 antimalarials/metabolites was performed using the internal standard (I.S.1 = trimipramine-D_3) method. Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared with plasma isolated from outdated transfusion blood (see Sections Sections 2.6.4 and 3.5, matrix effect).

Eight-point calibration standard curves were calculated and fitted either by $1/x$ or $1/x^2$ weighted quadratic regression, or quadratic log–log regression, when appropriate, of the peak-area ratio of antimalarials/metabolites to I.S., versus the concentrations of the respective antimalarials/metabolites to I.S. in each standard sample. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The calibration for the 14 antimalarials/ metabolites was established over the range reported in Table 1. The standard curve was chosen to cover the range of concentrations expected in patients.

2.6. Analytical method validation

The method validation procedure was based on the recommendations published on-line by the Food and Drugs Administration (FDA) [131] as well as on the recommendations of the Conference Report of the Washington Conference on “Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies” [132] and the Arlington Workshop “Bioanalytical Methods Validation – A revisit with a Decade of Progress” [133]. More recent recommendations from Matuzewski et al. were also considered [134,135].

2.6.1. Accuracy and precision

Replicate analysis ($n = 6$) of quality control samples at 4 concentrations (low (L), intermediate (I) medium (M) high (H), see Table 3) were used for the intra-assay precision and accuracy determination. Of importance, the concentration selected for the low (L) QC sample corresponds to 3 times the respective lower limits of quantification (i.e. the lower calibration level) kept in the finalized method, in accordance to the FDA recommendations [131]. For artemether, no intermediate QC control samples were used. The four concentrations were chosen to encompass the whole range of the calibration curve corresponding to the drug levels anticipated to occur in most patient samples. Inter-assay accuracy and precision were determined by repeated analysis performed on six different occasions. The concentration in each sample was determined using calibration standards prepared on the same day. The precision was calculated as the coefficient of variation (CV%) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy was calculated as the bias or percentage deviation between nominal and measured concentration.

During the initial routine analysis of patient samples, duplicate control samples at three concentration levels (I, M and H) were assayed. To comply with FDA recommendations [131] an additional QC at a concentration corresponding to 3 times the LOQs, designated low (L) QC sample, was thereafter added (see Table 3). The analytical series were considered valid and accepted only if the percentage of deviation (bias) between theoretical and back-calculated (experimental) concentrations for each calibration level and quality control samples were within ±15%, and <20% at the limit of quantification (defined as the lowest calibrator).

Table 3. Precision and accuracy of the assay for antimalarials/metabolites in human plasma with QC samples at low (L), intermediate (I), medium (M) and high (H) concentrations.

	Nominal concentration (ng/ml)	**Intra-assay (*n* = 6)**				**Inter-assay (*n* = 6)**			
		Concentration found (ng/ml)	**SD±**	**Precision CV %**	**Accuracy bias %**	**Concentration found (ng/ml)**	**SD±**	**Precision CV %**	**Accuracy bias %**
Piperaquine	6	6.2	0.5	7.9	3.7	5.8	0.4	6.8	−3.9
	30	33.5	2.5	7.3	11.6	29.4	3.0	10.2	−2.1
	300	333.5	16.8	5.0	11.2	307.7	22.1	7.2	2.6
	3000	3050.0	156.5	5.1	1.7	2913.7	188.3	6.5	−2.9
Desethyl-amodiaquine	0.9	1.0	0.1	5.9	6.0	0.9	0.0	3.7	1.9
	4.5	4.6	0.3	7.0	2.5	4.3	0.2	4.2	−3.9
	45	49.8	2.4	4.7	10.7	46.0	2.3	4.9	2.3
	450	458.9	38.5	8.4	2.0	422.8	29.7	7.0	−6.1
Chloroquine	7.5	7.1	0.3	4.8	−5.1	7.2	0.4	5.6	−3.8
	37.5	36.9	1.9	5.3	−1.7	35.2	1.4	3.9	−6.0
	375	422.2	23.5	5.6	12.6	398.4	19.4	4.9	6.2
	3750	3752.4	327.2	8.7	0.1	3519.5	286.2	8.1	−6.1
Amodiaquine	0.9	0.9	0.1	7.1	2.7	0.9	0.0	4.3	−2.1
	4.5	4.6	0.2	4.6	1.7	4.3	0.2	5.0	−4.5
	45	48.1	2.7	5.6	6.9	45.7	1.4	3.1	1.5
	450	456.8	12.7	2.8	1.5	434.5	42.4	9.8	−3.4

Table 3. Continued.

	Nominal concentration (ng/ml)	Intra-assay (*n* = 6)				Inter-assay (*n* = 6)			
		Concentration found (ng/ml)	SD±	Precision CV %	Accuracy bias %	Concentration found (ng/ml)	SD±	Precision CV %	Accuracy bias %
Pyronaridine	3	3.1	0.3	9.3	2.3	3.0	0.1	3.9	1.1
	7.5	7.4	0.6	8.3	−1.4	6.8	0.6	8.1	−9.0
	75	73.3	3.5	4.8	−2.3	73.7	3.3	4.5	−1.7
	750	775.6	35.2	4.5	3.4	748.5	63.6	8.5	−0.2
Quinine	7.5	7.6	0.6	7.3	1.0	7.2	0.3	3.7	−3.7
	37.5	39.6	2.6	6.7	5.6	35.4	2.7	7.7	−5.6
	375	416.6	17.0	4.1	11.1	381.4	26.8	7.0	1.7
	3750	3646.7	247.6	6.8	−2.8	3598.4	340.0	9.4	−4.0
Sulfadoxine	1.5	1.5	0.1	6.2	−0.5	1.5	0.1	6.0	−1.0
	7.5	7.6	0.5	6.3	0.7	7.2	0.5	6.8	−4.3
	75	76.8	4.3	5.6	2.5	76.6	4.1	5.4	2.1
	750	766.3	48.7	6.4	2.2	717.9	64.7	9.0	−4.3
Pyrimethamine	1.5	1.5	0.1	5.9	2.4	1.5	0.1	3.7	−1.2
	7.5	7.6	0.3	3.6	1.6	7.4	0.2	3.2	−0.9
	75	76.1	3.6	4.7	1.4	75.4	4.1	5.4	0.6
	750	796.1	20.3	2.6	6.1	731.3	30.5	4.2	−2.5

Table 3. Continued.

	Nominal concentration (ng/ml)	Intra-assay (*n* = 6)				Inter-assay (*n* = 6)			
		Concentration found (ng/ml)	SD±	Precision CV %	Accuracy bias %	Concentration found (ng/ml)	SD±	Precision CV %	Accuracy bias %
Mefloquine	7.5	7.4	1.0	13.3	−1.7	7.0	0.3	4.9	−6.2
	37.5	37.5	1.7	4.4	0.1	35.8	1.7	4.6	−4.5
	375	399.5	11.7	2.9	6.5	386.5	24.2	6.3	3.1
	3750	3775.0	90.5	2.4	0.7	3570.0	203.8	5.7	−4.8
Dihydroartemisinin	3	3.0	0.4	13.4	1.3	3.0	0.1	5.0	−0.1
	15	14.5	1.3	9.2	−3.6	14.9	0.9	5.9	−0.7
	150	162.0	8.6	5.3	8.0	147.4	8.0	5.4	−1.7
	1500	1643.0	65.1	4.0	9.5	1483.7	107.2	7.2	−1.1
Artesunate	6	5.8	0.4	6.6	−3.8	5.6	0.5	9.2	−7.0
	15	13.6	0.9	6.2	−9.1	13.8	0.8	5.6	−7.9
	150	147.4	4.2	2.8	−1.7	149.9	8.1	5.4	0.0
	1500	1580.5	96.3	6.1	5.4	1521.5	91.5	6.0	1.4
Artemether	15	14.4	1.7	11.8	−3.9	15.6	1.0	6.5	3.8
	150	155.2	17.6	11.4	3.5	152.1	9.1	6.0	1.4
	1500	1525.0	108.6	7.1	1.7	1467.4	120.0	8.2	−2.2

Table 3. Continued.

	Nominal concentration (ng/ml)	Intra-assay (*n* = 6)				Inter-assay (*n* = 6)			
		Concentration found (ng/ml)	SD±	Precision CV %	Accuracy bias %	Concentration found (ng/ml)	SD±	Precision CV %	Accuracy bias %
Desbutyl-lumefantrine	12	11.3	1.0	9.0	−5.4	11.3	0.8	6.8	−5.8
	30	29.4	4.2	14.3	−2.1	29.1	3.0	10.2	−3.1
	300	329.7	17.9	5.4	9.9	292.6	26.8	9.2	−2.5
	3000	3164.4	451.1	14.3	5.5	2909.7	367.4	12.6	−3.0
Lumefantrine	12	12.7	0.7	5.7	5.6	11.3	1.0	8.7	−5.4
	30	33.2	1.5	4.6	10.8	29.1	2.3	7.9	−2.9
	300	324.2	16.1	5.0	8.1	303.2	31.7	10.4	1.1
	3000	3390.9	267.0	7.9	13.0	3104.0	354.7	11.4	3.5

2.6.2. Lower limit of quantification and limit of detection

The lowest levels chosen for calibration curves were selected initially to reflect the lowest – clinically relevant – concentrations expected be occur in patients, based on published pharmacokinetic data. However, it was observed that our LC–MS/MS instrument was able to attain far higher sensitivity levels. Thus, LOQ values have been determined by establishing calibration curves using for the lowest calibration samples standard serial dilutions (3/4 and ½) of the low standard samples of our first calibration curves and were analysed in triplicates (i.e. those samples were used and *integrated* for the establishment of the calibration curves). Back-calculated values of the lowest calibration samples with a bias and CV% below ±20%, enables to determine the LOQ values, in accordance with the documents mentioned above [131,132]. The LLOQ concentrations were finally selected as the lowest levels of the calibration curves established during the analytical method validation. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level of a blank preparation.

2.6.3. Stability of antimalarials/metabolites

Stability studies of antimalarials/metabolites included:

(a) Stability of plasma spiked with antimalarials/metabolites kept at room temperature (RT) and in the fridge at +4 °C: the concentrations of antimalarials/metabolites were measured immediately after preparation and after being left at room temperature (RT) and at +4 °C up to 48 h. Antimalarials/metabolites concentrations variations were expressed as a percentage of the initial concentration measured at $T = 0$.

(b) Stability of plasma samples after multiple freeze-thaw cycles: QCs at I, M and H levels of antimalarials/metabolites underwent three freeze-thaw cycles. Frozen samples were allowed to thaw at room temperature for 3 h and were subsequently refrozen during approximately 24 h. Antimalarials/metabolites levels were measured in aliquots from the three consecutive freeze-thaw cycles.

In all experiments, room temperature corresponds to the usual temperature of 24–25 °C of the Hospital Laboratory at CHUV, Lausanne, Switzerland.

2.6.4. Matrix effect, extraction yield and overall recovery

In the initial step of method validation, the matrix effect was examined qualitatively by the simultaneous post-column infusion of the 14 antimalarials/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of 6 different blank plasma extracts. The standard solution of all analytes at medium QC concentrations, containing also 100 ng/ml for TPR (I.S.1) and 2000 ng/ml for ART (I.S.2), was infused at a flow rate of 10 µl/min during the chromatographic analysis of blank plasma extracts from 6 different sources. The chromatographic signals of each selected MS/MS transition were examined to ascertain that no signal perturbation (drift or shift) of the MS/MS signal was present at the analyte's retention time.

Subsequently, the quantitative determination of the matrix effect, and the determination of its variability were also assessed. Three series of QC samples at I, M, and H in duplicates were prepared as followed:

(A) Pure standard solutions samples in MeOH/buffer A 1:1 directly injected onto column.
(B) Plasma extract samples from 6 different sources, spiked with antimalarials/metabolites and I.S after extraction.
(C) Plasma samples from 6 different sources (same as in B) spiked with drug standards solution and I.S. before extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effect) was assessed by comparing the absolute peak areas of analytes either solubilised in MeOH-buffer (ammonium formate 20 mM, pH adjusted to 4.0 with formic acid) 1:1 (*A*), or added after (*B*) and before (*C*) extraction of 6 different batches of plasma, based on the recommendations proposed by Matuszeski et al. [135].

The *extraction yield* of antimalarials/metabolites and I.S. were calculated as the absolute peak-area response in processed plasma samples spiked with drugs *before* extraction I expressed as the percentage of the response of the same amount of drugs added into blank plasma extracts *after* the extraction procedure (*B*) (*C*/*B* ratio in %). The *matrix effect* was assessed as the ratio of the peak areas of analytes added into blank plasma extracts *after* the extraction procedure (*B*) to the peak areas of pure analytes solubilised in MeOH–ammonium

formate 20 mM pH 4.0 1:1 (*A*) (*B*/*A* ratio in %). The *overall recovery* of antimalarials/metabolites and I.S. was calculated as the ratio of absolute peak-area response of antimalarials either in processed plasma samples spiked with drugs *before* extraction (*C*) to the peak areas of analytes solubilised in MeOH–ammonium formate 20 mM pH 4.0 1:1 (*A*) (*C*/*A* ratio). Recovery studies were done with plasma from 6 different sources spiked with drugs at the concentrations reported in Table 5.

The results normalized with the signal of I.S. (i.e. B2 and C2), used as an index of actual injection volume are also reported in Table 5.

Finally, we have performed further *standard-addition* experiments using 16 patients samples (8 from Tanzania and 8 from Cambodia, on Coartem® and MF/AS ACTs regimens, respectively). Patients plasma samples were divided in two aliquots, one for direct analysis and the second was spiked with nominal amount of antimalarial drugs/ metabolites (diluted in plasma) prior to quantification. In the second aliquots, the experimental and expected concentrations (i.e. measured in patients in the first aliquot, *plus* added amount) were compared. The results are given in Table 6.

2.6.5. Analytical issues with endoperoxide antimalarials in hemolysed plasma samples

Recently, Lindegardh et al. reported the occurrence of analytical problems with an LC–MS/MS method for DHA and AS related to the compounds' potential reactivity with hemoglobin (Hb) and hemolytic products in clinical samples, and the deleterious impact of the presence of organic solvent during the extraction procedure [102]. In that context, we carried out the following additional experiments focusing specifically on endoperoxide-containing antimalarials:

(a) *Impact of the presence of organic solvent on the assay of endoperoxide drugs in hemolysed plasma*: Using our extraction procedure, we compared the intensity of ART (artemisinin = I.S. at 2000 ng/ml) either solubilized in MeOH (i.e. as in the I.S. solution used in our proposed extraction method (cf. Section 2.3.2) or in plasma, and then added to plasma in the absence (usual calibration) and in presence of 0.2% hemolysed red-blood cells (RBC) (of note, 0.2% RCB gives a dark orange appearance to plasma). The addition of 100 µl of ART solution (2000 ng/ml) to 200 µl of control

or hemolysed plasma yields a final ART concentration of 667 ng/ml. The solution of ART in plasma was prepared using a concentrated ART solution in MeOH:H_2O 1:1 further diluted 1/20 with plasma, yielding a MeOH content of 2.5% in the I.S. solution, and 0.6% in the final plasma sample. Plasma and methanolic working solutions of DHA, AS and AM were prepared in the same way and were also added to control plasma or hemolysed plasma (0.2% hemolysed RBC) to reach a final concentration of 500 ng/ml. Samples were vortex-mixed and processed according to the extraction method given in Section 2.4.3. The intensities of artemisinin derivatives in control and hemolysed plasma were compared with respect to the MeOH used for diluting working solutions.

(b) *Evaluation of extraction methodology*: We compared our extraction procedure (protein precipitation, supernatant evaporation and reconstitution in buffer/MeOH (Method A) cf. Section 2.4.3) with the method in Lindegardh et al. for DHA and AS (i.e. protein precipitation and direct supernatant injection, Method B) [102]. ART was added as a 100 µl methanolic solution (2000 ng/ml) to 200 µl plasma calibration samples. DHA, AS and AM calibration samples were prepared as reported in Section 2.3.2. The signal intensities for ART, DHA, AS and AM in plasma calibration samples were determined using both extraction methods in the absence (usual calibration) and in the presence of 0.2% hemolysed RBC. Signal intensities for endoperoxide antimalarials are expressed as the mean percentage of the calibration samples prepared in plasma with no RBC, used as control (100%). In addition, we also measured the signal intensity obtained for the I.S. trimipramine-D_3.

(c) *Experiments with malaria patients plasma samples*: While analysing clinical samples from malaria patients, we noticed that a few samples, some of them with overt hemolysis, showed a drop in the signal intensity (expressed as peak areas) of the I.S. ART in comparison to the calibration samples. Specifically, these very samples had been collected at least 18 h (mean 22 h) after the last AS intake and had therefore no detectable levels of AS and DHA left. Remnant plasma aliquots from these selected blank malaria samples ($n = 35$) were reanalyssed after the addition of DHA, AS and AM (500 ng/ml, prepared as in Section 2.3.2) using our proposed extraction methodology and the addition of ART 2000 ng/ml as I.S. The respective signal intensity of ART, DHA, AS and AM in these malaria samples were compared to those obtained after addition of the same solutions of DHA, AS and AM and the I.S. ART to 10 blank malaria patients samples without any signs of hemolysis, analysed in

duplicate, used as control (100%) (i.e.: hemolysis could be excluded in these malaria plasma samples because the signal intensity of the I.S. ART was the same as that measured in calibration levels analysed simultaneously).

2.6.6. Dilution effect

Some patient samples were found to contain drug concentrations exceeding the highest level of the calibration curve (see Table 1). To ascertain whether the dilution of these samples prior to a subsequent analysis could affect the accuracy of the drug determination, a blank plasma sample was spiked with antimalarials at a concentration exceeding by two-fold the highest calibration level. The sample was thereafter analysed in duplicate after a three-fold dilution to bring the concentration within the calibration range. Dilution was carried out with blank plasma. Calculated and expected concentrations were compared.

2.6.7. Selectivity

The assay selectivity was assessed by analysing extracts from ten batches of blank plasma extracts from different sources.

2.6.8. Memory effect

With instruments of increasing sensitivity, the carry-over or memory effect has emerged as a problem, potentially influencing the accuracy at low levels found at the end of the dosing period of observation. The carry-over effect was assessed by determining the peak area of analytes at the expected retention time in series of MeOH/buffer A 1:1 solution after the analysis of the highest calibration plasma sample. The signal was expressed as the percentage of the absolute signal in the highest calibrator.

3. Results and discussion

3.1. Chromatograms

The proposed method enables the simultaneous quantification by liquid chromatography coupled with tandem MS/MS in 200 µl plasma aliquots of most of the currently available and

newly introduced artemisinin-based antimalarial combinations and their active metabolites (Figure 1). A chromatographic profile of a calibration plasma sample containing all antimalarials/metabolites at concentrations corresponding to one fourth of the highest calibration levels is shown in Figure 2 (a and b) in the positive mode, during the 2 acquisition segments 0–7.5 and 7.5–17 min, respectively, using the Selected Monitoring Reaction Mode and the gradient program given in Section 2.4. The respective retention times of antimalarial drugs and the I.S. trimipramine-D_3 are reported in Table 2. The separation is satisfactory for all considered analytes. Importantly, amodiaquine and its active metabolite N-desethyl-amodiaquine are eluted at 4.51 and 4.75 min, respectively, and no reciprocal signal cross talk is observed at the chosen *m/z* transition at 328.3 → 283.0 *m/z* and 356.3 → 283.1 *m/z*, respectively.

Though all antimalarials/metabolites and the I.S. trimipramine-D_3 were eluted within 17 min, a relatively prolonged rinsing step of 4 min at a flow rate of 0.4 ml/min was introduced to eliminate the memory effect observed with the basic compounds in the initial set-up of the method. The rinsing step was followed by the column-conditioning step with the initial solvent composition (98/2 solvent A/solvent B) at a flow rate of 0.4 ml/min (3 min).

Figure 3 shows the signals at all selected *m/z* transitions when a single solution containing all antimalarials/metabolites/I.S. was continuously infused post-column directly into the MS/MS detector during the chromatographic analysis of six different blank plasma extracts. The signals at the *m/z* transition showed a remarkably similar pattern, with all traces being essentially superimposable. Even though no noticeable matrix effect (no drifts or shifts of the signals) was observed at the respective retention time of the antimalarials, metabolites and I.S. peaks (shown in the chromatographic profile) in this perfusion experiment, some matrix effects were, however, found as reported in the experiments below (see Section 3.5).

Figure 4 shows the chromatographic profiles of a plasma sample obtained from an 18-year-old female patient (TK010) from Tanzania receiving Coartem® (one tablet contains 20 mg arthemether and 80 mg lumefantrine, Novartis, Basel, Switzerland) divided in six doses of 4 tablets. The plasma level of artemether, DHA, desbutyl-lumefantrine and lumefantrine measured on Day 2, 9 h after last drug intake (i.e. fourth dose) were 17, 26, 80, and 9923 ng/ml, respectively. On Day 7, the levels of artemether and DHA were below the LOD while the long-acting drugs lumefantrine and its metabolite desbutyl-lumefantrine measured 106 h 35 min (ca 4.5 days) after the last drug intake (sixth dose) were 805 and 28 ng/ml,

respectively (chromatogram not shown). The chromatogram in Figure 5 was obtained from a 35-year-old male patient from Cambodia (CP046) on an ACT regimen of artesunate/mefloquine 600/1250 mg divided in 3 daily doses (200/500 mg on Days 0 and 1, and 200/250 mg on Day 2). The blood sample was taken 1 h 09 min after the first dose. Measured plasma concentrations were 134, 239 and 82 ng/ml for artesunate, DHA and mefloquine, respectively. Of note, the "blank" base-line plasma taken in this patient prior to the first dose administration was found to already contain 55 ng/ml of mefloquine, indicating that this patient had previously taken an antimalarial drug at his own initiative (chromatogram not shown).

Figure 6 shows the chromatographic profile from a plasma collected in a 46-year-old male patient from Tanzania (TK098) 12 h 47 min after his second dose of Coartem® showing a concentration of 7.1, 6.7, 16.1 and 3551 ng/ml of artemether, DHA, desbutyl-lumefantrine and lumefantrine, respectively. Interestingly, the analysis revealed that this patient had also previously taken quinine, sulfadoxine and pyrimethamine before the medical visit, with plasma levels of 1105, 22,899 and 4.9 ng/ml, respectively.

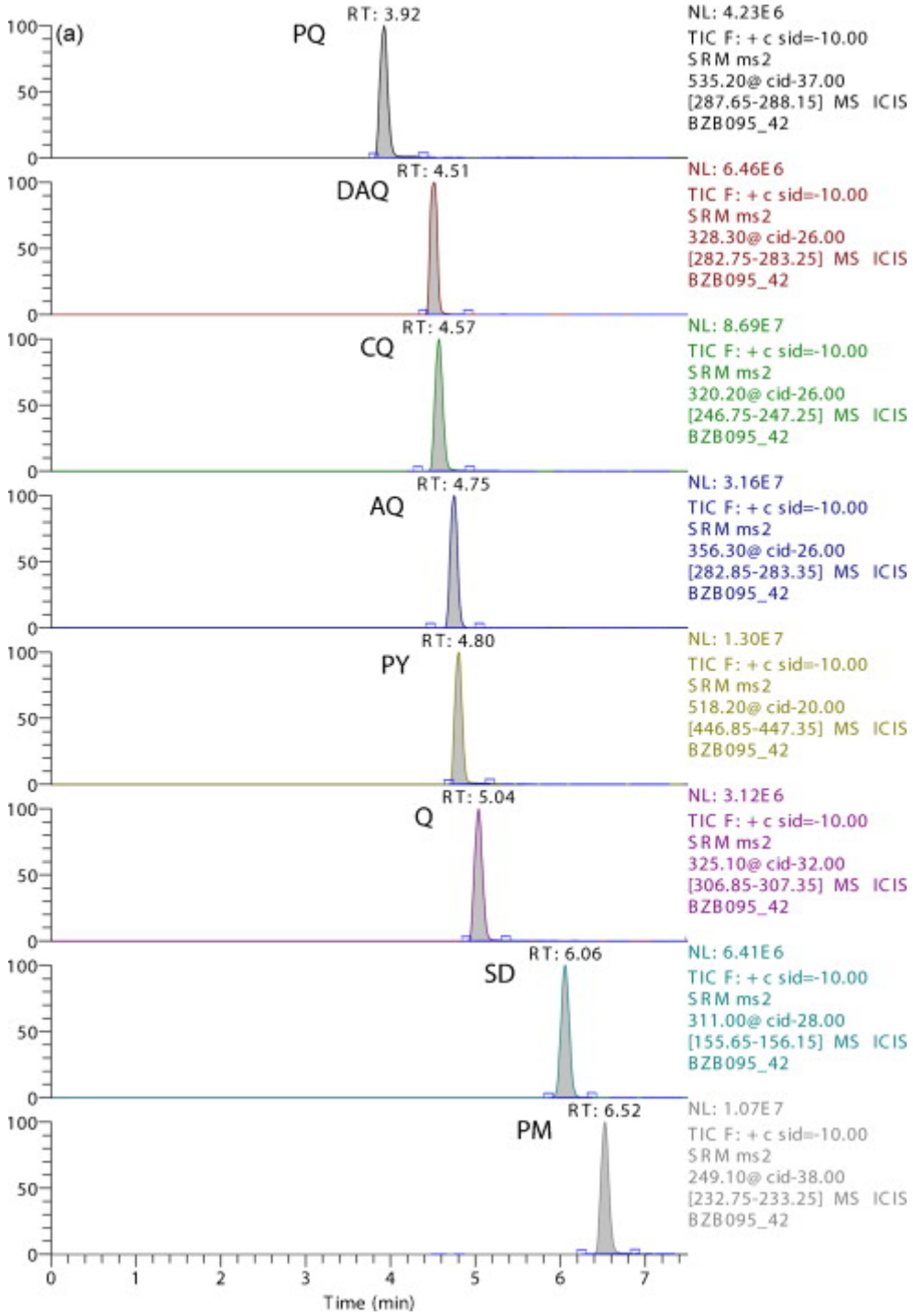

Figure 2. Chromatogram of the high quality control sample containing each antimalarial drugs at concentration reported in Table 1 (100 and 2000 ng/ml of internal standards), showing the first and second segments, at 0.0–7.5 and 7.5–17.0 min, respectively.

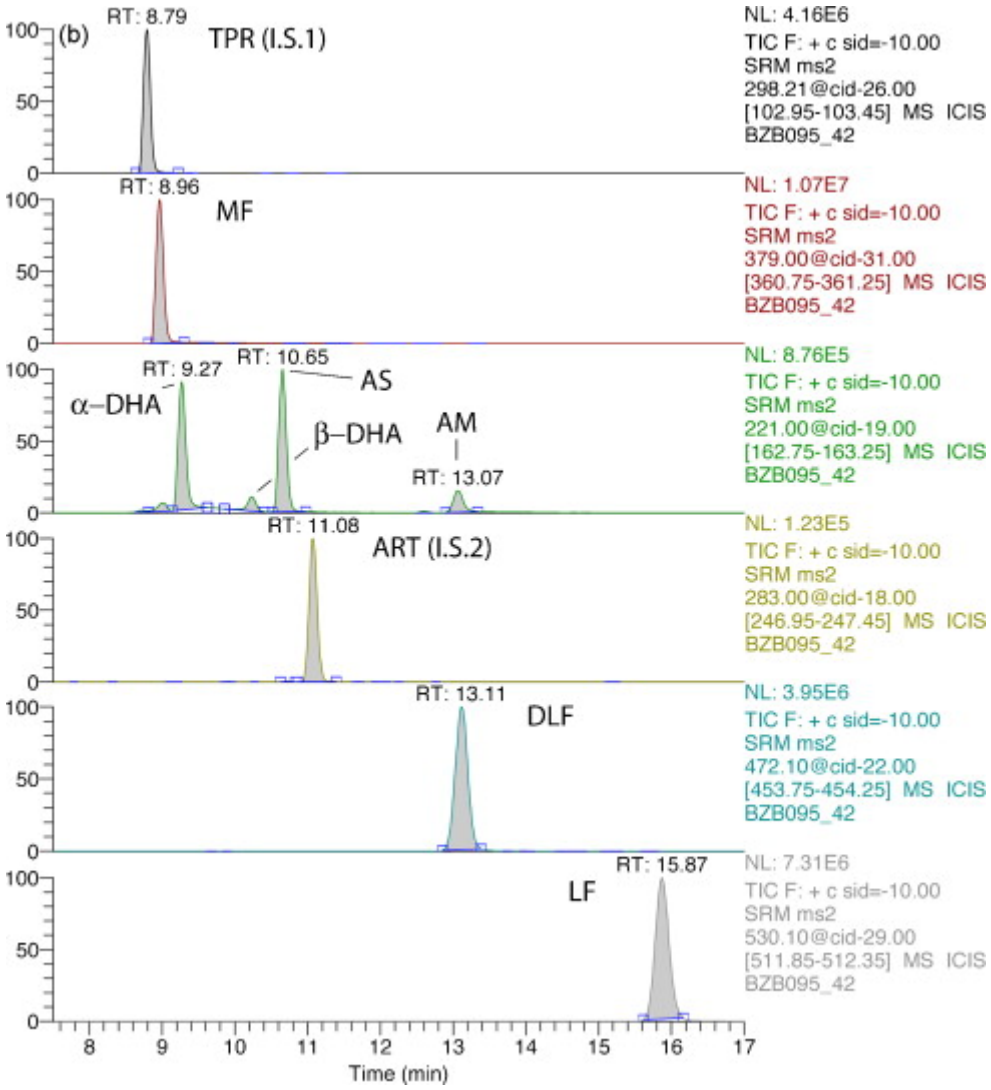

Figure 2. Continued.

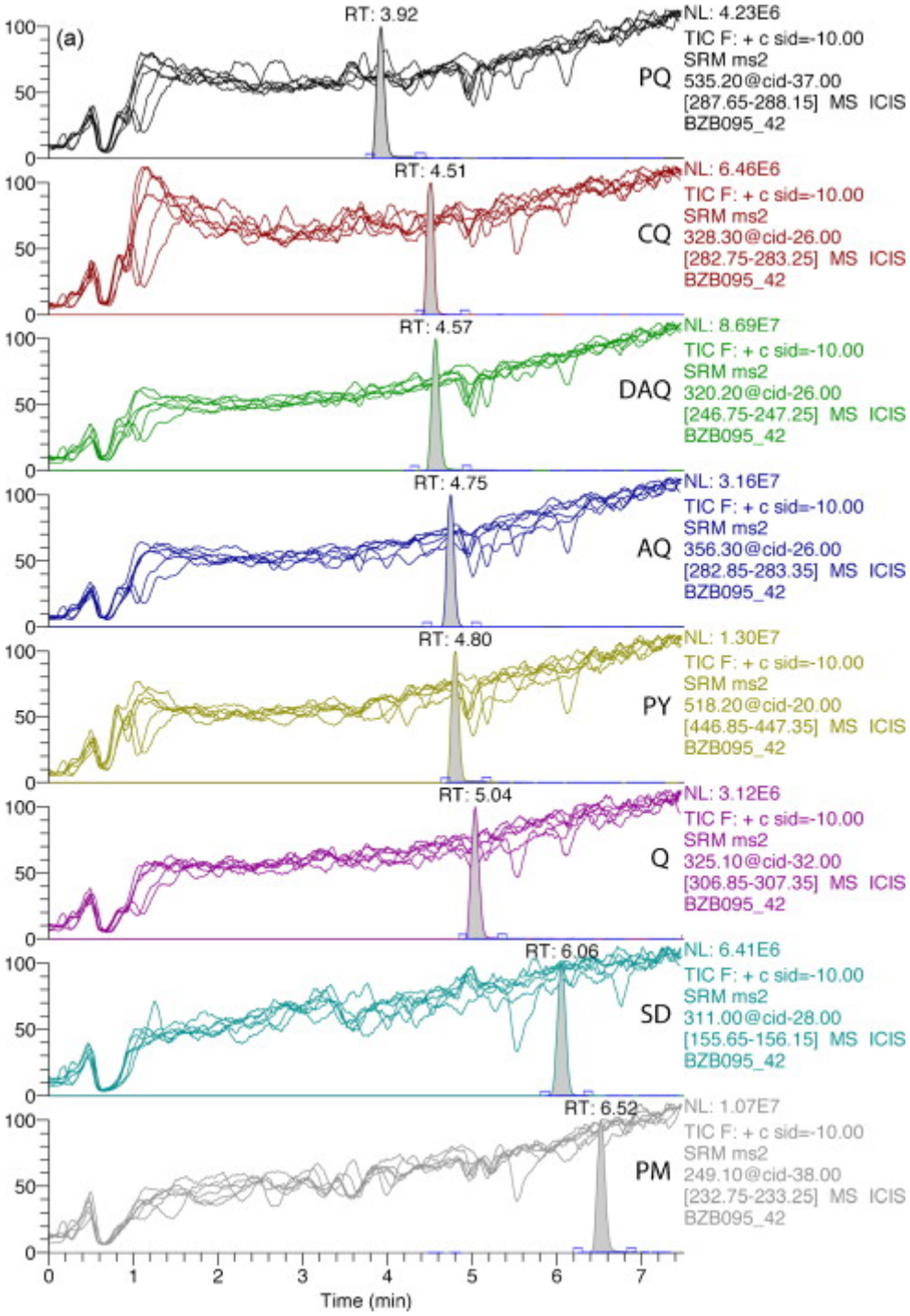

Figure 3. Chromatogram of six blank extract with post-column infusion of a calibration sample at medium QC concentration of each analysed drug and 100 and 2000 ng/ml of internal standards. Positive mode (first and second segments) is shown.

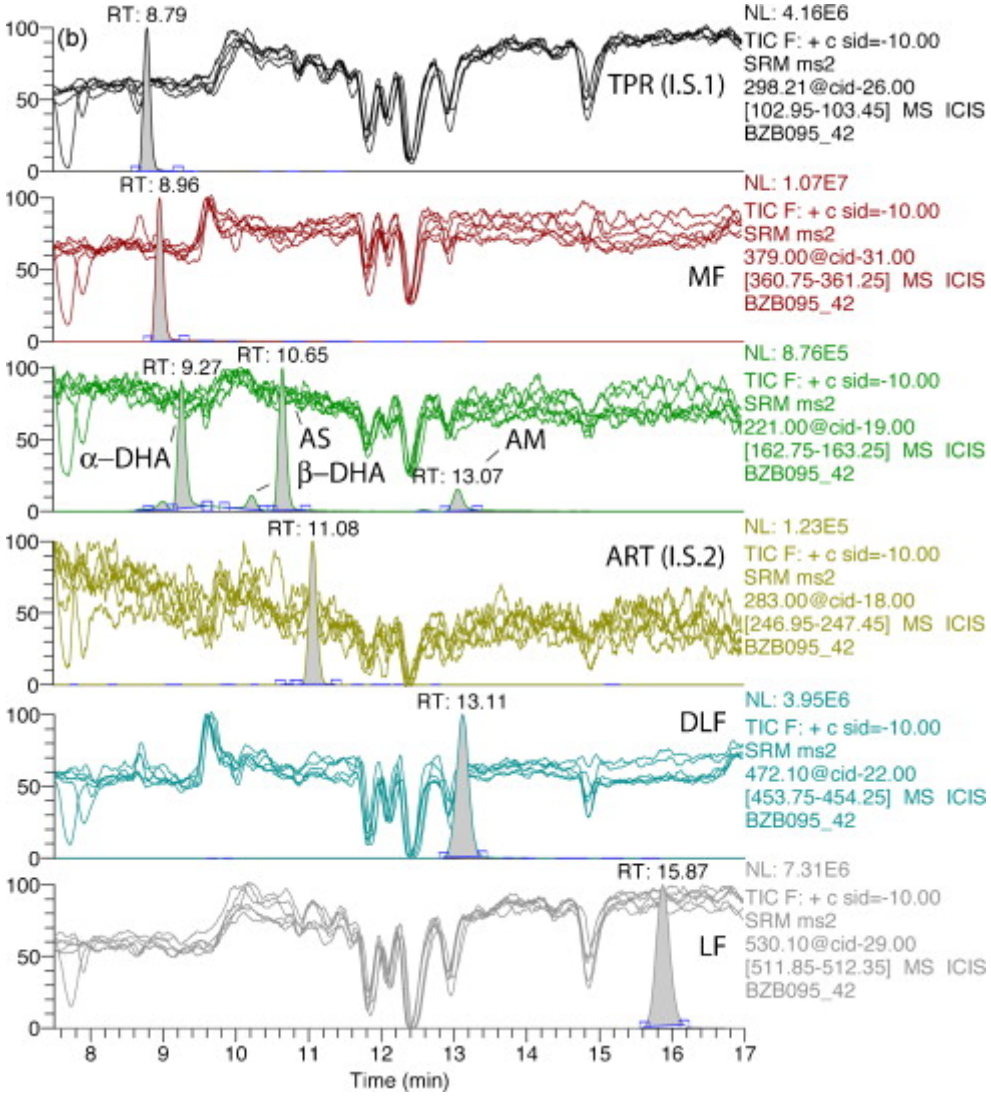

Figure 3. Continued.

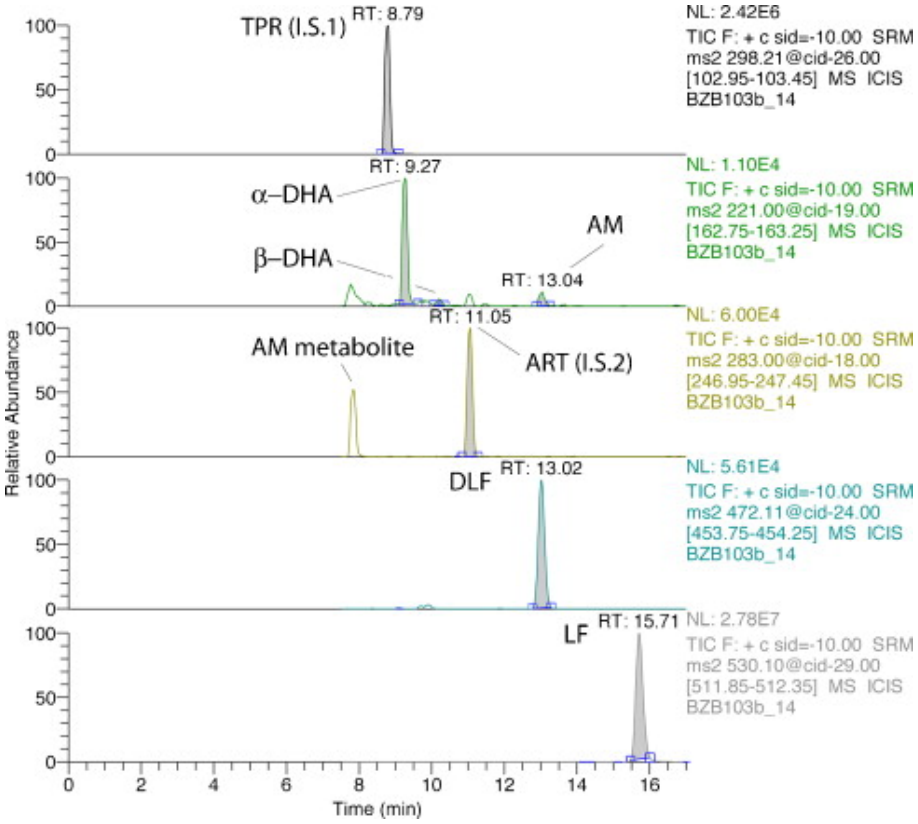

Figure 4. Chromatogram of plasma of a patient receiving artemether and lumefantrine (as Coartem® formulation; details in the text).

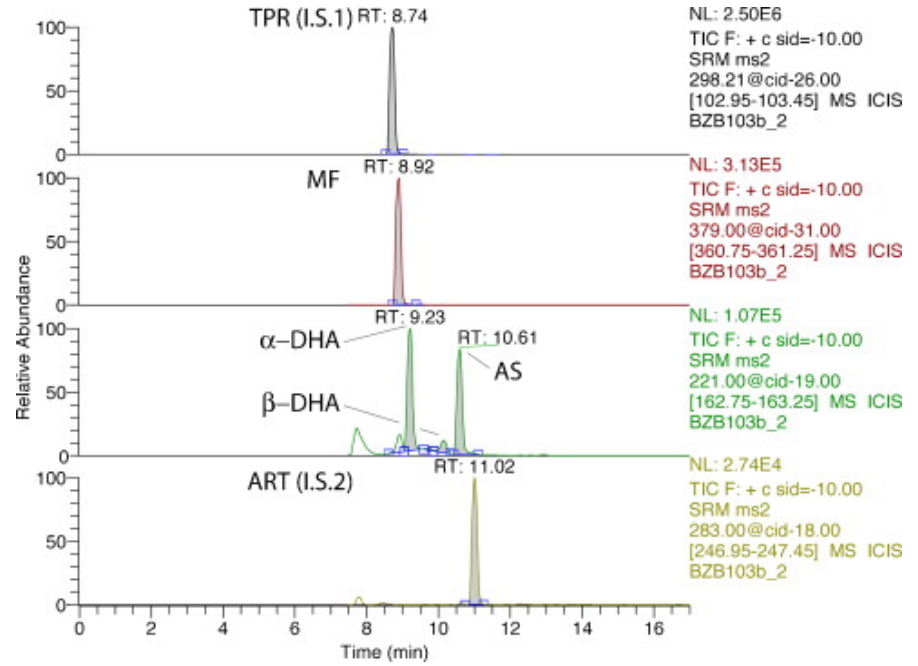

Figure 5. Chromatogram of plasma of a patient receiving artesunate and mefloquine (details in the text).

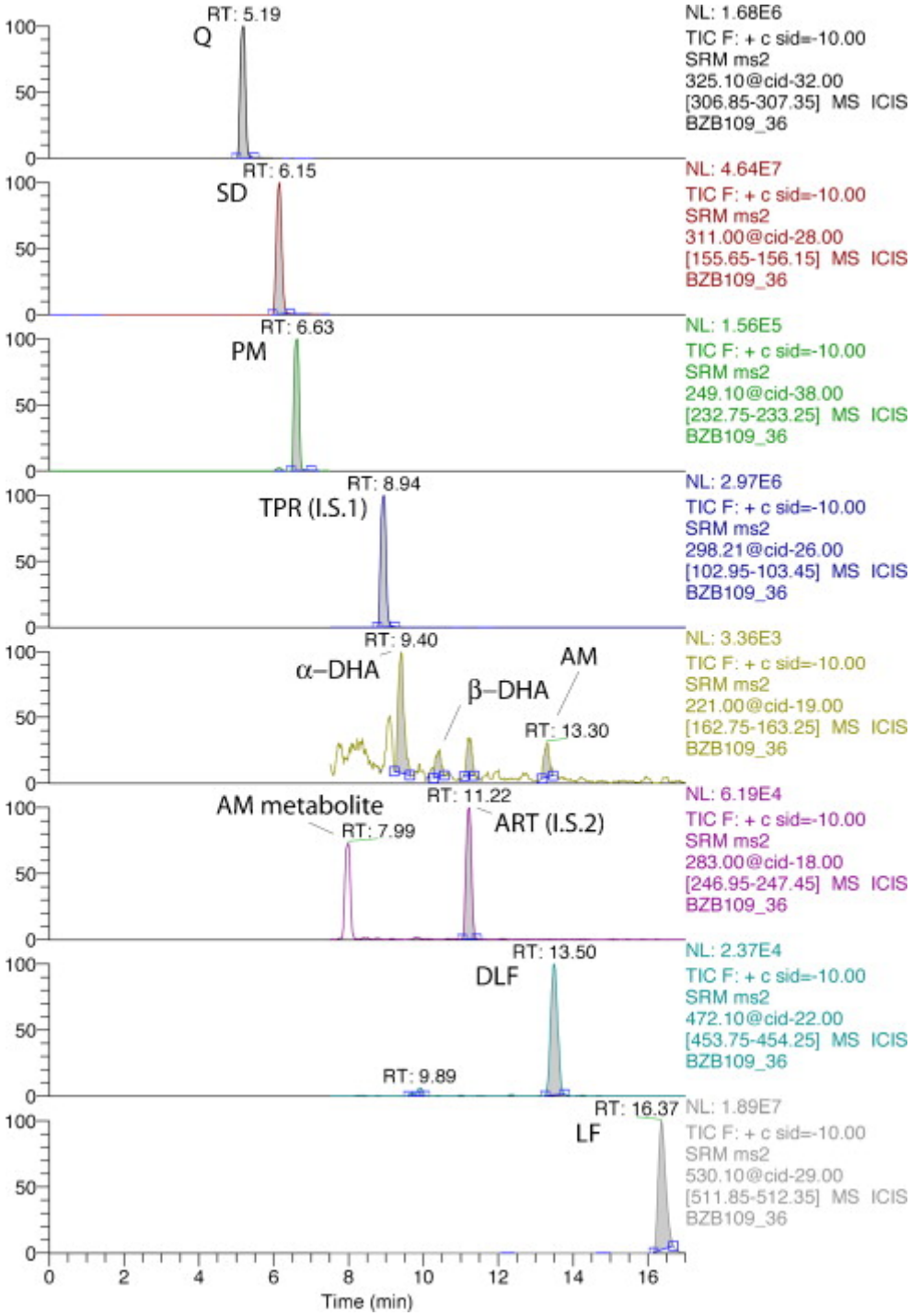

Figure 6. Chromatogram of plasma of a patient receiving artemether and lumefantrine (as Coartem® formulation; details in the text). Samples analysis reveals also the presence of quinine, and pyrimethamine/sulfadoxine (details in the text).

3.2. Internal standard and calibration curve

Ideally, deuterated analogues or homologues of the antimalarials or their metabolites would be the first-choice standards but these were not available to us and do not always compensate for the matrix effect [104]. Thus, a number of chemical compounds, unlikely to be found in patients but sharing some structural/chemical similarities with these antimalarials (i.e. aromatic heterocyclic amine linked to alkylamine, highly bound to plasma proteins), were evaluated as potential internal standards. Among them, trimipramine-D_3 was selected because it had a satisfactory chromatographic profile, with a negligible memory effect and, as a deuterated labeled compound, it is unlikely to be present in patients. For the quantification of the artemisinin derivatives AM, AS and DHA, the natural product artemisinin has been used as the I.S. in several assays [116] and was initially considered in our method as an additional I.S. for this class of compounds. It was found at an early stage of development that trimipramine-D_3 was also suited for artemisinin drugs, without affecting the accuracy and precision of the assay and with the advantage of a single I.S. for *all* antimalarials. In fact, ART, even though structurally related to DHA, AS and AM, was found *not* to closely mimic the reactivity of its hemisynthetic derivatives towards Hb (see below Section 3.8) and may spuriously affect analytical results when used as I.S. in the presence of slightly hemolytic plasma sample, such as those from some malaria patients. Moreover, previous/concomitant self-treatment with *Artemisia annua* herbal preparations (containing artemisinin) cannot be ruled out, and thus can also be detected with the proposed method. Having artemisinin as a second I.S. may be useful to identify situations (hemolysis) whereby the potential reactivity of hemoglobin towards endoperoxide-containing drugs may affect signal intensity and thus measured blood levels (see below Section 3.8) [136]. Interestingly, an AM metabolite – not yet identified – was detected at *ca* 8.0 min in patient's samples in the transition of *m/z* 283 → 247 chosen for artemisinin (Figure 4 and Figure 6).

For all antimalarials, calibration curves over the entire ranges of concentrations in Table 1 were satisfactory described by either $1/x^2$ or $1/x$ weighted quadratic regression, or quadratic log–log regression, of the peak-area ratio of antimalarial to I.S., *versus* the concentrations of the respective antimalarials/metabolites in each standard sample. The dynamic ranges can be considered satisfactory and span from 600 (AQ and DAQ) to 5000 (CQ, Q and MF). Over the considered concentration range, regression coefficient r^2 of the calibration curves were always greater than 0.99 with back-calculated calibration samples within ±15% (±20% at LLOQ).

3.3. Precision, accuracy, LLOQ and LOD

Precision and accuracy determined with the L, I, M and H QC samples are given in Table 3. The levels of control samples were selected to reflect the low, medium and high range of the calibration curves chosen to encompass the clinical range of concentrations found in patients' plasma. The mean intra-assay precision was similar over the entire concentration range and always less than 14.3%. Overall, the mean inter-day precision was good, with CVs within 3.1–12.6%. The intra-assay and inter-assay deviation (bias) from the nominal concentrations of each analysed antimalarials/metabolites QC were comprised between −9.1 and +13.0%, and −9.0 to +6.2%, respectively.

The results of the determination of LLOQ and LOD of antimalarials drugs in plasma are shown in Table 4. By analysing in triplicate plasma samples spiked with decreasing concentrations, the lowest achievable LOD among the considered antimalarials was obtained for DAQ at 10 pg/ml. The lowest LOQ was obtained for AQ and DAQ at 0.15 ng/ml, corresponding to an amount of 1.5 pg of drug into the 10 μl-injection volume.

Of note, the chosen levels of the calibration samples were selected initially to encompass the relevant range of concentrations presumably present in the plasma samples collected in this prospective field clinical study where antimalarials levels were determined in plasma samples taken at predetermined time after drug intake. With rare exceptions, all plasma drug levels in this study are above the lowest calibration levels. While validating the method, however, it was observed that the performance of our tandem MS/MS detector was such to attain lower detection and quantification limits, well below the clinically relevant range of antimalarial drugs concentrations established during the validation procedure. These results are reported in Table 4.

Table 4. Limit of detection and lower limit of quantification of antimalarials/metabolites.

	LOD (ng/ml)	LOQ (ng/ml)	Accuracy at LOQ (% bias)	Precision at LOQ (% CV)	Minimum quantifiable amount of drug on column (pg)	Lowest reported LOQ (ng/ml)	References
Piperaquine	0.5	1	2.0	15.2	10	1/1.5	Singhal et al. [128] / Tarning et al. [125]
Desethyl-amodiaquine	0.01	0.15	2.5	12.7	1.5	1.5	Chen et al. [107]
Chloroquine	0.2	1.25	3.5	2.1	12.5	2[a]	Singhal et al. [109]
Amodiaquine	0.04	0.15	3.1	7.8	1.5	0.15	Chen et al. [107]
Pyronaridine	0.25	0.75	4.7	13.7	7.5	5	Naik et al. [127]
Quinine	0.2	1.25	3.7	10	12.5	40.6	Damien et al. [106]
Sulfadoxine	0.08	0.25	8.2	10.5	2.5	0.78[b]	Sabarinath et al. [110]
Pyrimethamine	0.06	0.25	1.6	3.9	2.5	0.78[b]	Sabarinath et al. [110]
Mefloquine	0.07	1.25	2.4	7.8	12.5	50	Lai et al. [99]
Dihydro-artemisinin	0.5	0.75	−0.2	12.7	7.5	1/1.7/0.2[b]	Naik et al. [120] / Gu et al. [105] / Xing et al. [130]
Artesunate	0.5	1.5	5.5	9.7	15	1	Naik et al. [120]

Table 4. Continued.

	LOD (ng/ml)	LOQ (ng/ml)	Accuracy at LOQ (% bias)	Precision at LOQ (% CV)	Minimum quantifiable amount of drug on column (pg)	Lowest reported LOQ (ng/ml)	References
Artemether	3	5	1.1	18.6	50	5	Souppart et al. [116]
Desbutyl-lumefantrine	2	3	6.5	2.2	30	5/21	Hatz et al. [129] / Lindegardh et al. [95]
Lumefantrine	2	3	5.5	1.4	30	50/24	Hatz et al. [129] / Lindegardh et al. [95]

LOD: Limit of detection; LOQ: limit of quantification; SD: standard deviation.

[a] Dog plasma.

[b] Rat plasma.

Table 5. Matrix effect, extraction yield, overall recovery and process efficiency of antimalarials/metabolites: (a) without and (b) with normalization with I.S.

	Nominal conc. (ng/ml)	Mean peak area			Mean peak-area ratio		ME (%)	CV (%)	ext RE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A (*n* = 2)	*B* (*n* = 6)	*C* (*n* = 6)	B2	C2	*B/A*		*C/B*		C2/B2			C/A	
PQ	30	463,339	305,036	275,930	0.012	0.011	65.8	7.3	90.5	13.6	95.2	101.6	5.5	59.6	8.5
	300	5,903,171	4,944,385	4,911,089	0.193	0.202	83.8	1.8	99.3	3.0	104.5			83.2	2.4
	3000	38,309,081	36,613,858	36,582,684	1.431	1.504	95.6	1.1	99.9	1.6	105.1			95.5	0.9
DAQ	4.5	631,180	608,464	590,629	0.024	0.024	96.4	3.9	97.1	3.4	102.1	104.4	2.1	93.6	3.4
	45	6,212,975	5,989,574	6,055,713	0.234	0.249	96.4	2.1	101.1	3.4	106.4			97.5	2.3
	450	43,277,028	41,825,364	41,658,554	1.635	1.713	96.6	1.3	99.6	2.3	104.8			96.3	1.5
CQ	37.5	19,603,116	18,456,063	18,087,496	0.721	0.744	94.1	3.7	98.0	4.0	103.1	104.7	1.3	92.3	4.0
	375	156,759,550	153,125,711	153,404,821	5.986	6.309	97.7	2.8	100.2	3.0	105.4			97.9	3.8
	3750	682,764,100	707,475,085	709,536,225	27.655	29.178	103.6	2.4	100.3	2.1	105.5			103.9	2.0
AQ	4.5	3,819,259	3,603,097	3,681,960	0.141	0.151	94.3	6.3	102.2	5.9	107.5	107.8	4.3	96.4	6.6
	45	37,274,257	33,905,511	36,282,942	1.325	1.492	91.0	3.5	107.0	4.9	112.6			97.3	6.0
	450	265,582,976	246,574,259	242,340,858	9.638	9.966	92.8	3.4	98.3	2.8	103.4			91.2	2.5
PY	7.5	807,394	669,041	615,786	0.026	0.025	82.9	7.6	92.0	12.2	96.8	100.7	3.6	76.3	7.7
	75	10,183,026	10,459,753	10,342,889	0.409	0.425	102.7	2.5	98.9	5.2	104.0			101.6	4.3
	750	121,225,955	113,563,974	109,407,170	4.439	4.499	93.7	2.1	96.3	3.9	101.4			90.3	2.0
CQ	37.5	728,591	632,693	647,122	0.025	0.027	86.8	4.4	102.3	6.4	107.6	106.7	1.5	88.8	3.5
	375	5,544,075	477,7095	4,884,734	0.187	0.201	86.2	4.4	102.3	4.8	107.6			88.1	3.9
	3750	24,136,642	21,997,888	21,921,437	0.860	0.901	91.1	3.2	99.7	4.6	104.8			90.8	2.9

Table 5. Continued.

	Nominal conc. (ng/ml)	Mean peak area			Mean peak-area ratio		ME (%)	CV (%)	ext RE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A (*n* = 2)	*B* (*n* = 6)	*C* (*n* = 6)	B2	C2	*B/A*		*C/B*		C2/B2			C/A	
SD	7.5	1,544,599	1,206,270	1,231,883	0.047	0.051	78.1	2.6	102.1	4.9	107.4	106.4	2.3	79.8	2.5
	75	11,802,907	9,929,646	10,209,811	0.388	0.420	84.1	3.4	102.8	2.3	108.2			86.5	3.0
	750	58,601,536	55,876,683	54,986,658	2.184	2.261	95.4	2.0	98.4	3.0	103.5			93.8	1.4
PM	7.5	2,195,084	1,680,490	176,5491	0.066	0.073	76.6	1.6	105.1	3.9	110.5	108.8	2.6	80.4	2.3
	75	16,256,773	13,801,907	14,465,487	0.540	0.595	84.9	3.6	104.8	3.0	110.3			89.0	4.8
	750	97,559,988	90,336,998	90,589,054	3.531	3.725	92.6	3.4	100.3	2.4	105.5			92.9	3.1
MF	37.5	1,044,765	788,973	920,040	0.031	0.038	75.5	7.2	116.6	10.5	122.7	116.7	5.3	88.1	5.4
	375	12,396,638	8,437,387	9,383,194	0.330	0.386	68.1	10.0	111.2	12.7	117.0			75.7	9.5
	3750	120,129,330	85,365,515	89,507,628	3.337	3.681	71.1	6.2	104.9	5.9	110.3			74.5	8.9
DHA	15	53,938	42,190	40,960	0.002	0.002	78.2	11.3	97.1	6.0	102.1	100.9	4.1	75.9	10.2
	150	537,803	450,578	44,6951	0.018	0.018	83.8	5.3	99.2	13.5	104.4			83.1	6.0
	1500	478,0571	4,387,361	4,014,270	0.171	0.165	91.8	6.8	91.5	5.1	96.3			84.0	6.8
AS	15	75,870	72,481	74,813	0.003	0.003	95.5	10.7	103.2	5.3	108.6	104.2	3.6	98.6	13.0
	150	850,377	778,923	752,128	0.030	0.031	91.6	2.7	96.6	13.0	101.6			88.4	3.3
	1500	7,580,286	7,264,111	7,082,061	0.284	0.291	95.8	4.4	97.5	5.1	102.6			93.4	5.2
DLF	30	2,183,107	483,301	591,038	0.019	0.024	22.1	7.7	122.3	11.0	128.7	117.2	8.5	27.1	4.6
	300	26,958,705	5,901,924	6,192,902	0.231	0.255	21.9	7.1	104.9	11.8	110.4			23.0	11.7
	3000	244,686,731	54,557,187	58,430,410	2.133	2.403	22.3	9.9	107.1	12.4	112.7			23.9	5.5
AM	15	19,156	13,487	12,649	0.001	0.001	70.4	10.8	93.8	14.7	98.7	94.6	8.9	66.0	9.2
	150	216,555	163,681	155,920	0.006	0.006	75.6	6.7	95.3	8.5	100.2			72.0	7.4
	1500	1,928,062	1,657,750	1,337,153	0.065	0.055	86.0	4.6	80.7	6.6	84.9			69.4	4.1

Table 5. Continued.

	Nominal conc. (ng/ml)	Mean peak area			Mean peak-area ratio		ME (%)	CV (%)	ext RE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A (*n* = 2)	*B* (*n* = 6)	*C* (*n* = 6)	B2	C2	*B*/*A*		*C*/*B*		C2/B2			C/A	
LF	30	8,223,435	1,867,593	1,473,025	0.073	0.061	22.7	7.8	78.9	15.7	83.0	85.0	9.5	17.9	12.0
	300	73,501,143	21,100,338	18,827,212	0.825	0.774	28.7	8.7	89.2	13.4	93.9			25.6	10.6
	3000	636,041,562	185,392,142	137,587,637	7.247	5.658	29.1	6.7	74.2	14.1	78.1			21.6	8.3
TPR (I.S.)	100	28,002,971	25,582,553	24,317,108			91.4	2.2	95.1	2.7				86.8	2.3
ART (I.S.)	2000	696,817	657,912	626,607			94.4	5.9	95.2	5.6				89.9	3.6

A = peak area of standard solutions without matrix and without extraction (MeOH/buffer A 1:1), *B* = peak area of analytes spiked after extraction, *C* = peak area of analytes spiked before extraction, B2 = ratio of the peak area of the analyte and the I.S. spiked after extraction, C2 = ratio of the peak area of the analyte and the I.S. spiked before extraction, ME = matrix effect expressed as the ratio of the mean peak area of the analytes spiked after extraction (*B*) to the mean peak area of the same standard solution without matrix (*A*) multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. Ext RE = extraction procedure recovery calculated as the ratio of the mean peak area of the analytes spiked before extraction (*C*) to the mean peak area of the analytes spiked after extraction (*B*) multiplied by 100. Analysis RE = analysis recovery calculated as the ratio of the mean peak-area ratio of the analytes spiked before extraction (C2) to the mean peak-area ratio of the analytes spiked after extraction (B2) multiplied by 100. PE = process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (*C*) to the mean area of the same analyte standards (*A*) multiplied by 100

Table 6. Standard-addition experiments with DHA, mefloquine and lumefantrine in 16 patients plasma samples (8 from Tanzania and 8 from Cambodia).

	Patients samples no.	**Patient samples direct determination**	**Spiked with (ng/ml)**	**Concentration measured in piked sample**	**Expected**	**Deviation (%)**
Lumefantrine (Tanzania)	84.2	165	250	329	415	−20.7
	82.2	1431	250	1576	1681	−6.3
	96.2	823	250	1025	1073	−4.5
	92.1	974	250	1169	1224	−4.5
	85.1	3606	250	4119	3856	6.8
	96.1	2126	250	2276	2376	−4.2
	93.2	1509	250	1769	1759	0.5
	94.2	2534	250	2596	2784	−6.8
DHA (Tanzania)	84.2	122	125	252	247	2.0
	82.2	55	125	205	180	14.1
	96.2	16	125	152	141	7.4
	92.1	296	125	459	421	8.9
	85.1	0	125	121	125	−3.1
	96.1	152	125	295	277	6.6
	93.2	55	125	204	180	13.1
	94.2	135	125	257	260	−1.3

Table 6. Continued.

	Patients samples no.	Patient samples direct determination	Spiked with (ng/ml)	Concentration measured in piked sample	Expected	Deviation (%)
Mefloquine (Cambodia)	34	86	312.5	444	398	11.5
	25	100	312.5	419	413	1.7
	36	152	312.5	506	465	8.9
	53	193	312.5	573	505	13.4
	35	208	312.5	588	520	13.0
	64	252	312.5	576	565	2.0
	24	551	312.5	857	863	−0.7
	51	582	312.5	944	894	5.5
DHA (Cambodia)	34	617	125	814	742	9.6
	25	1340	125	1438	1465	−1.8
	36	359	125	493	484	2.0
	53	1607	125	1714	1732	−1.1
	35	645	125	850	770	10.5
	64	772	125	914	897	1.9
	24	1189	125	1375	1314	4.7
	51	1106	125	1281	1231	4.1

3.4. Stability of antimalarials/metabolites in plasma

The stability of antimalarials/metabolites QCs in human plasma samples left at *room temperature* (RT) was ascertained up to 48 h. The variation over time of each drug levels was mostly comprised within the ±15% of starting concentrations indicating that, taking into account the analytical variability, antimalarials are stable, with the notable but expected exception of DHA and AS showing at RT a significant decrease of −15, −48 and −75%, and −12, −23 and −48% from the initial levels after 8, 24 and 48 h at RT, respectively (Figure 7, RT). By contrast, DHA and AS levels were remarkably stable in plasma samples left during the same period in the fridge at 4 °C (Figure 7, 4 °C). This indicates that in field trials, after blood centrifugation without delay, plasma can be conveniently stored at 4 °C up to 48 h prior to final storage at −80 °C. This good stability of DHA and AS in the plasma at 4 °C was observed irrespectively of the presence of other antimalarials during the *in vitro* experiments. Interestingly in separate *in vitro* experiments at RT (data not shown), significant differences in the rate of decrease in AS and DHA levels were observed amongst the different plasma sources used in the *in vitro* experiments. Variability in plasma esterases concentrations or activity are probably involved in AS decay. During the analysis in the laboratory, plasma samples were never allowed to stay more than 1 h at room temperature prior to extraction, indicating that under such conditions the stability of antimalarials/metabolites in plasma is such that the accuracy is not likely to be notably affected.

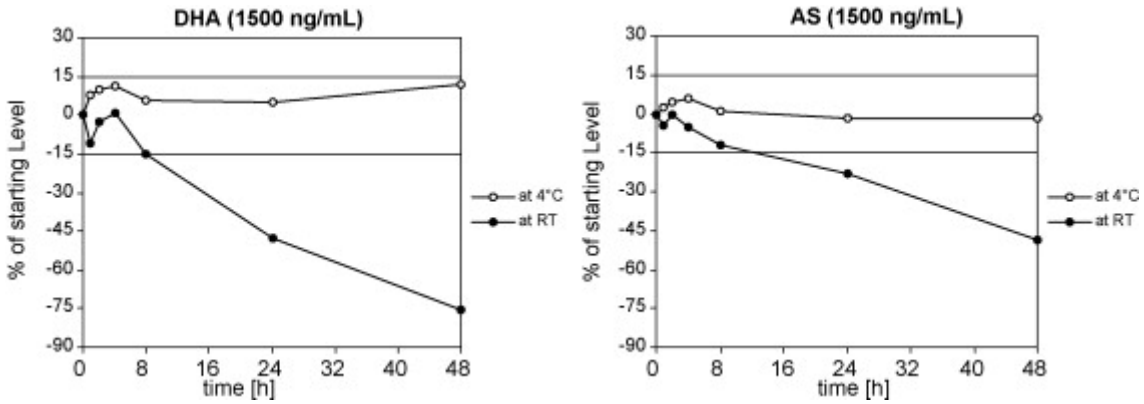

Figure 7. Comparison of stability of DHA and AS in plasma at RT and +4 °C (horizontal lines correspond to ±15% of initial values).

The variation of antimalarials/metabolites concentrations after one and two freeze-thaw cycles was less than −15.5% after 2 cycles. For some antimalarials (amodiaquine, quinine and sulfadoxine at the medium QC level; artemether, desbutyl-lumefantrine and lumefantrine at

the low QC concentrations) an apparent increase of about 12% was observed after 2 cycles. After 3 cycles, significant loss of lumefantrine (−19%) and desbutyl-lumefantrine (−30%) were observed for the high QCs. Thus, samples containing lumefantrine/desbutyl-lumefantrine are apparently vulnerable to multiple freeze-thaw cycles. When repetitive analyses of samples are considered, it is advised to distribute plasma aliquots in separate vials to be defrozen only once.

Of note, we have also investigated the stability of antimalarial drugs in dried plasma extracts samples (in case samples could be processed on-site up to the stage of the solid dried residues and shipped at room temperature), and in blood at +4 °C and at RT. However, the determination of antimalarials/metabolites stability was obscured by the antimalarial drug distribution in and out red-blood cells (RBC) which occurred for some antimalarials in these *in vitro* experiments. A comprehensive analysis of antimalarials stabilities in various biofluids, and antimalarials drugs handling in and out RBC, and the effect of temperature, is outside the scope of the present article and will be reported elsewhere.

3.5. Matrix effect and recovery

Among the solvent mixtures tested (mentioned in Section 2.4.2), the best overall recovery for the 14 antimalarials/metabolites was obtained after acetonitrile precipitation of plasma, followed by evaporation of the supernatant solution and reconstitution of the dried extracts in MeOH/buffer A 1:1 mixture.

Matrix effect was examined by the simultaneous post-column infusion of antimalarials/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blank plasma extracts from blood donors. As exemplified in Figure 3, no significant drifts or shifts of the selected transition signals were apparent during the chromatography of the six blank matrices at the retention time of the 14 compounds (Table 2). Of note, AM and DLF were eluted at 13.07 and 13.11 min just after a small drop in the selected transition signal appearing between 12.90 and 13.05 min which should therefore not affect AM and DLF signal intensity: this drop was reproducible between plasma batches and, because all calibration are prepared in plasma, will correct for any possible matrix effect.

Co-eluting matrix components may nevertheless reduce or enhance the ion intensity of analytes, possibly affecting the reproducibility and accuracy of the assay. Consequently, for the sake of validation, all standard calibrations and quality control samples have been prepared by spiking drugs in human plasma.

The assessment of the *matrix effect* (Table 5) was quantified as the peak-area response of analytes added to blank plasma extracts (i.e. *B*, drugs added *after* extraction), expressed as the percentage of the response of standard solution of drugs directly injected onto the column (*A*) (ratio *B*/*A*, in Table 5). A value above or below 100% for the matrix effect indicates an ionization enhancement or suppression, respectively. The results indicate that co-eluting plasma matrix components appear to reduce the ion intensity for DLF and LF to about 22% and 27% for DLF and LF, respectively. This is due to the fact that both LF and DLF in MeOH/buffer A (1:1) solution had comparatively a very intense signal. Accordingly, these high signal areas of pure LF and DLF negatively affect overall recovery and efficiency. However, the extraction recovery (*C*/*B*) was not affected as the results showed an excellent sensitivity, comparable to or even better than lumefantrine and desbutyl-lumefantrine LOQ values reported to date (Table 4, [95]). The problem of ion suppression for LF using LC–MS has been already reported producing notably spurious pharmacokinetic results necessitating samples re-analysis by HPLC UV [38]. Keeping this in mind, we put a lot of effort to optimize gradient elution for LF and DLF with a relatively "flat" gradient pattern in the second part of the program (i.e. 59 → 68% organic between 10 and 17 min) for controlling elution of DLF and LF at a time were the matrix effect would be minimal, or at least for which the variability would be reduced. Finally, the peak intensity obtained with pure piperaquine and pyronaridine standards (signals A) were obtained using a new LC column after conditioning and equilibration of long enough duration (i.e. after about 40 analyses). After prolonged use, some decrease in the intensity of pyronaridine and piperaquine occurred, without affecting, however, their excellent signal in spiked plasma samples.

The *analytical recovery* was calculated in a similar way, but considering drugs response/I.S. ratio instead, before (C2) and after (B2) the extraction procedure (ratio C2/B2, in Table 5). Taking into account the I.S. response enables to correct for the occurrence of variation over time in the MS/MS spectrometer performance and injection volume. As indicated in Table 5, the extraction recovery for the two I.S. trimipramine-D_3 and artemisinin was essentially identical and above 95%. The *analytical recoveries* achieved were always >78.1% for all

antimalarials. The overall *recovery* given in Table 5 (column *C*/*A*) was obtained as the peak-area response of analytes spiked into plasma samples *before* the extraction procedure I, such as calibration and control samples, expressed as the percentage of the peak area of pure drug standard solution (*A*) directly injected into the column. This overall *recovery* (i.e. process efficiency) takes into account the extraction yield and the matrix effect: for example, DHA has a mean matrix-mediated ionization decrease down to about 85% (Table 5, column ME) which, combined with a mean extraction yield of 96% (Ext RE, Table 5), gives an overall recovery (process efficiency, PE) around 82%. Overall, these results indicate that even though no apparent matrix effect was observed in the infusion experiment (Figure 3a and b), matrix components do influence the overall process efficiency. This was especially noticeable for lumefantrine and desbutyllumefantrine, requiring therefore the preparation of calibration and control samples in plasma matrix reflecting at best the composition of the samples to be analysed. Most importantly, it is not so much the matrix effect *per-se* that must be reduced than its variability. As shown in Table 5, the variability of the matrix effect of 6 different plasma matrices never exceeded 11.3%, demonstrating indeed that the proposed extraction procedure is able, if not to eliminate, at least to normalize and standardize the matrix effect.

Finally, our standard addition experiments (reported in Table 6) performed with DHA, lumefantrine and mefloquine using the malaria patient plasma itself indicate a good correspondence between experimental and expected concentrations in the spiked aliquots, with deviations comprised within ±15% in 31 out of 32 measurements. These experiments have therefore allowed to ascertain that (i) any possible unrecognized matrix effect (caused by differences in plasma composition due to altered physio-pathological conditions associated with malaria) is adequately either circumvented or normalized using our proposed extraction method and (ii) any unrecognized metabolites are not markedly influencing the analytes signal intensity, at least for the drugs for which clinical samples were available. This demonstration was especially important for lumefantrine, for which ion suppression has been previously reported to be an issue [38].

3.6. Dilution effect

After the three-fold dilution of the spiked plasma with antimalarials at a concentration exceeding by two-fold the high calibration level, the deviation (bias) from the expected concentrations was less than 12.5% for all drugs. This indicates that plasma samples

containing antimalarials above the high level of calibration can be adequately diluted with blank plasma prior to the LC–MS/MS analysis.

3.7. Analytical issues with artemisinin drugs in hemolysed samples

3.7.1. Impact of the presence of organic solvent on the assay of endoperoxide drugs in hemolysed plasma

With our extraction methodology, the signal intensity of artemisinin added as I.S. to hemolysed plasma (0.2% RBC) was decreased by ca. −20% in the presence of 0.2% hemolysed RBC, irrespective of whether the ART solution was added as a "free" aqueous/methanolic solution, or "shielded" as a protein-bound form solubilized in plasma ($p = 0.26$, Student *t*-test) (see Figure S1, upper left, MeOH *versus* plasma solution (Annex)). This indicates that, with our proposed method, the presence of MeOH in the ART solution does not account *per se* for the drop of ART measured in hemolysed plasma (see below). For DHA, AS and AM at 500 ng/ml, the nature of the solvent used for their solubilization in the working solutions has also little impact on the very small decrease in their signal intensities in hemolysed plasma (Figure S1) ($p = 0.6$, 0.62, 0.43, for DHA, AS and AM, respectively, solubilized in MeOH *versus* in plasma). Of note, the higher reactivity of ART towards Hb is unlikely to be explained by differences in the degree of plasma protein binding which is similar for ART, DHA and AM (85–88%, 93%, 95–98%), respectively [137-139]. Indeed, AS, even though significantly less bound to plasma proteins (62–81% [139]) was no more reactive than DHA and AM. Thus, the higher reactivity of ART with Hb is most probably related to intrinsic differences related to its chemical characteristics.

3.7.2. Evaluation of sample extraction methodology

The results of the comparative study of the stability of arteminisin compounds in calibration samples in the presence or absence of Hb, are shown in Figure S2, using either (A) our proposed method (protein precipitation, supernatant evaporation and reconstitution) or (B) protein precipitation and direct supernatant injection. Again, the signal of artemisinin was similarly decreased by *ca* 20–25% ($p < 0.001$ Student *t*-test) in the presence of 0.2% hemolysed RBC with both extraction methods (Figure S2: ART, upper left, method A *versus* B). Using method B (direct supernatant injection) also the signals of DHA, AS and AM were

significantly decreased in hemolysed plasma in comparison to those added to pure control plasma ($p < 0.001$, $p < 0.01$ and $p < 0.02$, for DHA, AS and AM, respectively). By contrast, with method A (supernatant evaporation and reconstitution), DHA, AS and AM were found to be remarkably stable in the presence of 0.2% RBC: their signal intensities in hemolysed calibration samples were not significantly different from controls and within the analytical variability (mean difference compared to control non-hemolysed plasma: −2.3% ($p = 0.17$); −2.9% ($p = 0.16$), and −1.3% ($p = 0.73$) for DHA, AS and AM, respectively) (Figure S2: columns A). These experiments indicate that, in the presence of Hb in plasma, the evaporation and reconstitution in buffer/MeOH after protein precipitation used in our method appears to be essential steps to eliminate components affecting DHA, AS, and AM signal intensities during the subsequent LC–MS/MS analysis. These results confirm Lindegardh et al. [102] finding that the analysis of DHA and AS with direct plasma supernatant injection after plasma protein precipitation was particularly vulnerable to the presence of Hb and RBC components. Other factors, including the chromatographic conditions (stepwise flow gradient program on reverse-phase column whereby DHA and AS are eluted with a 100% organic solvent (MeOH/I 75:25) washing) may have contributed to the size of the effect seen. The inherent vulnerability of this method was then corrected by the same group in a follow-on paper by a preliminary clean-up by SPE on reverse-phase packing material [101].

The signal of trimipramine-D_3 (TPR) was insensitive to the presence of Hb, using our methodology (Figure S2 upper right: TPR method A), but decreased (as well as piperaquine, data not shown) when using the direct supernatant injection (Method B). This substantiates the choice of TPR as the I.S. for all compounds and highlights the critical importance of the evaporation step and reconstitution in buffer/MeOH after protein precipitation to the standardization of sample extracts injected into the LC–MS/MS.

3.7.3. Experiments with malaria patients plasma samples

In Figure S3, the hemolysed malaria patients samples have been classified according to the percentage reduction in signal intensity of the I.S. ART, used as a surrogate for the presence of Hb in comparison to the mean signal intensity of ART in 10 blank malaria patients samples without hemolysis. In the addition experiments performed with hemolysed plasma samples from malaria patients, the intensity of ART (I.S., 667 ng/ml) as well as DHA, AS and AM spiked at 500 ng/ml are shown in Figure S3. A significant drop in ART signal intensity was

visible, as expected (see above), and was more pronounced (down to 65%) with increasing evidence of hemolysis in comparison to control ($p < 0.0001$). Of note, visual examination of plasma samples with a −25% to −50% reduction in ART signal were frankly red. By contrast, the DHA did not appear to be significantly affected by Hb or possible hemolytic compounds in the group of hemolysed malaria patient samples showing a reduction in ART signal of as much as −15 to −25% ($p = 0.16$, in comparison to control). In the same latter group of samples, AS and AM were slightly more sensitive to the presence of Hb (Figure S3) and their signal intensities were reduced to 91 ± 9% and 92 ± 7% of that of non-hemolysed controls patient samples, a small, albeit significant, decrease ($p < 0.01$). However, given the ±15% allowances in analytical variability these decreases are unlikely to impact to a clinically relevant extend the accuracy of bioanalysis of AS and AM of samples in case of the presence of low levels of Hb. Further detailed investigations on the stability of endoperoxide antimalarials in the presence of Hb are ongoing in our laboratory but are outside the scope of the present report.

3.8. Selectivity

No peaks from endogenous compounds were observed at the drugs retention time in any of ten blank plasma extracts evaluated. The product ion monitoring was chosen, given its relative abundance, while avoiding possible structural analogies with other drugs or fragments analysed. Every channel was simultaneously observed, and we never saw any selectivity problems or *cross talk* signal at the retention time of interest between acquisition channels. This was examined in details for instance for the *m/z* transition chosen for amodiaquine and N-desethylamodiaquine, and for DHA/AS/AM and artemisinin.

3.9. Memory effect

A critical issue for the analysis of some basic antimalarials (PQ, PY, DLF, LF) analysis was their tendency to get adsorbed by reverse-phase octadecyl-based chromatographic packing materials, resulting in a “memory” effect. Most of the carry-over could be eliminated by programming a relatively prolonged rinsing period after each analysis. With this approach, the carry-over effect ascertained by sequential injection of 10 µl of MeOH/buffer A 1:1 solution after the analysis of the *high calibrator* was demonstrated to be minimal. After the first following injection, the memory effect was below 0.1% except for piperaquine, lumefantrine,

desbutyl-lumefantrine, chloroquine, pyronaridine and quinine which showed signals corresponding to a carry-over of 0.219, 0.193, 0.161, 0.127, 0.125 and 0.106% of the peak areas of the highest calibration levels, respectively. In the following second and third injection of the blank solution, the highest observed carry-over corresponds to 0.098 and 0.07% of the high calibration levels, respectively. Thus, by measure of precaution, the analyses of 3 blank samples were programmed after the *high calibration samples*, prior to the first analysis of patients' samples.

3.10. Clinical applications

This analytical method proved reliable and sensitive for monitoring concentrations of antimalarial/metabolites in plasma isolated from patients (see examples in Figure 4, Figure 5 and Figure 6). It is being used in a field study done by the Swiss Tropical Institute in collaboration with the National Center for Parasitology, Entomology and Malaria Control and the Pasteur Institute of Cambodia, and with the Ifakara Health Institute in Tanzania. This study addresses the influence of host pharmacogenetic factors on plasma concentrations of antimalarials and their consequences on clinical responses and toxicities. Our method is being applied to the measurement of plasma levels of artesunate, DHA, mefloquine and piperaquine and artemether, DHA, lumefantrine, desbutyl-lumefantrine.

4. Conclusion

This analytical method offers advantages over previously published methods in terms of convenience (a single extraction procedure for 14 major antimalarials and metabolites reducing significantly the analytical time), sensitivity, selectivity and throughput. Mass spectrometry has already been used in malaria, but so far limited to single or chemically related compounds, and in some cases compromised by ion suppression, a recently identified problem [103,104]. Here, ion suppression and potential matrix effects have been comprehensively investigated (see Table 5 and Figure 3) following the FDA's guidelines [131] and the recommendations of Matuszewski et al. [135].

Developing an analytical method for the simultaneous determination of as many as 14 antimalarials/metabolites in the same chromatographic run was a challenge because current antimalarial drug combinations (ACTs) comprise both basic lipophilic drugs and structurally

unrelated artemisinin derivatives with distinctly different chromatographic behaviour, lipophilicity and ionization efficiency. Among the various column types tested (i.e. X-Terra, Symmetry, etc.) the Atlantis-d18 (Waters) performed best in terms of retention time and peak shape reproducibility and robustness. The use of this column permitted in particular a reliable chromatographic elution of pyronaridine and piperaquine, which tended to be eluted unretained (i.e. running near the solvent front) onto some of the columns tested, or appearing as erratic broad-shape peaks, which precluded accurate quantification. Careful control of the solution A (pH 2.8) with addition of 0.5% formic acid and of the gradient elution program of the mobile phase is mandatory for standardizing each drug's peak shape and retention time. Retention time reproducibility is particularly important, as two segments of data acquisition are programmed in the positive mode detection for the simultaneous analysis of antimalarials/metabolites in the same run. Given the number of analytes in the proposed method, an increase in the number of MS/MS transitions recorded simultaneously would result in reduced sensitivity because of the time required for data acquisition at each of the selected MS/MS transition channels. To circumvent this limitation, two consecutive sets of MS/MS transitions in the positive mode were programmed during the analytical run (see Section 2.4).

The lack of deuterated antimalarial derivatives may be regarded as a limitation of our analytical method. However, they are generally not accessible; making them available would resolve questions on matrix effect interferences and, more generally, facilitate analytical routine. So far though, to the best of our knowledge, of the nearly 30 LC–MS/MS methods published for the assay of single or a few chemically related or unrelated antimalarials, only very few have used deuterated analogues as I.S., and this only the more recent antimalarials [101,102,104,140,141].

More generally, with the LC–MS/MS technology, one will always be confronted with the residual uncertainty as to the potential influence of unknown, or known, but generally not available, metabolites of antimalarial drugs. Making standard substances (e.g. relevant metabolites, available deutered I.S.) available through a centralized warehouse would be extremely useful for analytical standardization and cross-validation worldwide. This is probably an aspect worth being addressed by initiatives like the World Antimalarial Resistance Network (WARN) [40].

A solvent at pH 4.0 for the reconstitution of dried plasma residues was suited to both the basic antimalarials (expected to be in their protonated form as their pK_a's range 8–9) and artemisinin compounds, which were found to be stable at this pH. Acidic pH (i.e. EtOH-0.1% glacial acetic acid 1:1) has already been used to solubilise artemether and DHA [116].

Concerning recovery, the volume of MeOH and buffer pH 4.0 1:1 chosen for the reconstitution of dried extracts aimed at achieving the lowest possible limit of quantification given the wide differences in the solubilities in organic solvents and buffers, polarity and ES ionization performance of the 14 analytes. Given the large number of compounds, this 1:1 ratio was a compromise that takes into account the clinically relevant plasma target concentrations for each antimalarial and metabolite. Overall, the precision and the accuracy of the low calibration sample were, for all antimalarials, within the ±20% limit recommended by the FDA and the Washington and Arlington Conference Reports [131-133]. Most importantly, the LLOQs were far below the clinically relevant levels obtained in patients, and either similar to (piperaquine, DHA, artesunate, arthemether) or better than (pyronaridine, mefloquine, desethyl-amodiaquine, desbutyl-lumefantrine, lumefantrine) previous methods (see Table 4, and references cited therein). The proposed method enables to quantify, depending on the drug analysed, as little as 1.5–12.5 pg on-column, thus allowing to detect the "tail" of the levels produced by the long-acting drugs (lumefantrine, piperaquine, pyronaridine, quinine, mefloquine, sulfadoxine-pyrimethamine), and to quantify drug levels at critical time-points, such as for instance 7 days after treatment start (which was found to correlate with exposure and treatment outcome in the case of lumefantrine) [40], and references cited therein]. The availability of very sensitive LC–MS/MS methods is critical to determining accurately the terminal half-life of slowly eliminated antimalarials, such as piperaquine [142].

With regard to the instability of endoperoxide antimalarials in the presence of hemoglobin, our experiments done with control and hemolysed plasma from uninfected and malaria patients indicate that artemisinin added as I.S. appears to be comparatively more vulnerable than DHA, AS and AM to the presence of hemoglobin. This makes ART unsuited as a formal I.S. in case of hemolytic samples but a sensitive surrogate for hemolysis. In fact, with an alternate methodology recently reported elsewhere by our group [136], ART may indeed be used as I.S. for the analysis of hemolysed plasma samples: since artemisinin compounds are reported to react with (ferrous) Fe^{2+} – but not ferric (Fe^{3+}) – heme from Hb [141], the addition

of sodium nitrite, a known methemoglobin-forming agent [143] to hemolysed plasma samples prevent most of the apparent DHA or AS degradation in hemolysed samples and, importantly, to the same proportion as ART added as I.S.

For these reasons, and since stable isotopes-labeled compounds were not available as I.S. to us during the course of this analytical development, we had trimipramine-D_3 as I.S. for this broad-range analysis, as it does not react with Hb and is particularly well suited for the other antimalarial drugs considered in this analysis (i.e. lipophilic alkylamines).

The main shortcoming of LC–MS/MS is the high acquisition and maintenance costs, which makes it unaffordable for many clinical or academic research laboratories, particularly in malaria endemic countries, thus limiting its spread and applicability. It is therefore certainly of importance to also address the problem of the more appropriate procedure for samples collection in field conditions. In that context, we have demonstrated that all drugs (i.e. including artemisinin drugs) are stable for up to 48 h in *plasma* stored at 4 °C (Figure 7, 4 °C). Therefore, samples can be collected in the field, and easily kept and transported to the nearest laboratory where they can be frozen and stored until assayed or further shipped to their final destination.

This broad-range LC–MS/MS method is suited for detecting drug use in a population (e.g. upon enrolment in a trial or to assess real antimalarial drugs consumption) and measuring drug exposure in treated patients and will help improving our understanding of antimalarial drugs pharmacokinetics, efficacy and toxicity. This is of particular relevance in this era of antimalarial combination therapies and co-morbidities (notably HIV co-infection and potential drug-drug interactions with concomitant medication, notably antiretroviral drugs.) Building upon previous analytical and population pharmacokinetics expertise with antiretrovirals [144,145] a better knowledge on the actual antimalarials plasma exposure and more precise definition of the pharmacokinetic/pharmacodynamic relationships will ultimately result in a more precise definition of the therapeutic ranges for the different drugs in different patient populations. The methodology presented here allows the measurement of total antimalarial drugs levels in plasma whereas the site of their antimalarial activity is expected to be mostly within erythrocytes. To that endeavour, we are currently attempting to further develop the LC–MS/MS assay to measure free and total levels in whole blood and red-blood cells.

CHAPTER 2

Single nucleotide polymorphisms in cytochrome P450 isoenzyme and *N*-acetyltransferase-2 genes in malaria patients from Cambodia and Tanzania – potential explanation for the lower efficacy of artemether–lumefantrine in Cambodia

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Abstract

One of the most important control measures in malaria is prompt diagnosis and treatment with an effective and safe combination of antimalarial drugs. The analysis of genetic polymorphisms in drug metabolizing enzymes is a step towards a broader understanding of inter-individual differences in pharmacokinetic and pharmacodynamic profiles and consequential treatment failures and adverse drug reactions. We analysed in this study allele frequencies of single nucleotide polymorphisms (SNPs) in genes encoding enzymes involved in the metabolism of antimalarials, namely cytochrome P450 isoenzymes (*CYP*) and *N*-acetyltransferase-2 (*NAT2*), in samples from Cambodia and Tanzania. Major differences were found in allele frequencies of *CYP2D6*, *CYP3A4*, *CYP3A5*, and *NAT2*. The wide difference in *CYP3A4*1B* allele frequency between the two populations presents a potential explanation for the lower efficacy of artemether–lumefantrine in Cambodia and highlights the importance of pharmacogenetic considerations in the decision-making process of first-line treatment policies for specific populations.

Introduction

One of the most important control measures in malaria is prompt diagnosis and treatment with an effective and safe combination of antimalarial drugs. Both drug efficacy and safety are strongly dependent on the achievement of appropriate circulating concentrations under treatment, with insufficient exposure associated with a risk of failure and resistance emergence, and too high levels with a risk of toxicity.

The analysis of genetic polymorphisms in genes encoding proteins and enzymes involved in drug absorption, distribution, metabolism, elimination and action is a step towards a broader understanding of inter-individual differences in pharmacokinetic and pharmacodynamic profiles and consequential treatment failures and adverse drug reactions. It has been stated that polymorphism of drug-metabolizing enzymes have by far the highest impact on inter-individual differences in drug response [47]. Depending on the alleles an individual is carrying, the metabolism can be altered. Certain enzyme polymorphisms can enhance drug metabolism, whereas others abolish or decrease drug metabolism, and frequencies of such polymorphisms vary among different ethnic groups [50,51]. As a consequence, ethnicity may have a major impact on drug metabolism and hence drug efficacy and safety. This might be of

particular interest for antimalarial drugs where a rapid onset of the antiparasitic effect (sufficient C_{max} and short t_{max}) and a slow elimination (long $t_{½}$) to protect against recrudescence are required for successful treatment. A deeper insight in the inter-population variability in the profile of genes encoding enzymes responsible for antimalarial drug metabolism could facilitate the selection of appropriate first-line treatment for uncomplicated malaria in a specific population.

For this study, we have chosen two populations reported to differ in their clinical response to current antimalarial treatment, namely to artemisinin-based combination therapies (ACTs). In Tanzania, the *in vivo* efficacy of ACTs have been reported to be excellent [146], whereas from Cambodia decreasing efficacies were reported with artemether-lumefantrine [147]. We have investigated single nucleotide polymorphisms (SNPs) in genes of the phase I cytochrome P450 enzyme family (*CYP*) and the phase II *N*-acetyltransferase-2 (*NAT2*) in malaria patients from Cambodia and Tanzania. Both, *CYP* and *NAT2* are involved in the metabolism of various antimalarial drugs. The responsible enzymes with established polymorphisms are *CYP2A6* and *CYP2B6* for the artemisinins, *CYP2C8* for amodiaquine, chloroquine and dapsone, *CYP2C9* and *NAT2* for dapsone and sulfamethoxazole, *CYP2C19* for dapsone and proguanil, *CYP2D6* for chloroquine and halofantrine, *CYP3A4* for the artemisinins, chloroquine, dapsone, halofantrine, lumefantrine, mefloquine, primaquine and quinine, and *CYP3A5* for *β*-arteether, artemether, chloroquine, mefloquine, quinine and sulfadoxine [37,44,52,59-79]. However, for some antimalarial drugs (i.e. piperaquine, pyrimethamine and pyronaridine) the metabolic pathway is still not very well known, or they are barely metabolized at all (i.e. atovaquone and doxycycline) [53-58].

Materials and methods

During an *in vivo* drug efficacy study in patients with uncomplicated malaria of all age, venous blood samples (anticoagulated using EDTA) were obtained after informed consent from 125 patients in Northern and Western Cambodia (64 in 2007 at Phnom Dék Health Centre, Rovieng district, Preah Vihear province, and 61 in 2008 at Pramoy Health Centre, Veal Veng district, Pursat province) and 149 patients in Central Tanzania (in 2008 at Kibaoni Health Centre, Kilombero district, Morogoro region).

Study population in Cambodia

In Cambodia more than 90 % of the people are Khmer ethnic. The ethnic minorities include Chinese, Chams (Muslim descendants of Cham refugees who fled to Cambodia after the fall of Champa), Khmer Loeu (collective term for Mon-Khmer or Austronesian speaking hill tribes), and Vietnamese. In the Preah Vihear and Pursat province Khmer Loeu people form the most represented minority and they tend to live in separate villages.

Study population in Tanzania

The study in Tanzania was conducted at Kibaoni Health centre in Ifakara town in Kilombero districts. The town lies in the flood plain of the Kilombero river valley at approximately 36.41 degrees East and 8.8 degrees South. The total population of the study area is approximately 90 000. The inhabitant of the Kilombero river valley live in widely scattered households in the rice field plains. Most people are subsistence farmers of various ethnic groups and recently highly mobile pastoralist ethnic groups , i.e. Masaai, Barbaig and Sukuma, have immigrated in the area.

Laboratory procedures

Whole blood samples from Cambodia where immediately stored in liquid nitrogen for one week and then transferred into a -80°C freezer. In Tanzania, samples were kept on ice for no longer than 6 h after withdrawal and then stored in a -80°C freezer. Genomic DNA was extracted from 200 µl whole blood using the QIAamp 96 DNA Blood Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions.

Pharmacogenetic analysis

Target genes with SNP known to alter enzyme activity involved in the metabolism of antimalarial drugs were selected for the analysis. This information was obtained from the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se/), and the Consensus Human Arylamine *N*-Acetyltransferase Gene Nomenclature (http://louisville.edu/medschool/pharmacology/NAT.html). Selected target sequences containing the respective SNPs in *CYP* and *NAT2* genes were amplified by polymerase chain reaction (PCR)

using primers shown in Table 1. The following SNPs were selected: *CYP2A6*2* (479T>A, L160H), *CYP2B6*5* (1459C>T, R487C), *CYP2B6*6* (only 516G>T, Q172H, also called *CYP2B6*9*), *CYP2C8*3* (only 416G>A, R139K), *CYP2C9*3* (1075A>C, I359L), *CYP2C9*5* (1080C>G, D360E), *CYP2C19*3* (636G>A, W212X), *CYP2D6*4* (1846G>A, splicing defect), *CYP2D6*10* (100C>T and 4180G>C, P34S and S486T), *CYP2D6*17* (1023C>T and 2859C>T, T107I and R296C), *CYP3A4*1B* (-392A>G), *CYP3A5*3* (6986A>G, splicing defect), *NAT2*5* (341T>C, I114T), *NAT2*6* (590G>A, R197Q), *NAT2*7* (857G>A, G286E), and *NAT2*14* (191G>A, R64Q). The reverse primer for *CYP2D6*10* (100C>T) and the forward primer for *CYP2D6*17* (1023C>T) contained a mismatch at the second position at the 3' end in order to avoid amplification of the pseudogene *CYP2D7*. For the PCR 1 µl of purified DNA was mixed with 24 µl of the PCR master mix. The PCR master mix contained 2.5 µl 10 × reaction buffer B (Solis BioDyne, Estonia), 2.5 µl 10 × solution S (Solis BioDyne, Estonia), 2.5 µl primer mix (see Table 1, each primer 10 µM in 1 × Tris-EDTA, Operon Biotechnologies GmbH, Germany), 2 – 3 µl $MgCl_2$ 25mM (according to Table 1, Solis BioDyne, Estonia), 2.5 µl dNTP mix (each nucleotide 2mM in Tris-HCl 10 mM, pH 7.4, GE Healthcare, Switzerland) and 0.4 µl FIREPol DNA polymerase 5 U/µl (Solis BioDyne, Estonia) and dH_2O (Milli-Q Advantage A10, Millipore AG, Switzerland) was added up to 24 µl. The PCR protocol was 3 min at 96 °C followed by 40 cycles (30 sec at 96 °C, 1 min 30 sec at 56 – 64 °C according to Table 1 and 1 min 30 sec at 72 °C) with a final elongation for 10 min at 72 °C. The PCR products were then sent to Macrogen, Ltd., Korea for purification and sequencing using the PCR primers highlighted in bold and listed in Table 1.

Data analysis

Sequences were analysed using the ABI Prism AutoAssembler version 1.4.0 (Applied Biosystems) for assembly. The genotype of each patient was then assessed visually. Hardy-Weinberg equilibrium was tested using the chi-square Hardy-Weinberg equilibrium test calculator for biallelic markers of the Online Encyclopedia for Genetic Epidemiology studies (http://www.oege.org). Differences of allele frequencies between populations were tested using 2 × 2 tables and Fischer's exact test. A Bonferroni correction for multiple comparisons was performed for both tests and a *P*-value of <0.003 was considered significant. The fixation index (F_{ST}) was calculated using Arlequin version 3.1 [148] in order to measure population differentiation based on the investigated SNPs.

Table 1. Primers, $MgCl_2$ [µl] for the master mix and annealing temperature [°C] used to amplify target sequence in cytochromes P450 isoenzyme genes and *N*-acetyltransferase 2 genes.

SNP	Primer		$MgCl_2$ [µl]	T [°C]
*CYP2A6*2*	Forward	**5′-TCTCTCTCTCTACCTCGACAT-3′**	3	64
	Reverse	5′-GTTCCTCGTCCTGGGTGTT-3′		
*CYP2B6*5*	Forward	**5′-CCAGAAGACATCGATCTGAC-3′**	2	64
	Reverse	5′-TCTCTCAGAGGCAGGAAGTT-3′		
*CYP2B6*6*	Forward	**5′-TGAGTGATGGCAGACAATCACA-3′**	2	64
	Reverse	5′-CAAGTTGAGCATCTTCAGGAACT-3′		
*CYP2C8*3*	Forward	**5′-CTAAAGGACTTGGTAGGTGCA-3′**	2	64
	Reverse	5′-CAGGATGCGCAATGAAGACC-3′		
*CYP2C9*3/*5*	Forward	**5′-CTGGTTTATGGCAGTTACACATT-3′**	2	64
	reverse	5′-GAGAAAGTCCAGTTAAACTGCC-3′		
*CYP2C19*3*	forward	**5′-GGGAATTCATAGGTAAGATATTA-3′**	2	56
	reverse	5′-GGAGTGATATAAGCACGCTTTG-3′		
*CYP2D6*4*	forward	**5′-CCGCCTTCGCCAACCACT-3′**	2	64
	reverse	5′-CCCTGCAGAGACTCCTCGGT-3′		
*CYP2D6*10* (100C>T)	forward	**5′-CCCATTTGGTAGTGAGGCAGGT-3′**	2	64
	reverse	5′-CCCCTTCTCAGCCTGGCTTCTTG-3′		
*CYP2D6*10* (4180G>C)	forward	5′-AGCCACCATGGTGTCTTTGCT-3′	2	64
	reverse	**5′-TTGCCCTGAGGAGGATGATC-3′**		
*CYP2D6*17* (1023C>T)	forward	**5′-CGCGAGGCGCTGGTGACCAA-3′**	2	64
	reverse	5′-CCAGCTCGGACTACGGTCATCAC-3′		
*CYP2D6*17* (2850C>T)	forward	**5′-GACTCTGTACCTCCTATCCACGTCA-3′**	2	64
	reverse	5′-TCCCTCGGCCCCTGCACTGTTT-3′		
*CYP3A4*1B*	forward	**5′-CTCACCTCTGTTCAGGGAAAC-3′**	2	64
	reverse	5′-ATGGCCAAGTCTGGGATGAG-3′		
*CYP3A5*3*	forward	**5′-TGGAGAGTGGCATAGGAGATAC-3′**	2.5	64
	reverse	5′-CCATACCCCTAGTTGTACGACACA-3′		
NAT2	forward	5′-GGGATCATGGACATTGAAGCATATT-3′	2	64
	reverse	**5′-ACGTGAGGGTAGAGAGGATATCTG-3′**		

Primers highlighted in **bold** were used for sequencing.

Ethical approval

All the applied protocols were approved by the ethics committee of the two cantons of Basel (Ethikkommission beider Basel) and the responsible local authorities (Medical Research Coordination Committee of the National Institute for Medical Research in Tanzania and National Ethics Committee for Health Research in Cambodia). Blood samples were obtained after written informed consent in the local language (Khmer or Swahili) from the participants or their responsible guardians.

Results

We have sequenced amplified PCR fragments flanking relevant SNPs in *CYP* and *NAT2* genes from 125 Cambodian and 150 Tanzanian malaria patients. Allele frequencies were calculated for each population and each SNP (Tables 2 and 3) however, sequence data were not always available for all samples.

Most allele frequencies were found to be in Hardy-Weinberg equilibrium. However, in the Tanzanian study population *CYP2B6*5* and *CYP2D6*4* and in the Cambodian study population *CYP2B6*5*, *CYP2C9*3*, *CYP2D6*10* (4180G>C), and *CYP2D6*17* (2850C>T) were found not to have Hardy-Weinberg proportions.

No mutation was observed in both populations for *CYP2A6*2* and *CYP2C8*3*. Furthermore, in Tanzania no mutation was observed in *CYP2C9*3* but the mutation was found at a frequency of 6.56% in Cambodia. The reverse was observed for *CYP2C9*5* and in *CYP2D6*4* which were not mutated in Cambodia whilst both genes were found mutated at low frequencies in Tanzania (0.76% and 4.1%, respectively). *CYP2D6*17* showed no mutation in Cambodia but was found at high frequencies mutated in Tanzania (20.26%) (Table 2).

High frequencies of mutated alleles were observed in both populations for *CYP2B6*6, CYP2D6*10*, and *NAT2*6,* whereas *CYP2B6*5* and *CYP2C19*3* were found only at low frequencies mutated in both populations.

Major differences in frequencies of mutated alleles between both populations was observed for *CYP2D6*10* (60.56% to 6.62%), *CYP3A5*3* (64.52% to 18.40%), and *NAT2*7* (20.49% to 2.36%) all showing high frequencies of mutation in Cambodia but low frequencies in Tanzania. In contrast, *CYP2D6*17* (7.69% to 60.64%), *CYP3A4*1B* (3.72% to 73.51%), *NAT2*5* (6.56% to 36.36%), and *NAT2*14* (2.48% to 14.54%) showed lower mutation frequencies in Cambodia than in Tanzania.

Differences in frequencies were found significant in 11 SNPs and no significant differences were observed in 7 SNPs (Tables 2 and 3). The F_{ST}- value was 0.16033 at 5 % significance level.

Table 2. Allele frequencies in cytochrome P450 isoenzyme genes in Cambodia and Tanzania.

		Cambodia				Tanzania				P_F
		n	**% SNP**	χ^2	P_χ	***n***	**% SNP**	χ^2	P_χ	
*CYP2A6*2*	T/T	123	0.00			148	0.00			
	T/A	0				0				
	A/A	0				0				
*CYP2B6*5*	C/C	116	3.31	66.52	<0.003	145	1.69	93.91	<0.003	0.108
	C/T	2				1				
	T/T	3				2				
*CYP2B6*6*	G/G	38	38.30	0.93	0.335	56	34.30	2.31	0.129	<0.003
	G/T	40				47				
	T/T	16				18				
*CYP2C8*3*	G/G	75	0.00			69	0.00			
	G/A	0				0				
	A/A	0				0				
*CYP2C9*3*	A/A	109	6.56	13.38	<0.003	131	0.00			<0.003
	A/C	10				0				
	C/C	3				0				
*CYP2C9*5*	C/C	122	0.00			129	0.76	0.01	0.920	0.268
	C/G	0				2				
	G/G	0				0				
*CYP2C19*3*	G/G	117	2.82	0.10	0.752	139	0.71	0.01	0.920	0.049
	G/A	7				2				
	A/A	0				0				
*CYP2D6*4*	G/G	74	0.00			126	4.10	37.07	<0.003	0.007
	G/A	0				5				
	A/A	0				3				
*CYP2D6*10* (100C>T)	C/C	19	60.56	2.30	0.129	119	6.62	0.31	0.578	<0.003
	C/T	33				16				
	T/T	38				1				
*CYP2D6*10* (4180G>C)	G/G	17	71.31	9.78	<0.003	13	73.59	1.79	0.181	0.065
	G/C	36				49				
	C/C	69				80				

Table 2. Continued.

		Cambodia				Tanzania				P_F
		n	**% SNP**	χ^2	P_χ	***n***	**% SNP**	χ^2	P_χ	
*CYP2D6*17* (1023C>T)	C/C	66	0.00			74	20.26	0.02	0.888	<0.003
	C/T	0				37				
	T/T	0				5				
*CYP2D6*17* (2850C>T)	C/C	46	7.69	10.92	<0.003	9	60.64	1.10	0.294	<0.003
	C/T	4				19				
	T/T	2				19				
*CYP3A4*1B*	A/A	87	3.72	0.14	0.708	8	73.51	0.39	0.532	<0.003
	A/G	7				55				
	G/G	0				71				
*CYP3A5*3*	A/A	16	64.52	0.02	0.888	98	18.40	1.39	0.238	<0.003
	A/G	56				39				
	G/G	52				7				

n indicates the number of patients, % SNP the mutated allele frequency in percent, χ^2 the result from the Hardy-Weinberg equilibrium test and P_χ the one-tailed *P*-value. P_F is the *P*-value of Fischer's exact test for differences between populations.

Table 3. Allele frequencies in *N*-acetyltransferase 2 gene in Cambodia and Tanzania.

		Cambodia				Tanzania				P_F
		n	**% SNP**	χ^2	P_χ	***n***	**% SNP**	χ^2	P_χ	
*NAT2*5*	T/T	106	6.56	0.60	0.439	58	36.36	0	1.000	<0.003
	T/C	16				66				
	C/C	0				19				
*NAT2*6*	G/G	44	39.75	0.01	0.920	84	26.69	3.51	0.061	<0.003
	G/A	59				49				
	A/A	19				15				
*NAT2*7*	G/G	78	20.49	0.24	0.624	141	2.36	0.09	0.764	<0.003
	G/A	38				7				
	A/A	6				0				
*NAT2*14*	G/G	115	2.48	0.08	0.777	107	14.54	7.42	0.006	<0.003
	G/A	6				27				
	A/A	0				7				

n indicates the number of patients, % SNP the mutated allele frequency in percent, χ^2 the result from the Hardy-Weinberg equilibrium test and P_χ the one-tailed *P*-value. P_F is the *P*-value of Fischer's exact test for differences between populations.

Discussion

In this study we found alleles that showed similar frequencies in both populations and others that varied greatly in malaria patients from Cambodia and Tanzania. The F_{ST}-value implies that the degree of differentiation among the two populations is moderate. The finding that the majority of samples was found to be in Hardy-Weinberg equilibrium proves that sampling was unbiased. The few cases where population data were found not to be in Hardy-Weinberg proportion are most likely due to the small sample size (and thus might represent chance findings) or due to the Wahlund effect, i.e. reduction in heterozygosity due to highly diverging subpopulations.

Alleles showing major differences were of *CYP2D6, CYP3A4, CYP3A5,* and *NAT2*. This is in accordance with previous reports showing that allele frequencies of *CYP2D6* and *NAT2* vary considerably between continents or even countries [149,150]. There is little information available on allele frequencies of *CYP* and *NAT2* gene polymorphisms in Tanzanian or Cambodian populations. Sistonen *et al*. investigated *CYP2D6* haplotype frequencies in single populations and found in Cambodia no polymorphism in *CYP2D6*4* and *CYP2D6*17* genes but 54.5 % mutated *CYP2D6*10* [150] which is comparable with our data. A study on *CYP2D6* genotypes in Tanzania also reported a low allele frequency for *CYP2D6*4* (1 %) and intermediate frequency for *CYP2D6*17* (17 %) [151], the latter showing a much higher frequency in our study. The small number of available sequence data for some of the *CYP2D6* loci was probably due to rather short PCR products or a low PCR efficiency. Short PCR products tend to fail sequencing due to stuttering at the beginning of sequencing. The PCR primers were initially designed for use in a DNA microarray and no attention had been paid to the length of the PCR product. It is also possible that mismatches incorporated into the primer to avoid amplification of the pseudogene *CYP2D7* might reduce PCR efficiency. In future studies primers, especially for *CYP2D6,* should be redesigned.

The observed low frequency of *CYP2C19*3* confirms previous findings from Tanzania where no *CYP2C19*3* alleles were found [152]. The allele frequencies we report for *CYP2B6*6* (516G>T), *CYP3A4*1B* and *CYP3A5*3* are very similar to those previously described for three Tanzanian populations. One study from Zanzibar reported allele frequencies of 32.0 % for *CYP2B6*6* (516G>T), 69.2 % for *CYP3A4*1B* and 15.8 % for *CYP3A5*3,* and two studies from mainland Tanzania reported allele frequencies of 73.2 % for *CYP3A4*1B* and 19.0 % for *CYP3A5*3* [152-154]. A comparison of our allele frequencies with frequency data

for Asians and Africans (available for *CYP2A6*2*, *CYP2B6*5*, *CYP2B6*6* (516G>T), *CYP2C8*3*, *CYP2C9*3*, *CYP2C19*3*, *CYP3A4*1B*, and *CYP3A5*3*) on the National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism (SNP) Homepage showed high concordance [155].

The major differences between the Cambodian and Tanzanian population in allele frequencies were observed for *CYP2D6*10*, *CYP3A4*1B*, and *CYP3A5*3*. *CYP3A4* has been implicated in metabolizing lumefantrine, and *CYP3A4* and *CYP3A5* are involved in the metabolism of artemether [61]. Epidemiological studies reported an association of the *CYP3A4*1B* allele with risk of higher grade of prostate cancer and risk of developing secondary acute myoblastic leukaemia following treatment with epipodophyllotoxins due to altered metabolism of testosterone and the chemotherapeutic [156,157]. A study on tacrolimus concluded that increased doses should be given to *CYP344*1B* carriers [158] and similar recommendations were given in another study on docetaxel [159]. These findings have initiated discussions about whether interindividual variability in hepatic *CYP3A4* activity is due to genetic factors. The author of the original prostate cancer study repeatedly defended the theory that there might be a downstream effect of the *CYP3A4*1B* polymorphism on some metabolic pathways that could be physiologically relevant [160,161]. Other authors doubted the described relationship between *CYP3A4*1B* allele and the phenotype [162-170]. The debate could not even be ended by an extensive review on the subject [171]. Keshava *et al.* concluded that (i) there is a lack of evidence to date that the major polymorphic variants in *CYP3A4* have any association with *CYP3A4* activity, (ii) that there is evidence for a correlation between *CYP3A4*1B* and early life events associated with breast cancer risk and (iii) that the five prostate cancer studies reviewed did not provide convincing support for a direct role of *CYP3A4*1B* in prostate carcinogenesis. Some authors suggested that a linkage disequilibrium between *CYP3A4*1B* and *CYP3A5*1A* (and thus increased *CYP3A5* expression) could be the actual cause of the altered metabolism [163,168,172]. Therefore, it is interesting to observe a high allelic frequency of the mutated *CYP3A4*1B* and low frequency of the wild type *CYP3A5*1* in Tanzania and *vice versa* in Cambodia. Lumefantrine, the long-acting component of the most widely used ACT in Africa is metabolized by *CYP3A4*. The artemisinin component (i.e. artemether) is metabolized by *CYP3A4* and *CYP3A5* and hence differences in the efficacy of artemether–lumefantrine (AL) might be explained by pharmacogenetics. Studies in Cambodia on efficacy of AL showed cure rates of only 71.1% [147]. These high treatment failure rates could not be explained by food intake nor could

parasite resistance be demonstrated using molecular markers [147,173]. Thus, one could speculate that host factors might influence treatment outcome. With our findings we can hypothesize that Cambodian subjects probably metabolize AL differently than African patients and that therapeutic plasma levels may not have been sustained long enough in Cambodian subjects [174]. The fact that AL may be of limited use as alternative treatment against malaria in Cambodia led to the continued use of artesunate–mefloquine (AM). In 1982, the first mefloquine resistant parasites were found along the Thai-Cambodian border [175]. Due to high levels of resistance against mefloquine [176], AM virtually represents a monotherapy and might have a non negligible effect on the emergence of the reduced susceptibility to artemisinins observed in South-East Asia [177-184]. Therefore, it seems important to deepen our understanding of the treatment failures under AL in Cambodia. The impact of altered drug metabolism needs to be confirmed by pharmacokinetic studies in malaria patients and we intend to analyze this genotype-phenotype association in the patients in a recently conducted study (AL2002 study).

This highlights the need to include pharmacogenetic data in the evaluation of antimalarial treatment regimens in specific ethnic groups. Should the above findings be confirmed it would be essential to gather molecular data for the selection of an appropriate first-line treatment for uncomplicated malaria in specific populations.

CHAPTER 3

A microarray based system for the simultaneous analysis of single nucleotide polymorphisms in human genes involved in the metabolism of antimalarial drugs

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Abstract

Background

In order to provide a cost-effective tool to analyse pharmacogenetic markers in malaria treatment, we compared DNA microarray technology with sequencing of polymerase chain reaction (PCR) fragments to detect single nucleotide polymorphisms (SNPs) in a larger number of samples.

Methods

The microarray was developed to affordably generate SNP data of genes encoding the human cytochrome P450 enzyme family (*CYP*) and *N*-acetyltransferase-2 (*NAT2*) involved in antimalarial drug metabolisms and with known polymorphisms, i.e. *CYP2A6, CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, *CYP3A5*, and *NAT2*.

Results

For some SNPs, i.e. *CYP2A6*2*, *CYP2B6*5*, *CYP2C8**3, *CYP2C9*3/*5*, *CYP2C19*3*, *CYP2D6*4* and *NAT2*6/*7/*14*, agreement between both techniques ranged from substantial to almost perfect (kappa index between 0.61 and 1.00), whilst for other SNPs a large variability from slight to substantial agreement (kappa index between 0.39 and 1.00) was found, e.g. *CYP2D6*17* (2850C>T), *CYP3A4*1B* and CYP3A5*3.

Conclusion

The major limit of the microarray technology for this purpose was lack of robustness and with a large number of missing data or with incorrect specificity.

Background

Drug action depends on how drugs are metabolized and differences in activity of metabolizing enzymes can significantly contribute to the efficacy of drugs [47,185]. This might also be true for drugs given to treat malaria. We intended to analyse single nucleotide polymorphisms (SNPs) in genes encoding enzymes implicated in metabolizing antimalarial drugs in order to determine the contribution of these enzymes to the pharmacokinetics of the specific drugs. Standard methods to detect SNPs, such as the polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) approach [186,187] are usually time consuming, expensive and/or not suitable for use in resource-poor countries. Here we compare an alternative, DNA microarray based [188] technique to detect SNPs in a larger sample size with sequencing. The microarray was developed to affordably generate SNP data of genes encoding the human cytochrome P450 enzyme family (*CYP*) and *N*-acetyltransferase-2 (*NAT2*) involved in antimalarial drug metabolisms. The respective enzymes with known polymorphisms are *CYP2A6* and *CYP2B6* for the artemisinins, *CYP2C8* for amodiaquine, chloroquine and dapsone, *CYP2C9* and *NAT2* for dapsone and sulfamethoxazole, *CYP2C19* for dapsone and proguanil, *CYP2D6* for chloroquine and halofantrine, *CYP3A4* for the artemisinins, chloroquine, dapsone, halofantrine, lumefantrine, mefloquine, primaquine and quinine, and *CYP3A5* for artemether, *β*-arteether, chloroquine, mefloquine, quinine and sulfadoxine [37,44,52,59-79]. For certain antimalarial drugs (piperaquine, pyrimethamine and pyronaridine) the metabolic pathway is yet not well known, while others (atovaquone and doxycycline) are barely metabolized at all [53-58].

Methods

Sequencing

Samples were collected in the context of an *in vivo* treatment study (CHAPTER 6) assessing the effect of pharmacogenetics on the pharmacokinetic profile of antimalarials in malaria patients from Cambodia (n = 125) and Tanzania (n = 149). Target sequences in *cyp* and *nat2* genes of these samples were amplified using PCR. The PCR primers used are listed in Table 1 and the full protocol has been described elsewhere (CHAPTER 2). The amplified regions contained SNPs which are known to alter the function of enzymes involved in the metabolism of antimalarial drugs (for target loci and effect of the SNP see Table 1). PCR products were purified and sequenced by Macrogen (Macrogen Ltd., Korea). ABI Prism AutoAssembler

version 1.4.0 (Applied Biosystems) was used for assembly and analysis of sequences. The genotype of each patient was then assessed visually. Aliquots of the same PCR products were used for primer extension and microarray analysis.

Table 1. Primers used to amplify target sequences in chytochromes P450 isoenzymes and *N*-acetyltransferase 2 genes.

SNP	**Primer**	
*CYP2A6*2* (479T>A, L160H)	forward	**5′-TCTCTCTCTCTACCTCGACAT-3′**
	reverse	5′-GTTCCTCGTCCTGGGTGTT-3′
*CYP2B6*5* (1459C>T, R487C)	forward	**5′-CCAGAAGACATCGATCTGAC-3′**
	reverse	5′-TCTCTCAGAGGCAGGAAGTT-3′
*CYP2B6*6* (516G>T, Q172H)	forward	**5′-TGAGTGATGGCAGACAATCACA-3′**
	reverse	5′-CAAGTTGAGCATCTTCAGGAACT-3′
*CYP2C8*3* (416G>A, R139K)	forward	**5′-CTAAAGGACTTGGTAGGTGCA-3′**
	reverse	5′-CAGGATGCGCAATGAAGACC-3′
*CYP2C9*3/*5* (1075°>C, I359L / 1080C>G, D360E)	forward	**5′-CTGGTTTATGGCAGTTACACATT-3′**
	reverse	5′-GAGAAAGTCCAGTTAAACTGCC-3′
*CYP2C19*3* (636G>A, W212X)	forward	**5′-GGGAATTCATAGGTAAGATATTA-3′**
	reverse	5′-GGAGTGATATAAGCACGCTTTG-3′
*CYP2D6*4* (1846G>A, splicing defect)	forward	**5′-CCGCCTTCGCCAACCACT-3′**
	reverse	5′-CCCTGCAGAGACTCCTCGGT-3′
*CYP2D6*10* (100C>T, P34S)	forward	**5′-CCCATTTGGTAGTGAGGCAGGT-3′**
	reverse	5′-CCCCTTCTCAGCCTGGCTTCTTG-3′
*CYP2D6*10* (4180G>C, S486T)	forward	5′-AGCCACCATGGTGTCTTTGCT-3′
	reverse	**5′-TTGCCCTGAGGAGGATGATC-3′**
*CYP2D6*17* (1023C>T, T107I)	forward	**5′-CGCGAGGCGCTGGTGACCAA-3′**
	reverse	5′-CCAGCTCGGACTACGGTCATCAC-3′
*CYP2D6*17* (2850C>T, R296C)	forward	**5′-GACTCTGTACCTCCTATCCACGTCA-3′**
	reverse	5′-TCCCTCGGCCCCTGCACTGTTT-3′
*CYP3A4*1B* (-392A>G)	forward	**5′-CTCACCTCTGTTCAGGGAAAC-3′**
	reverse	5′-ATGGCCAAGTCTGGGATGAG-3′

Table 1. Continued.

SNP	Primer	
*CYP3A5*3* (6986A>G, splicing defect)	forward	**5′-TGGAGAGTGGCATAGGAGATAC-3′**
	reverse	5′-CCATACCCCTAGTTGTACGACACA-3′
*NAT2*5/*6/*7/*14* (341T>C, I114T / 590G>A, R197Q / 857G>A, G286E / 191G>A, R64Q)	forward	5′-GGGATCATGGACATTGAAGCATATT-3′
	reverse	**5′-ACGTGAGGGTAGAGAGGATATCTG-3′**

Primers highlighted in **bold** were used for sequencing. SNP positions are indicated in brackets; they were obtained from the Home Page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se/), and the Consensus Human Arylamine *N*-Acetyltransferase Gene Nomenclature (http://louisville.edu/medschool/pharmacology/NAT.html).

Patient selection for DNA microarray validation

Of 274 patients from Cambodia and Tanzania, 96 were selected (out of practical reason, i.e. 96-well plate) for validation of the microarray. Patients were assorted by the number of successfully sequenced SNPs and then their ID number. For all 96 selected patients at least 16 out of 18 SNPs have been successfully sequenced.

Extension control and elimination of non-incorporated nucleotides.

As extension control for the microarray, amplified nested PCR product from the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) from strains 3D7 (wildtype at loci *pfcrt76* and *pfcrt97*) and K1 (mutation at locus *pfcrt76* and wildtype at *pfcrt97*) was mixed in a ratio of 55%:45%. Primers and PCR conditions have been described elsewhere [188].

To eliminate non-incorporated nucleotides prior to primer extension, all nested PCR products of one blood sample were pooled and 10 µl of the pooled PCR products and 0.5 µl of the extension control mix were digested with 8 U shrimp alkaline phosphatase (SAP) and 4 µl 10 × SAP buffer (both Amersham Biosciences, Freiburg, Germany) in a reaction volume of 48 µl for 1 h at 37°C. SAP was inactivated by incubating samples for 15 min at 90°C.

Primer extension and denaturation

Since the microarray scanner used only supported dual-fluorescence measures simultaneously and because of the large similarity of the *cyp* genes, a strategy of three parallel reactions with different primer and dye combinations had to be applied. Different extension primer mixes (I, II and III) were prepared according to Table 2, each with a total volume of 320 µl containing the corresponding primers (62.5 nM final concentration of each primer, Operon Biotechnologies GmbH, Germany) diluted in 1 × Tris-EDTA (TE). Afterwards, three extension mixes (I, II and III) were prepared (final volume of 8 µl) containing 2 U of HOT TERMIPol® DNA Polymerase (Solis BioDyne, Estonia), 1.8 µl of 10 × Reaction Buffer C (Solis BioDyne, Estonia), 2.5 mM $MgCl_2$, and 0.625 µM of the corresponding Cy3- and Cy5-labeled ddNTPs (Perkin Elmer, Schwerzenbach, Switzerland); ddNTP mixes are listed in Table 3. Then, 12 µl SAP digested PCR product of each patient were mixed with 8 µl of the extension mix I, II or III, respectively. The following primer extension protocol was used: 1 min at 94°C followed by 40 cycles of 10 sec at 94°C followed by 40 sec at 50°C. The three mixes of each patient were then pooled again in a 96 well plate and mixed with 10 µl of the hybridization buffer. The hybridization buffer contained 37.5 µM EDTA pH 8.0, 7 pM of two differently labelled positive hybridization controls 5'-GCCTCCACGCACGTTGTGATATGTA-[Cy3]-3' and 5'-CTGTGACAGAGCCAACACGCAGTCT-[Cy5]-5' (Operon Biotechnologies GmbH, Germany), and 3% Sodium Dodecyl Sulfate (SDS). The plate was incubated for 1 min at 94°C and immediately chilled on ice for 2 min.

Microarray production and microarray hybridization

Aldehyde-activated ArrayIt® SuperAldehyde 2 glass slides with SuperMask™ 16 (EBN European Biotech Network, Dolembreux, Belgium) were used. Oligonucleotides (Operon Biotechnologies GmbH, Germany) corresponding to the antisense DNA of the extension primers, extension controls (Table 2) and positive hybridization controls were spotted onto the microarrays in triplicate. The spotting was done by the DNA Array Facility of the Center for Integrative Genomics, University of Lausanne, Switzerland, using solutions of 50 µM oligonuclotide in 180 mM phosphate buffer (pH 8.0). All oligonucleotides had a C7-aminolinker attached to the 3' end. Anchor oligonucleotides prelabeled with Cy3 and Cy5 and four oligonucleotides with a random sequence were added as positive and negative controls, respectively. Of the pooled and denatured primer extension reaction mixture 35 µl were

transferred to one well of the microarray, and 6 µl 20 × Standard Saline Citrate (SSC) were added. In each well, representing a single microarray, DNA from one patient was hybridized. Hybridization was carried out in a humid chamber at 50°C for 90 min. After hybridization, the slide was washed at room temperature in 2 × SSC with 0.2% SDS for 10 min, followed by a wash with 2 × SSC for 10 min, and a final wash with 2 × SSC plus 2% ethanol for 2 min. These three steps represent the first wash step. Slides were dried with compressed air.

Table 2**.** Extension primers of three parallel mixes.

SNP	**Primer**	**Mix**
*CYP2C8*3.1*	5′-GGAATTTTGGGATGGGGAAGA-3′	I
*CYP2A6*2*	5′-GCTTCCTCATCGACGCCC-3′	I
*CYP2D6*4*	5′-CCGCATCTCCCACCCCCA-3′	I
*CYP2D6*10* (100C>T)	5′-ACGCTGGGCTGCACGCTAC-3′	I
*CYP2D6*17* (2859C>T)	5′-AGCTTCAATGATGAGAACCTG-3′	I
*CYP2D6*17* (1023C>T)	5′-CCCGAAACCCAGGATCTGG-3′	I
*CYP3A5*3*	5′-TGGTCCAAACAGGGAAGAGATA-3′	I
*CYP3A4*1B*	5′-CATAAAATCTATTAAATCGCCTCTCTC-3′	I
*E.CYP2C9*3*	5′-TGCACGAGGTCCAGAGATAC-3′	II
*E.CYP2C9*5*	5′-CAGGCTGGTGGGGAGAAG-3′	II
*E.CYP2B6*6* (516G>T)	5′-AGATGATGTTGGCGGTAATGGA-3′	II
*E.CYP2D6*10* (4180G>C)	5′-GTGTCTTTGCTTTCCTGGTGA-3′	II
pfcrt76	5′-TTTGTTTAAAGTTCTTTTAGCAAAAATT-3′	II
pfcrt97	5′-GTTTTGTAACATCCGAAACTCA-3′	II
*NAT2*7*	5′-GTGCCCAAACCTGGTGATG-3′	III
*NAT2*14*	5′-TTGATCACATTGTAAGAAGAAACC-3′	III
*CYP2C19*3*	5′-AGGATTGTAAGCACCCCCTG-3′	III
*CYP2B6*5*	5′-TACCCCCAACATACCAGATC-3′	III
*NAT2*5*	5′-CTTCTCCTGCAGGTGACCA-3′	III
*NAT2*6*	5′-TATACTTATTTACGCTTGAACCTC-3′	III

Table 3. Labelled ddNTPs used for the three parallel extension mixes.

ddNTP and label	Mix
ddATP Cy3 ddCTP Cy3 ddGTP Cy5 ddUTP Cy5	I
ddATP Cy5 ddCTP Cy3 ddGTP Cy5 ddUTP Cy3	II
ddATP Cy3 ddCTP Cy5 ddGTP Cy5 ddUTP Cy5	III

Data acquisition

Microarrays were scanned at 635 nm and 532 nm using an Axon 4100A fluorescence scanner (Bucher Biotec AG, Basel, Switzerland). After the first scan slides were washed and scanned again and after each wash, Cy3 and Cy5 images were acquired and analyzed using the Axon GenePix Pro software (version 6.0). An in house developed perl script based on Kestler's statistics module [189] was used to call SNPs based on probe signal intensities. The script calculates receiver operating characteristic (ROC) curves using signal intensity values from the set of positive and negative controls for each hybridization. Hybridization specific thresholds that maximize both sensitivity and specificity were then used to make SNP calls.

Data comparison

SNP data gathered from sequencing and from microarray analysis were compared for agreement. The kappa index was interpreted based on the criteria of Landis and Koch [190]. Hardy-Weinberg equilibrium was tested using the chi-square Hardy-Weinberg equilibrium

test calculator for biallelic markers of the Online Encyclopedia for Genetic Epidemiology studies (http://www.oege.org).

Ethical approval

All the applied protocols were approved by the ethics committee of the two cantons of Basel (Ethikkommission beider Basel, EKBB) and the responsible local authorities (i.e. in Tanzania from the Institutional Review Board of the Ifakara Health Institute and the National Institute for Medical Research Review Board and in Cambodia from the National Ethics Committee for Health Research). Blood samples were collected following written informed consent in the respective local language (Khmer or Swahili) from the participants or their guardians.

Results

We tested the agreement between results obtained from sequencing and from the microarray on 18 SNPs within eight *cyp* isoenzyme genes and *nat2 genes* from 26 Cambodian and 70 Tanzanian malaria patients. The results are summarized in Table 4. For some SNPs agreement ranged from substantial to almost perfect, whilst for other SNPs a large variability from slight to substantial agreement was found. Where applicable, Chi-square Hardy-Weinberg equilibrium tests for the allele frequencies acquired by the microarray showed that most SNPs were significantly ($P < 0.01$) out of equilibrium (Table 5). Exceptions were *CYP2D6*17* (1023C>T) ($P = 0.50$) for Tanzania after the 1st wash; *CYP2C8*3* ($P = 0.92$), *CYP2C9*3* ($P = 0.92$), *CYP2C9*5* ($P = 0.92$), *CYP2D6*4* ($P = 0.92$) and *CYP3A5*3* ($P = 0.16$) after the 1st wash and *CYP3A5*3* ($P = 0.01$) and *NAT2* ($P = 0.07$) after the 2nd wash for Cambodia.

Table 4. Comparison of SNP data acquired either by sequencing or DNA microarray technology.

SNP	Country	1st wash			2nd wash			Agreement
		κ	*n*	%	*κ*	*n*	%	
*CYP2A6*2*	Cambodia	0.87	24	88.9	0.92	19	70.4	Substantial to almost perfect
	Tanzania	0.83	58	82.9	0.61	53	75.7	
*CYP2B6*5*	Cambodia	0.94	27	100.0	0.94	27	100.0	Almost perfect
	Tanzania	0.92	67	95.7	0.86	67	95.7	
*CYP2B6*6*	Cambodia	0.42	26	96.3	0.42	27	100.0	Fair to moderate
	Tanzania	0.35	63	90.0	0.40	59	84.3	
*CYP2C8*3*	Cambodia	0.97	20	74.1	1.00	20	74.1	Substantial to almost perfect
	Tanzania	0.90	39	55.7	0.79	38	54.3	
*CYP2C9*3*	Cambodia	0.92	26	96.3	0.94	26	96.3	Almost perfect
	Tanzania	0.90	67	95.7	0.82	64	91.4	
*CYP2C9*5*	Cambodia	0.98	27	100.0	1.00	27	100.0	Almost perfect
	Tanzania	0.93	67	95.7	0.86	70	100.0	
*CYP2C19*3*	Cambodia	1.00	27	100.0	1.00	26	96.3	Almost perfect
	Tanzania	0.90	67	95.7	0.83	66	94.3	
*CYP2D6*4*	Cambodia	0.98	26	96.3	1.00	26	96.3	Almost perfect
	Tanzania	0.91	63	90.0	0.88	65	92.9	
*CYP2D6*10* (100C>T)	Cambodia	0.59	24	88.9	0.64	20	74.1	Moderate to substantial
	Tanzania	0.72	31	44.3	0.76	27	38.6	
*CYP2D6*10* (4180G>C)	Cambodia	0.40	21	77.8	0.50	17	63.0	Fair to moderate
	Tanzania	0.45	45	64.3	0.51	38	54.3	
*CYP2D6*17* (1023C>T)	Cambodia	0.86	19	70.4	1.00	6	22.2	Moderate to almost perfect
	Tanzania	0.59	37	52.9	0.49	37	52.9	
*CYP2D6*17* (2850C>T)	Cambodia	0.29	7	25.9	0.20	6	22.2	Slight to substantial
	Tanzania	0.66	20	28.6	0.59	21	30.0	
*CYP3A4*1B*	Cambodia	0.00	6	22.2	0.00	5	18.5	Slight to substantial
	Tanzania	0.76	23	32.9	0.69	22	31.4	
*CYP3A5*3*	Cambodia	0.39	21	77.8	0.42	17	63.0	Fair to substantial
	Tanzania	0.73	61	87.1	0.65	58	82.9	

Table 4.

SNP	Country	1st wash			2nd wash			Agreement
		κ	*n*	%	*κ*	*n*	%	
*NAT2*5*	Cambodia	0.32	14	51.9	0.38	8	29.6	Fair to moderate
	Tanzania	0.47	37	52.9	0.53	28	40.0	
*NAT2*6*	Cambodia	0.70	26	96.3	0.73	25	92.6	Substantial
	Tanzania	0.74	62	88.6	0.71	62	88.6	
*NAT2*7*	Cambodia	0.72	25	92.6	0.70	22	81.5	Substantial
	Tanzania	0.75	55	78.6	0.67	49	70.0	
*NAT2*14*	Cambodia	1.00	27	100.0	1.00	27	100.0	Substantial to almost perfect
	Tanzania	0.76	62	88.6	0.74	61	87.1	

Data was aquired in 27 Cambodian and 70 Tanzanian malaria patients. *K* indicates the kappa index. N is the number and % the percentage of samples with results for both techniques.

Table 5. Chi-square Hardy-Weinberg equilibrium tests for SNP data acquired by DNA microarray technology.

SNP	Cambodia				Tanzania			
	1st wash		2nd wash		1st wash		2nd wash	
	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
*CYP2A6*2*	23.00	<0.01	7.29	<0.01	23.57	<0.01	34.02	<0.01
*CYP2B6*5*	N.A.		N.A.		54.39	<0.01	32.76	<0.01
*CYP2B6*6*	N.A.		N.A.		16.90	<0.01	17.41	<0.01
*CYP2C8*3*	0.01	0.92	N.A.		8.22	<0.01	39.35	<0.01
*CYP2C9*3*	0.01	0.92	N.A.		22.08	<0.01	11.13	<0.01
*CYP2C9*5*	0.01	0.92	N.A.		14.69	<0.01	14.30	<0.01
*CYP2C19*3*	N.A.		N.A.		18.22	<0.01	9.98	<0.01
*CYP2D6*4*	0.01	0.92	N.A.		20.87	<0.01	18.55	<0.01
*CYP2D6*10* (100C>T)	24.00	<0.01	7.39	<0.01	31.00	<0.01	27.00	<0.01
*CYP2D6*10* (4180G>C)	13.67	<0.01	N.A.		11.94	<0.01	38.00	<0.01
*CYP2D6*17* (1023C>T)	11.90	<0.01	7.00	<0.01	0.45	0.5	35.34	<0.01
*CYP2D6*17* (2850C>T)	7.35	<0.01	10.00	<0.01	37.66	<0.01	30.03	<0.01
*CYP3A4*1B*	N.A.		N.A.		N.A.		24.00	<0.01
*CYP3A5*3*	1.98	0.16	6.39	0.01	13.36	<0.01	18.74	<0.01
*N.A.T2*5*	8.41	<0.01	3.32	0.07	13.83	<0.01	13.70	<0.01
*N.A.T2*6*	N.A.		N.A.		33.29	<0.01	12.04	<0.01
*N.A.T2*7*	9.97	<0.01	8.63	<0.01	30.21	<0.01	24.82	<0.01
*N.A.T2*14*	N.A.		N.A.		32.08	<0.01	19.30	<0.01

χ^2 indicates the result from the Hardy-Weinberg equilibrium test, *P* the one-tailed *P*-value, and N.A. that Chi-square could not be calculated because the allele frequency was either 0% or 100%.

Discussion

Comparison of data generated by microarray analysis with sequencing showed that the performance of the DNA microarray is limited. For some SNPs, i.e. *CYP2A6*2*, *CYP2B6*5*, *CYP2C8**3, *CYP2C9*3/*5*, *CYP2C19*3*, *CYP2D6*4* and *NAT2*6/*7/*14*, agreement ranged from substantial to almost perfect (kappa index between 0.61 and 1.00), whilst for other SNPs a large variability from slight to substantial agreement (kappa index between 0.39 and 1.00) was found, e.g. *CYP2D6*17* (2850C>T), *CYP3A4*1B* and *CYP3A5*3*. No clear trend in rise or decline of agreement between methods was visible from the 1st to the 2nd wash of the slide and thus it remains ambiguous whether repeated washing improves the performance of the microarray. However, agreement tends to be lower among the Cambodian samples. A possible explanation is the strong dependence of kappa on true prevalence of the SNPs [191] and the dissimilar distribution of the SNPs of interest in Cambodians and Tanzanians [192]. Furthermore, among most of these SNPs a considerable number of patient samples failed to yield a signal on the microarray (e.g. *CYP2D6*17* (2850C>T) and CYP *CYP3A4*1B*). The trend was clearly higher in samples from Tanzania, which might also be due to a lower quality of DNA arising from suboptimal storage conditions after blood withdrawal. Sequencing data agreed with published reference sequences from public sources (Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee, http://www.cypalleles.ki.se/) and thus seemed to be truthful.

Because *cyp*s evolved out of a single ancestor [185,193], they show very close sequence similarities, which in turn makes it difficult to design gene specific primers, in particular extension primers that have to be designed at a defined position. It therefore became almost impossible to develop a single multiplex PCR and thus the microarray method described here is time consuming and laborious. This is in contrast to a similar microarray developed for the analysis of drug resistance associated SNPs in *Plasmodium falciparum* genes that permits the simultaneous analysis of many SNPs in hundreds of samples in a very short time period (approximately 15 h for four 96-well plates) with significantly reduced costs compared to other systems [188].

Furthermore, the costs of sequencing have decreased considerably during the last years and this trend may well continue. On the other hand, the costs of microarray reagents (especially Cy3- and Cy5-labeled ddNTPs that are used in three combinations) and glass slides for arraying have increased and are unlikely to decrease over time. So the costs for the microarray

thechnology are not considerably lower anymore and therefore, overall costs of both methods have become comparable.

While SNP analysis microarray has been successfully used to analyse point mutations in drug resistance associated genes in *Plasmodium* [188,194], it seems to fail with closely related genes such as the human *cyp* genes. For the latter, sequencing appears to be a more reliable method.

Conclusions

Although microarray allows the simultaneous determination of many SNPs, the lack of robustness for the described approach here prohibits its wide use in pharmacogenetics and sequencing occurs the more reliable technique. With the availability of large sequencing capacities world wide, molecular-epidemiological studies using sequencing for a limited number of SNPs in *CYP* genes in a large population are feasible.

CHAPTER 4

Residual antimalarials in malaria patients from Tanzania – implications on drug efficacy assessment and spread of parasite resistance

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Abstract

Background

Repeated antimalarial treatment for febrile episodes and self-treatment are common in malaria-endemic areas. The intake of antimalarials prior to participating in an *in vivo* study may alter treatment outcome and affect the interpretation of both efficacy and safety outcomes. We report the findings from baseline plasma sampling of malaria patients prior to inclusion into an *in vivo* study in Tanzania and discuss the implications of residual concentrations of antimalarials in this setting.

Method and Findings

In an *in vivo* study conducted in a rural area of Tanzania in 2008, baseline plasma samples from patients reporting no antimalarial intake within the last 28 days were screened for the presence of 14 antimalarials (parent drugs or metabolites) using liquid chromatography-tandem mass spectrometry. Among the 148 patients enrolled, 110 (74.3%) had at least one antimalarial in their plasma: 80 (54.1%) had lumefantrine above the lower limit of calibration (LLC = 4 ng/mL), 7 (4.7%) desbutyl-lumefantrine (4 ng/mL), 77 (52.0%) sulfadoxine (0.5 ng/mL), 15 (10.1%) pyrimethamine (0.5 ng/mL), 16 (10.8%) quinine (2.5 ng/mL) and none chloroquine (2.5 ng/mL).

Conclusions

The proportion of patients with detectable antimalarial drug levels prior to enrolment into the study is both surprising and worrying. Artemether–lumefantrine was expected to be available only at government health facilities and sulfadoxine–pyrimethamine only for intermittent preventive treatment in pregnancy (IPTp). Self-reporting of previous drug intake is unreliable and thus screening for the presence of antimalarial drug levels should be considered in future *in vivo* studies to allow for accurate assessment of treatment outcome. Furthermore, persisting sub-therapeutic drug levels of antimalarials in a population could promote the spread of drug resistance. The knowledge on drug pressure in a given population is important to monitor standard treatment policy implementation.

Introduction

The intake of antimalarial drugs prior to inclusion in an *in vivo* study may interfere with the estimation of treatment outcomes (for both efficacy and safety) due to the presence of residual antimalarials. The standard World Health Organization (WHO) protocol for monitoring antimalarial drug efficacy does not exclude patients with a history of previous antimalarial drug use or the presence of antimalarial drugs in the urine or blood [195]. Nonetheless, it is customary in clinical studies to record the occurrence of previous drug intake at screening as reported by the patient, parent or guardian. Two studies in Africa investigated self-reporting of drug intake [196,197], and both concluded that it is inaccurate. A more objective indication on the drug use in a study population would be obtained by screening the urine or blood for the presence of antimalarial drugs. There are studies on residuals of antimalarials that have been used in past policies, i.e. chloroquine (CQ) or sulfadoxine–pyrimethamine (SP), in urine or blood in the general population or patients [198-211]. However, to our knowledge, there is no study on the presence of lumefantrine in malaria patients seeking medical care.

Policy makers in malaria endemic countries are faced with the difficult problem of ensuring easy and early access to effective and high quality antimalarials, while preventing their uncontrolled and unnecessary use, which would increase drug pressure on the parasites and encourage parasite resistance. Thus, knowledge of drug use in a specific area could help decisions makers to assess how treatment policies are implemented.

Here we report the findings of the analysis of baseline samples from patients with *Plasmodium falciparum* malaria recruited in an *in vivo* study in Tanzania. Samples were analysed for the presence of 14 currently in-use antimalarials in a single run using a liquid chromatography-tandem mass spectrometry assay [212].

Methods

Study area and population

The study was performed in a rural area with moderate to high malaria transmission intensity (Kilombero district, Morogoro region, Tanzania) during the main rainy season from March to May 2008. At the time of the study, artemether–lumefantrine (AL) had recently been introduced as first-line treatment and was only available at government health facilities to

ensure controlled prescription. Before 2006, the official first-line treatment in Tanzania was SP, which had in turn replaced CQ in 2001. In 2008, amodiaquine, SP and quinine were widely available in the private sector in the study area. Artesunate, dihydroartemisinin, and halofantrine could also be found sporadically in a few drug shops (Alba S *et al*., in preparation). In the private sector these drugs could be purchased over the counter without a doctor's prescription.

Febrile patients were recruited at the Kibaoni Health Center, 6 km from Ifakara down town, that serves a population of 26,261. The population lives in villages with good coverage of government health facilities and licensed drug stores (pharmacies, part II drug stores [*duka la dawa baridi*] and Accredited Drug Dispensing Outlets [ADDO; *duka la dawa muhimu*]) [213,214]. A map of the villages of residence of the patients included in the study with location of health facilities and drug dispensing outlets is presented in Figure 1.

Clinical procedures

The trial was designed to assess the effects of the individual pharmacogenetic profiles on the disposition of standard antimalarials (to be reported elsewhere). It was based on the standard WHO protocol for *in vivo* testing. Suspected malaria cases were screened by rapid diagnostic test Paracheck Pf®, Orchid Biomedical Systems, India) for the presence of *Plasmodium falciparum*. Parasite count and specification of *Plasmodium* were done by microscopy. The patients with a positive result were then seen by a clinical officer, who invited them to participate in the study if they did not present with danger signs of complicated malaria or severe concomitant illness, and if they reported not having taken antimalarials in the previous 28 days. The latter information was checked against the patient's care log book when available. Consenting patients had a baseline sample (Day 0, 4.5 mL venous blood collected in an EDTA Vacutainer®; Becton, Dickinson and Company, USA) taken to check for potential residual antimalarials and correct pharmacokinetic analyses later on. Treatment with the standard first-line treatment AL was then initiated, according to body weight and age category.

Laboratory procedures

Blood samples were kept on ice for no longer than 6 h after bleeding (venipuncture) and then aliquoted into whole blood, plasma and pellet and immediately stored at -80°C. Plasma concentrations of 14 antimalarial drugs and their metabolites, i.e. artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, lumefantrine, desbutyl-lumefantrine, piperaquine, pyronaridine, mefloquine, chloroquine, quinine, pyrimethamine and sulfadoxine, were determined by liquid chromatography coupled to tandem triple stage mass spectrometry (LC-MS/MS) [212]. The lower limits of the calibration range (LLC) in our method were selected as the lowest levels of the calibration curves, which confidently provide a bias and CV% below ± 20%, in accordance to FDA recommendations [131]. All samples were analyzed twice. First, quantitative measurement was performed using calibration and quality control samples; then, for confirmation, qualitative assessment was repeated using a new chromatographic column that had not been exposed to any antimalarial drugs. In order to exclude contamination and false positive results, a large set of blank controls was analyzed prior to the clinical samples on the new column, checking for the absence of specific MS/MS signals of the antimalarials investigated.

Data management and analysis

Summary statistics, Chi-square tests, multivariate analysis and graphs of residual plasma concentrations of antimalarials found prior to treatment were produced using Stata® (version 10.1 “intercooled”, Stata Corporation, College Station, TX, USA). Logistic regression analysis was used to investigate the influence of body weight, sex and distance from health facilities or pharmacies on the presence of antimalarials at study entry. The distance between patient home and health facility or pharmacy was defined as “close” or “far” depending on the distance in kilometers, also taking into account ease of access, i.e. main road and river (according to Figure 1). In the first multivariate analysis we considered Bondeni, Kapolo, Katrini, Kibaoni, Kibaoni HC, Kikwawila, Kilolelo, Kining’ina, Machipi, Maendeleo, Makelo, Mbasa, Mchonjoi, Michenga C, Milola S, Muungano, Nakafulu, Sakamaganga B, Station, and Viwanja Stini as “close” and Lungongole, Kilama A, and Kilama B as “far”. In the second analysis we also classified Kilolelo, Kining’ina, Machipi, Makelo, and Michenga C as “far” because the flooding of the Lumemo river might have been an obstacle during the

rainy season. We also evaluated the contribution of SP alone, AL alone or both, using likelihood ratio tests.

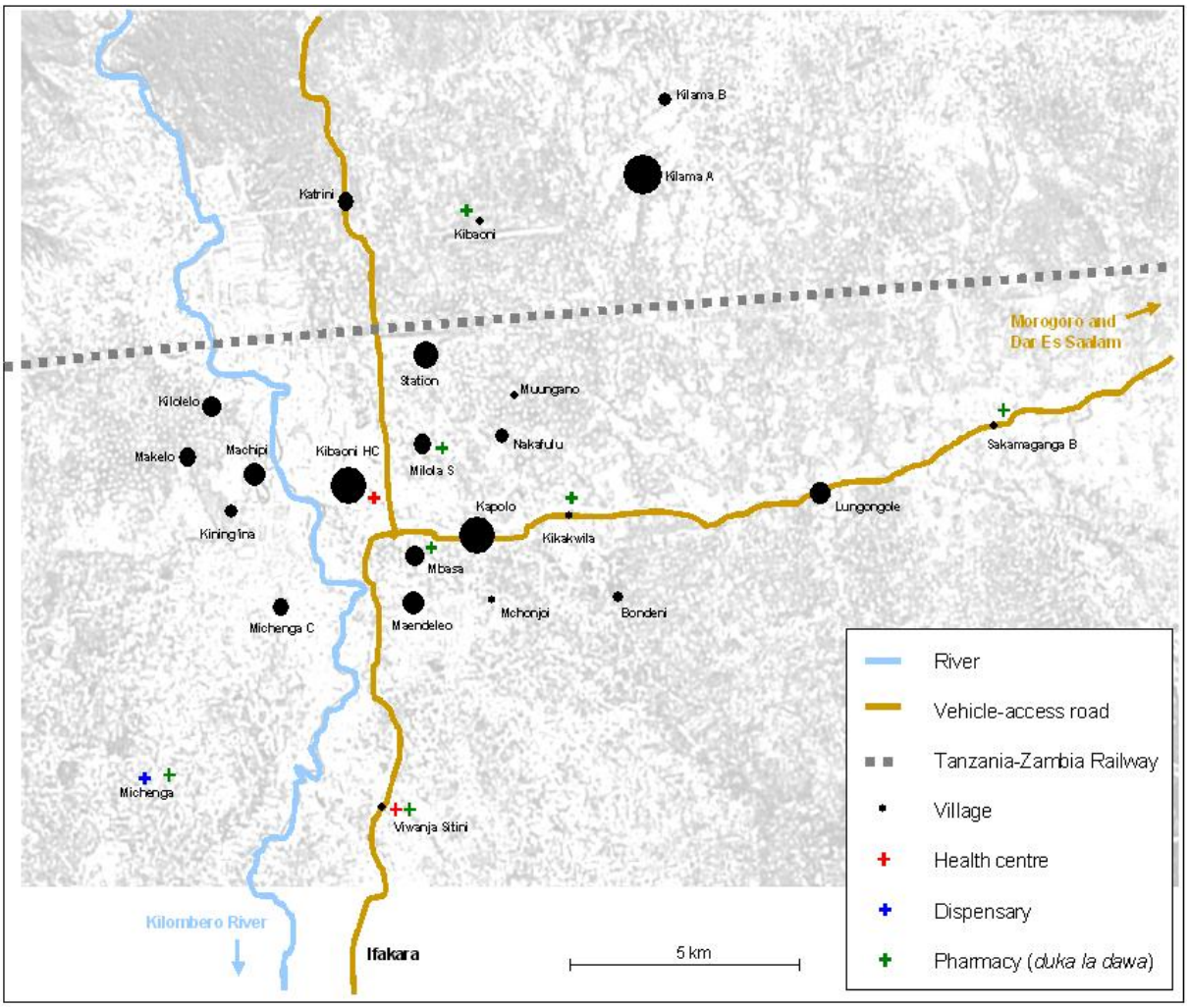

Figure 1. Villages of residence of the patients included in the study.

Estimation of time of drug intake for lumefantrine

To estimate the probable timing of drug intake, we compared the plasma concentrations of lumefantrine at baseline (C_0) and on Day 7 (C_7) after a complete treatment with AL for the same patients. We included only patients for whom we had both samples and who complied with the three-day, six-dose AL treatment schedule. Assuming a terminal elimination half-life of $t_{½}$ = 3.3 days for lumefantrine, an inter-individual variability of 40% [61] and a similar dosage on pre-study exposure and during the study, a back-calculation was done to estimate the intake time before baseline sampling:

$$\text{intake time} = \ln(C_7 / C_0) \cdot t_{½} / \ln(2) + 7 \quad \text{[days]}$$

The variability on $t_{½}$ was used to estimate a 90% confidence interval around this intake time, considering plausible inter-individual variations in elimination rate [61].

Bayesian back-calculations for sulfadoxine

A Bayesian estimation of the most likely drug intake time was also attempted from individual sulfadoxine plasma concentration data, with a minimization approach using the "Solver" implemented in Microsoft Excel®. Patients were assumed to have taken a single dose of sulfadoxine (combined with pyrimethamine) according to body weight: 1500 mg for >45 kg, 1000 mg for 31–45 kg, 750 mg for 21–30 kg, 500 mg for 11–20 kg and 250 mg for 5–10 kg. Approximate averages of pharmacokinetic parameters, inter-individual variability and intra-individual (residual) variability were derived from a literature review (Tables 1–4). These parameters were used to back-calculate the most likely time for dose intake, expected to produce the observed concentration according to a standard one-compartment model. The variability was used to estimate a 90% confidence interval around this intake time, considering plausible variations in clearance, distribution volume and measurement/modeling error [215]. Similar calculations were not attempted for other antimalarials, as their dosage forms are more heterogeneous (lumefantrine and quinine), their population pharmacokinetic parameters are less well characterized (lumefantrine) and their half-lives are shorter (pyrimethamine, quinine).

Ethical approval

All the applied protocols and related documents were approved by the Ethikkommission beider Basel (EKBB), the Institutional Review Board of the Ifakara Health Institute and the National Institute for Medical Research Review Board. Blood samples were obtained after written informed consent in Swahili from the participants or their responsible guardians.

Table 1. Review of pharmacokinetic studies of sulfadoxine.

Study	Number of subjects	Samples per subject	Sex	Age [y]	Body weight [kg]	Condition
Mansor *et al*. [216]	10	18	males	22–30	51–60	Healthy adults
Obua *et al*. [217]	83	6	both	2.5	11.9 ± 2.7	*Falciparum* malaria
Green *et al*. [218]	33	7	females	15–30	47.2–71.0	*Falciparum* malaria, pregnancy, HIV positiv
Trenque *et al*. [219]	89	3	both	0–14	3–59	Congenital toxoplasmosis
Barnes *et al*. [220]	290	8	both	14	<103	*Falciparum* malaria
Edstein *et al*. [221]	7		both	Adults		Healthy adults
Corvaisier *et al*. [222]	32	5	both	0.2–2.1	5–13.4	Congenital toxoplasmosis
Obua *et al*. [223]	9	14	both			Healthy adults

Table 2. Review of pharmacokinetic studies of sulfadoxine (continued).

Study	Analytic method	Sample type	Dose [mg]	Regimen	PK approach	Compartments
Mansor *et al*. [216]	GC	Plasma	1000	single dose	AUTOAN-NONLIN	2
Obua *et al*. [217]	HPLC-UV	Whole blood on filter paper	250 or 500	one dose daily for three days	NONMEM	2
Green *et al*. [218]	HPLC-UV	Whole blood	1500	single dose	log-linear regression	1
Trenque *et al*. [219]	HPLC-UV	Plasma	25/kg	two or three times a month	NONMEM	1
Barnes *et al*. [220]	HPLC-MS	Whole blood on filter paper	25/kg, ≤1500	single dose	WinNonlin	1
Edstein *et al*. [221]		Whole blood	500	single dose		
Corvaisier *et al*. [222]	HPLC-UV	Plasma	25/kg	one dose every 10 days	NPEM	1
Obua *et al*. [223]	HPLC-UV	Plasma	1500	single dose	WinNonlin	1

Table 3. Review of pharmacokinetic studies of sulfadoxine (continued).

Study	Volume of distribution (*V*)		Clearance (*CL*)		Comment
	[L]	[L/kg]	[mL/h/kg]	[L/h]	
Mansor *et al*. [216]	30.90[a]			0.084 ± 0.013	Mean ± S.D.
Obua *et al*. [217]		Central: 0.13 ± 47%; peripheral: 1.6 ± 0%		0.023 ±33%	Estimate ± CV%
Green *et al*. [218]	15.0 (12.1–16.0)	0.24 (0.021–0.27)	1.01 (0.82–1.61)	0.066 (0.048–0.092)	Median (IQR)
Trenque *et al*. [219]		2.07 ± 0% for 11 kg [b]		0.0108 ± 16.3% for 11 kg [c]	Estimate ± CV%
Barnes *et al*. [220]		0.40 (0.29–0.56)	1.85 (1.15–2.89)		Median (IQR)
Edstein *et al*. [221]		0.25 ± 0.03	0.79 ± 0.15		Mean ± S.D.
Corvaisier *et al*. [222]		0.393 ± 85%	2.07[d]		Mean ± CV%
Obua *et al*. [223]		0.15 (0.12–0.18)	0.39 (0.30–0.56)		Median (range)
Overall		0.25 ± 33%	1 ± 33%		

[a] Derived from clearance and plasma half-life ($t_{½}$); [b] Corrected by a factor of $(BW / 11\ \text{kg})^{0.72}$ for other body weight (BW) values; [c] Corrected by a factor of $(BW / 11\ \text{kg})^{0.64}$ for other body weight (BW) values; [d] Derived from clearance and elimination rate constant (λ)

Table 4. Review of pharmacokinetic studies of sulfadoxine (continued).

Study	Absorption rate constant (k_a) [h^{-1}]	Terminal half-life ($t_{½}$) [h]	Additive residual variability [µg/L]	Proportional residual variability	Comment
Mansor *et al*. [216]	1.30 ± 1.10	255 ± 61			Mean ± S.D.
Obua *et al*. [217]	0.30		2.60	27%	Estimate ± CV%
Green *et al*. [218]		148 (121–193)			Median (IQR)
Trenque *et al*. [219]	0.055	139[a]	8.70	31%	Estimate ± CV%
Barnes *et al*. [220]		161 (105–218)			Median (IQR)
Edstein *et al*. [221]					Mean ± S.D.
Corvaisier *et al*. [222]	1.659 ± 94%	132 ± 40%			Mean ± CV%
Obua *et al*. [223]	0.32 (0.29–0.62)	229 (139–272)			Median (range)
Overall	1.5 ± 0%	173	0	30%	

[a] Derived from $V/CL \times \ln(2)$

Results

A total of 1672 patients of all age were screened, of whom 389 (23%) had a positive malaria test and 150 were eligible and willing to participate in the *in vivo* study. Two patients (one from the Kibaoni HC area and one from Kining'ina) were excluded from the analyses (venipuncture unfeasible in one patient; treatment initiated before baseline sampling in the other one), leaving 148 patients with a valid baseline sample, of whom 64 (43.2%) were male and 84 (56.8%) female (3 (2.0%) pregnant in 3rd trimester). Patients' ages ranged from 1 to 78 years (median 9 years). 51 (34.5%) patients were children under the age of 5, and 94 (63.5%) were <12 years old.

The presence of antimalarial drug was detected in the plasma of 111 (75.0%) patients: 80 (54.1%) had lumefantrine above the lower limit of calibration (LLC = 4 ng/mL), 7 (4.7%) desbutyl-lumefantrine (LLC = 4 ng/mL), 77 (52.0%) sulfadoxine (LLC = 0.5 ng/mL), 15 (10.1%) pyrimethamine (LLC = 0.5 ng/mL), 16 (10.8%) quinine (LLC = 2.5 ng/mL) and none chloroquine (LLC = 2.5 ng/mL) or any other antimalarials tested. Summary statistics are shown in Table 5, and box plots of residual plasma concentrations are represented in Figure 2. Among the 111 patients with residual drug concentrations, 57 (38.5%) had more than one drug (note that parent drug and metabolite or combined regimens such as SP are considered as one): 43 (29.1%) had both lumefantrine and SP, 6 (4.1%) had both lumefantrine and quinine, 1 (0.7%) had both SP and quinine, and 7 (4.7%) had all three agents. The presence of residual antimalarials in plasma was significantly more frequent among children under 5 years of age (86.3%, $\chi^2 = 5.82$, $P = 0.016$) than among older children and adults (68.0% altogether).

For lumefantrine, among the 59 eligible patients the median plasma concentration (range) was 18.3 ng/mL (4.4–181.8 ng/mL) on Day 0 and 413 ng/mL (37.3–1402 ng/mL) on Day 7. This means that, to account for the levels observed on Day 0, a similar dosage level should have been administered at a median of 21 days (interquartile range 17–24 days, whole range 11–29 days) before study entry. In 2 patients (3%) this estimate was >28 days. The variability in $t_{½}$ translates into 90% confidence intervals extending from 74% to 144% of estimates (median).

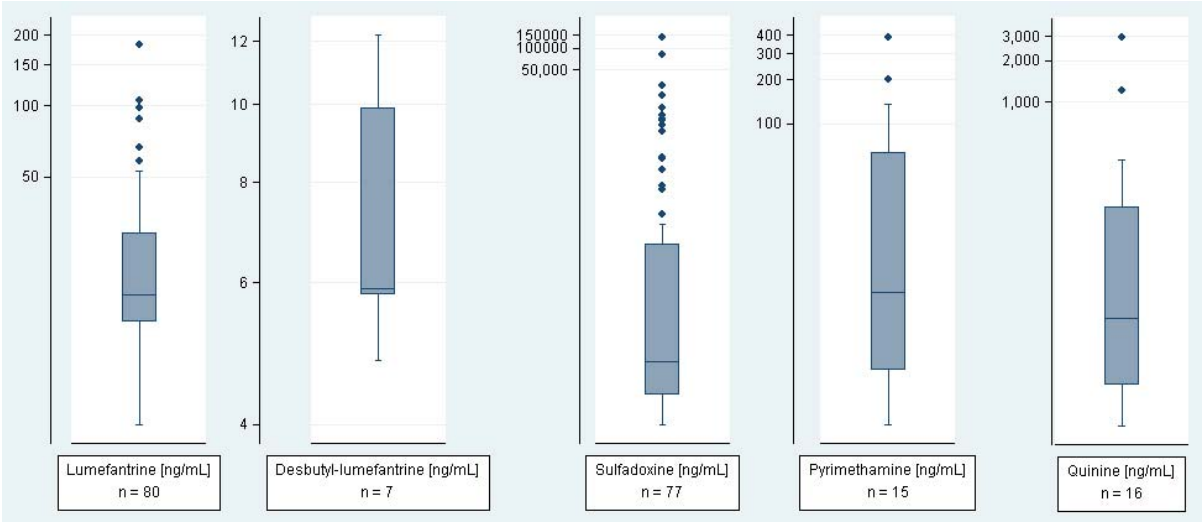

Figure 2. Residual plasma concentrations of antimalarials found prior to treatment in 148 malaria patients. Number of patients (n), median, 25th and 75th percentile, lower and upper adjacent values, and outside values are shown for lumefantrine, desbutyl-lumefantrine, sulfadoxine, pyrimethamine and quinine on a logarithmic scale [ng/mL].

Table 5. Residual plasma concentrations of antimalarials found prior to treatment in 148 malaria patients [ng/mL].

Antimalarial	Patients (%)	Mean	Median	Minimum	Maximum
Lumefantrine	80 (54.1)	25.3	15.8	4.4	181.8
Desbutyl-lumefantrine	7 (4.7)	7.3	5.9	4.8	12.2
Sulfadoxine	77 (52.0)	4’480.2	4.4	0.6	138'887.5
Pyrimethamine	15 (10.1)	56.8	7.1	0.9	391.3
Quinine	16 (10.8)	318.0	26.3	4.4	2'947.2

No artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, piperaquine, pyronaridine, mefloquine, or chloroquine was found.

Back-estimation of the most likely times for sulfadoxine intake indicated a median of 108 days before blood sampling at study entry (interquartile range 67 to 121 days, whole range <1 to 130 days). Two patients had concentrations >78 mg/mL, compatible with same day intake. In 70 patients (91%), the estimate exceeded 28 days. The evaluation of uncertainty around individual dose intake times showed 90% confidence intervals extending from 49% to 202% of estimates (median).

The investigation of the influence of body weight, sex and distance to health facilities or pharmacies on the probability of residual antimalarials at study entry (details not presented) showed only a significant relationship between body weight and residual AL levels (likelihood ratio $\chi^2 = 9.06$, $P = 0.03$ in the first analysis; likelihood ratio $\chi^2 = 9.60$, $P = 0.02$ in the second analysis), patients with lower body weight being more likely to show residual AL levels. However, this was not the case for SP or SP and AL taken together. Furthermore, neither sex nor distance from health facilities or pharmacies showed a significant effect on residual levels of SP, AL or both at study entry.

Discussion

This is the first study investigating the presence of a range of antimalarials in the plasma of African malaria patients on enrollment into an *in vivo* study. The measurement of 14 antimalarial drugs currently in-use allowed a comprehensive assessment of drugs available in the community under study.

Artemether–lumefantrine

Three in four patients had detectable plasma concentrations of antimalarials at the time of enrolment into the study, and in a majority of cases the agent detected was lumefantrine/ desethyl-lumefantrine – indicating that they had taken AL, which was supposedly available only at government health facilities to ensure controlled prescription of first-line treatments. Assuming that the patients had taken a three-day, six-dose AL treatment regimen, most patients (97%) must have taken the drug within 28 days prior to treatment. Furthermore, it is also possible that patients might have taken a sub-therapeutic dose of AL more recently. However, as indicated by the wide variability in elimination half-lives, these values represent only rough estimates. Nevertheless, these findings suggest that at least half of the patients included into our study had taken AL, mostly within the previous month (one month corresponds roughly to the limit of evaluation of past exposure, considering assay LLC for lumefantrine). The Day 7 values observed in this study are comparable with those of a study in Thai patients [median plasma concentration (range) of lumefantrine was 528 ng/mL (49–5175 ng/mL) after 6 doses of AL over 60 h according to body weight [224]] and in patients from Bangladesh [860.3 ng/mL (53.8–6215.0 ng/mL) [225]]. Due to the short half-

life of the artemisinin component, e.g. 45 min for dihydroartemisinin, it is likely that none of the patients had taken a co-formulated ACT (e.g AL) within the last 24 hours [226].

Sulfadoxine–pyrimethamine

SP was still found in approximately half of the patients, although it had been officially abandoned as first-line treatment since 2006. Assuming that the patients with residual SP in their plasma had taken a single dose of sulfadoxine according to body weight, most patients must have taken the drug ~3.5 months (median 108 days) prior blood withdrawal. However, 2 patients had sulfadoxine plasma concentrations indicating very recent exposure, and another 8 exposure during the last 4 weeks. Furthermore, it is also possible that patients might have taken a sub-therapeutic dose of SP more recently. These estimates are approximate, as indicated by the wide confidence interval explained by the fair degree of inter-individual variability in clearance, volume of distribution and residual error. Nevertheless, these findings are sufficient to conclude that a significant number of patients had taken SP for a previous febrile episode, which is contrary to the standard treatment recommendations. The LC-MS/MS method used for the determination of sulfadoxine (LLC = 0.5 ng/mL) would theoretically make it possible to detect traces of sulfadoxine up to 4 months (127 days) after a single dose of 25 mg/kg of sulfadoxine.

Chloroquine and quinine

This study tends to confirm that CQ, replaced by SP as first-line treatment in 2001, has been effectively withdrawn. On the other hand, one tenth of the patients were found with quinine in their plasma. Quinine has an elimination half-life of 16–18 h in malaria patients [227]. After a treatment with 10 mg/kg of quinine dihydrochloride administered 8-hourly orally for 7 days, the plasma quinine levels had fallen to below 0.4 μg/mL in almost all patients 40 h after the last dose on Day 7 [228]. Thus, we infer that most patients with quinine levels in our study must have taken the last quinine dose not more than 2 days before reporting at the health facility.

High numbers of patients with residual antimalarials

Why was the number of patients with residual antimalarials so high? Through the demographic surveillance system (DSS) data and a treatment seeking survey in the Ifakara area it was found that approximately 8% of children had fever in the previous 2 weeks when seen between January and April 2008 (Alba S *et al*., personal communication). An epidemiological study which used the Explanatory Model Interview Catalogue (EMIC) reported that 87.5% (78.2-93.8%) of all fevers in children in our study area were treated with one of the recommended antimalarials (at that time SP, amodiaquine or quinine) [207]. Based on these data, one would expect 14% of children to have had an antimalarial treatment in the preceding month (i.e. $(0.08 \times 2) \times 0.875$), which is much lower than the proportion of patients with residual antimalarial plasma levels in this study (86.3% for under 5s, 68% for older children and adults). This large discrepancy could be either due to poor recall of the study subjects in the epidemiological survey or a selection bias. The latter could have arisen either because we captured (i) only patients preferentially seeking antimalarial treatment at a health facility instead at drug shops (~76.3% of children under the age of 5 years receiving treatment according to the epidemiological study), (ii) patients who are more susceptible to repeated infections (and hence repeated treatments) or are more exposed to infection, or (iii) patients with easy access to treatment. However, similar results were found in two study sites in rural Cambodia (CHAPTER 5).

Reliability of medical history

Whatever the reason is for the large number of malaria patients with antimalarials in their blood at study baseline, the fact remains that these patients are the usual subjects investigated in *in vivo* studies and clinical trials. All patients included in the study reported not having taken antimalarials in the previous 28 days. Entry criteria based on self-reporting of previous drug intake (poor recall) or information recorded in the care log book (self-treatment not documented) are thus unreliable at least in this population and for lumefantrine.

Potential bias in drug safety and efficacy assessment

Previous drug intake may affect the current treatment in several ways. Higher drug exposure resulting from cumulative levels may lead to better efficacy or more toxicity. The parasites causing the disease at the time of enrolment may be less sensitive population selected by the previous treatment. Thus, previous antimalarial intake may interfere with the outcome of the treatment under investigation, and this study shows that only baseline drug concentration measurement in the blood can reliably be used for the purpose. Our LC-MS/MS assay covers 14 antimalarials in a single run. We can confidently exclude a lack of specificity and false positives as we included blank plasma samples as negative controls and systematically repeated the measurement on a new chromatographic column. Furthermore, we have demonstrated that all drugs are stable for up to 48 h in plasma stored at 4 °C [212]. Therefore, even in settings, where no LC-MS/MS instrument is available, samples can be collected in the field, and easily kept and transported to the nearest laboratory where they can be frozen and stored until assayed or further shipped to their final destination.

High drug pressure as risk factor for the spread of drug resistance

There is abundant literature on the effects of inadequate antimalarial treatment on the emergence and spread of resistance. Here, we do not know if the residual drug levels found were from a full or incomplete treatment, if the person had parasites at the time of the previous drug intake, and whether the parasites causing the current episode are the same or a new infection. Be it as it may, the residual levels were not enough to control parasite replication and clinical symptoms. This means that these parasites have been exposed to inadequate drug levels for some time. The chances of drug resistant parasites to be selected depends on several factors, and is higher for patients with no immunity (e.g. young children), drugs with long residence times and resistance being conferred through single point mutations, and for infections with a large parasite biomass [229]. These patients had a mean baseline parasite biomass of ~9×10^{10} (ranging from ~1×10^{8} to ~6×10^{11}, data not shown), values which are in line with those reported for symptomatic cases in malaria-endemic areas [230], and were exposed to drug concentrations which were likely to be in the selective window [45].

The findings of this study must be confirmed in other settings as they have potential implications for both clinical research and surveillance (treatment efficacy and safety outcome) and control (pharmaco-epidemiology, adherence to policy).

CHAPTER 5

Residual antimalarials at admission in malaria patients from Cambodia – indication of drug pressure

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Abstract

The border area between Thailand and Cambodia has been known as the origin of antimalarial drug resistance for the past 30 years. There is an active and highly diverse market in antimalarials in this area, and knowledge on drug use in this specific area will be useful in evaluating the impact of the ongoing interventions undertaken to increase access to appropriate treatment with artemisinin-based combination therapy and to contain the spread of drug resistance. Here we report the findings from the analysis of baseline samples from *falciparum* malaria patients recruited in two *in vivo* studies for the presence of 14 currently in-use antimalarials in a single run using a liquid chromatography-tandem mass spectrometry assay. More than 50% of patients had residual drug concentrations above the lower limit of calibration of at least on antimalarial at admission. Among the drugs detected were the currently used first-line drugs mefloquine (25% and 35% of patients) and piperaquine (15% of patients), the first-line drug against *vivax* malaria chloroquine (25% and 41% of patients), and the former first-line drug quinine (5% and 34% patients). The findings suggest that there is a high drug pressure in the area and many people still seek treatment in the private and informal sector where accurate treatment following the first-line treatment policies is not guaranteed. Thus, efforts to contain emergences and spread of parasite resistance against new antimalarials by promotion of comprehensive behavioural change, communication, community-based mobilization and advocacy along the Thai-Cambodian border should be maintained.

Introduction

A few years ago, the World Health Organization (WHO) recommended that conventional monotherapies such as chloroquine (CQ), amodiaquine (AQ) or sulfadoxine–pyrimethamine (SP) should be replaced by artemisinin-based combination therapies (ACTs) for treating falciparum malaria and today ACTs are used as first-line treatment throughout the world [4,182,231,232]. Initiatives to scale-up control interventions and eliminate malaria are critically dependent on the sustained efficacy of ACTs. However, there is recent worrying evidence of reduced response to artemisinins arising on the Thai-Cambodian border [177-184,233,234]. This area has long been known as the origin of antimalarial drug resistance. Parasites carrying resistance markers against CQ, SP, and mefloquine (MQ) were first found here before appearing elsewhere in the world [179,181,235]. In Cambodia, because of poor

transportation and public health infrastructure, antimalarials were made available in the private sector to increase patients' access to the drug. While instrumental to reaching out for more patients, this also led to uncontrolled use of these drugs. A recent study of malaria treatment–seeking behaviour in Cambodia showed that more than 80% of the patients initially sought treatment from private providers and pharmacies or consumed drugs on their own [236]. Drugs from the private sector are often of substandard quality and the drug providers do not emphasize adherence. These key factors lead to treatment failure and development of resistance against CQ, SP and MQ in this area and it is conceivable that the availability of artemisinin monotherapies, incorrect dosages and poor drug quality must have affected response to artemisinin derivatives too [178].

Several studies and programs are currently focusing on improving the availability of quality assured artemisinin-based combinations. In a study on access to ACTs in remote areas of Cambodia [237] it was shown that in the private sector up to 26% of patients received CQ, 22% an artemisinins monotherapy and 12% quinine (Q). Treatments from private practitioners contained artemisinins monotherapy in 34%, CQ in 11% and Q in 13% of cases. Similar findings were reported for public health facilities, where artesunate (AS) monotherapy and CQ each accounted for 11% of the treatments given. Provision of free diagnosis and treatment through trained village malaria worker were found to be effective means of increasing ACT coverage in the studied settings. In 2008, a baseline outlet and household survey was conducted in Cambodia in the frame of ACTwatch [238]. The survey assessed levels and trends in the availability, price and volume of antimalarials, providers' perceptions and knowledge of antimalarial medicines at different outlets, and consumer treatment-seeking behaviour and volumes of specific antimalarials consumed. The findings, which are to be presented by the end of 2009, are expected to provide and promote evidence and recommendations for policy makers on methods to increase availability and decrease the consumer price of quality assured artemisinin-based combination therapies through the private sector. WHO and several key partners from the ministries of health and academia currently work on containing the spread of artemisinin-resistant malarial parasites along the Thai-Cambodian border. One of the key objectives of the collaboration is supporting the containment and elimination of artemisinin-tolerant parasites through comprehensive behavioural change, communication, community-based mobilization and advocacy [177,184]. Knowledge of drug use in this specific area could help evaluating the impact of the interventions undertaken.

This study aims at collecting information about circulating antimalarials in two Cambodian provinces with different levels of drug resistance and thus also recommended first-line treatments. Here we report the findings from the analysis of baseline samples from falciparum malaria patients recruited in two *in vivo* studies for the presence of 14 currently in-use antimalarials in a single run using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay [212].

Materials and methods

Study area and population

The study was performed in two rural areas with moderate transmission intensity (Praeh Vihear district, Cambodia) during the main rainy season from October 2007 to February 2008 and July to October 2008. AS–MQ has been the first-line treatment for *Plasmodium falciparum* infection since 2000 and was available at government health facilities as well as in private structures through social marketing program (Malarine). CQ has been used as first-line treatment for *P. vivax* infection. AS, dihydroartemisinin (DHA), and Q can easily be found in the private sector without a doctor's prescription. At the time of the study, DHA–piperaquine (DHA–PPQ) had recently been introduced as first-line treatment in Veal Veng district because of low clinical efficacy of the AS–MQ regimen and was only available at government health facilities to ensure controlled use.

Clinical procedures

The trial was designed to assess the effect of the pharmacogenetic profile on the pharmacokinetics of standard antimalarials (to be reported elsewhere). It was based on the standard WHO protocol for *in vivo* testing [239].

Phnom Dék Health Centre, Rovieng district, Preah Vihear province, Cambodia

The study was performed from October 2007 to February 2008. Suspected malaria cases were screened by microscopy. Total number of patients screened was 234 and out of these 67 (29%) were infected with *P. falciparum* and 74 (32%) with *P. vivax*. No mixed infections

were detected. The patients with a positive slide for *P. falciparum* were then seen by the clinician who invited them to participate in the study if they were older than 6 months, not pregnant or lactating and did not present with danger signs of complicated malaria or any other severe concomitant illness. After informed consent by the patient, the baseline sample (Day 0, 4.5 mL venous blood collected in an EDTA Vacutainer) was taken, hematocrit was measured, filter paper sample and a thin and thick smear were taken. Then the national standard first-line treatment introduced in 2000 [178], i.e. AS (Arsumax, Sanofi-Aventis, France) and MQ (Eloquine, Medochemie Ltd, Cyprus), was given according to age.

Pramoy Health Centre, Veal Veng district, Pursat province, Cambodia

The study was performed from July to October 2008. Suspected malaria cases were screened by microscopy. Total number of patients screened was 287 and out of these 82 (29%) were infected with *P. falciparum* and 50 (17%) with *P. vivax*. No mixed infections were detected. The patients with a positive slide for *P. falciparum* were then seen by the clinician who invited them to participate in the study if they were older than 6 years, not pregnant or lactating and did not present with danger signs of complicated malaria or any other severe concomitant illness. After informed consent by the patient, the baseline sample (Day 0, 4.5 mL venous blood collected in an EDTA Vacutainer) was taken, hematocrit was measured, filter paper sample and a thin and thick smear were taken. Then the treatment with DHA-PPQ (Duo-Cotecxin, Zhejiang Holley Nanhu Pharmaceutical Co., Ltd, China) was given according to age.

Laboratory procedures

Blood samples were immediately aliquoted into whole blood, plasma and pellet and stored in liquid nitrogen. The samples were transferred into a -80 °C freezer within one week. Plasma concentrations of 14 antimalarial drugs and their metabolites, i.e. artemether, AS, DHA, AQ, *N*-desethyl-AQ, lumefantrine, desbutyl-lumefantrine, PPQ, pyronaridine, MQ, CQ, Q, pyrimethamine and sulfadoxine, were determined by liquid chromatography coupled to tandem triple stage mass spectrometry (LC-MS/MS) [212]. The lower limits of the calibration range (LLC) in our method were selected as the lowest levels of the calibration curves, which confidently provide a bias and CV% below ± 20%, in accordance to FDA recommendations [131]. All samples were analyzed twice. First, quantitative measurement was performed using

calibration and quality control samples; then, for confirmation, qualitative assessment was repeated using a new chromatographic column that had not been exposed to any antimalarial drugs,. In order to exclude contamination and false positive results, a large set of blank controls was analyzed prior to the clinical samples on the new column, checking for absence of specific MS/MS signals of the antimalarials investigated.

Data management and analysis

Summary statistics and graphs of the residual plasma concentrations of antimalarials found prior to treatment in the study population were produced using Stata (version 10.1 "intercooled", Stata Corporation, College Station, TX, USA).

Estimation of dose intake time for mefloquine and piperaquine

To estimate the probable timing of drug intake, we compared the plasma concentrations of MQ in patients from Preah Vihear and PPQ in patients from Pursat at baseline (C_0) and on Day 14 (C_{14}) after a complete treatment with the respective drug among the same patients. We included only patients for whom we had both samples and who complied with the three-day treatment schedule. Assuming a mean terminal elimination half-life of $t_{½}$ = 15 (90% CI: 12.93–17.07) days for MQ [240,241] and $t_{½}$ = 27.8 (total range 10.2–216) days for PPQ [242], and a similar dosage on pre-study exposure and during the study, a back-calculation was done to estimate the intake time before baseline sampling:

$$\text{intake time} = \ln(C_{14} / C_0) \cdot t_{½} / \ln(2) + 14 \ \text{[days]}$$

Similar calculations were not attempted for the other antimalarials (i.e. CQ and Q), as we did not have the respective C_0 and C_{14} after a standardized treatment in the study patients.

Results

At Phnom Dék Health Centre, 64 patients were eligible and willing to participate in the *in vivo* study. 38 (59.4%) were male and 26 (40.6%) female; age ranged from 2 to 57 years (median age 18 years). The presence of antimalarial drug was detected in the plasma of 33

(51.6%) patients: 16 (25.0%) had MQ above the lower limit of calibration (LLC = 2.5 ng/mL), 16 (25.0%) CQ (LLC = 2.5 ng/mL), and 3 (4.7%) Q (LLC = 2.5 ng/mL) and none any other antimalarials tested. Summary statistics are shown in Table 1 and box plots of residual plasma concentrations are presented in Figure 1. Of the 32 patients with residual drug concentrations, 3 (9.1%) had more than one drug: 2 patient had CQ and MQ and 1 CQ and Q. For MQ, the 12 of the above 16 patients who had also a value on Day 14, had a median plasma concentration (range) of 52.8 ng/mL (12.6–987 ng/mL) on Day 0. One patient had a plasma concentration above the approximate *in vivo* minimum inhibitory concentration (MIC) of mefloquine for resistant *P. falciparum*, i.e. 500 ng/mL [243,244]. On Day 14, the median plasma concentration was 757 ng/mL (392–1135 ng/mL). This means that a similar dosage level should have been administered at a median of 68 days (interquartile range 47–79 days, whole range 17–102 days) before enrolling into the study, to account for the levels observed on Day 0. For 2 patients (17%) this estimate was <28 days. As there were only 3 late parasitological failures (LPFs) on Day 42, correlation between Day 0 concentration and treatment failure was not assessed. The variability in $t_{½}$ translates into 90% confidence intervals extending from 89% to 111% of estimates (median).

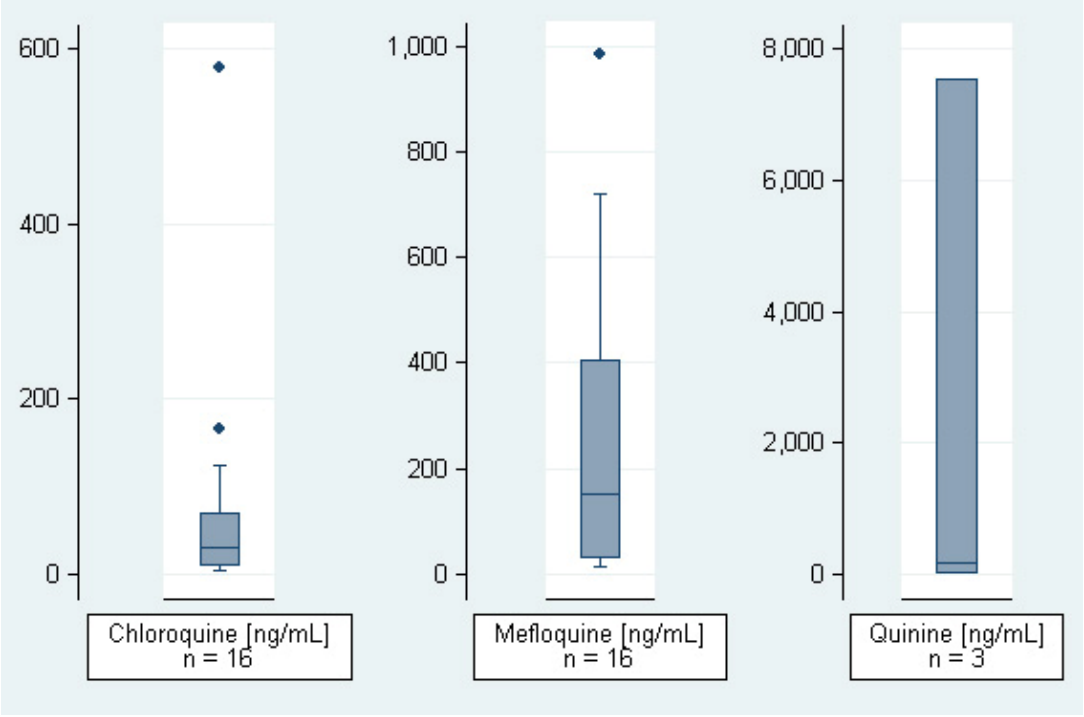

Figure 1. Residual plasma concentrations of antimalarials found prior to treatment in 64 malaria patients from Preah Vihear. Number of patients (n), median, 25th and 75th percentile, lower and upper adjacent values, and outside values are shown for chloroquine, mefloquine and quinine [ng/mL].

Table 1. Residual plasma concentrations of antimalarials found prior to treatment in 64 malaria patients from Preah Vihear [ng/mL].

Antimalarial	Patients	Mean	Median	Minimum	Maximum
Mefloquine	16	260.3	152.5	12.6	987.0
Chloroquine	16	75.8	30.1	4.1	579.7
Quinine	3	72.1	4’312.7	3.5	7’550.8

No artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, desbutyl-lumefantrine, lumefantrine, piperaquine, pyronaridine, sulfadoxine or pyrimethamine was found.

At Pramoy Health Centre, 61 patients were eligible and willing to participate in the *in vivo* study. 38 (62.3%) were male and 23 (37.7%) female; age ranged from 7 to 53 years (median age 18 years). The presence of antimalarial drug was detected in the plasma of 42 (68.9%) patients: 25 (41.0%) CQ, 21 (34.4%) MQ, 9 (14.7%) PPQ (LLC = 2 ng/mL), and 2 (3.3%) Q and none any other antimalarials tested. Summary statistics are shown in Table 2 and box plots of residual plasma concentrations are in Figure 2. Of the 42 patients with residual drug concentrations, 12 (28.6%) had more than one drug: 1 (8.3%) patient had MQ and PPQ, 1 (8.3%) CQ and PPQ, 2 (16.7%) MQ and Q, 5 (41.7%) CQ and MQ, and 3 (25.0%) CQ, MQ and PPQ. For PPQ, among the 7 eligible patients the median plasma concentration (range) was 7.9 ng/mL (6.1–22.9 ng/mL) on Day 0 and 28.5 ng/mL (18.8–56.3 ng/mL) on Day 14. This means that a similar dosage level should have been administered at a median of 67 days (interquartile range 53–81 days, whole range 15–93 days) before enrolling into the study, to account for the levels observed on Day 0. In 1 patients (14%) was this estimate shorter than 28 days. As there was only 1 late parasitological failures (LPFs) on Day 28, correlation between Day 0 concentration and treatment failure was not assessed. The variability in $t_{½}$ (lowest and highest value measured for $t_{½}$, [242]) translates into intervals extending from 50% to 632% of estimates (median).

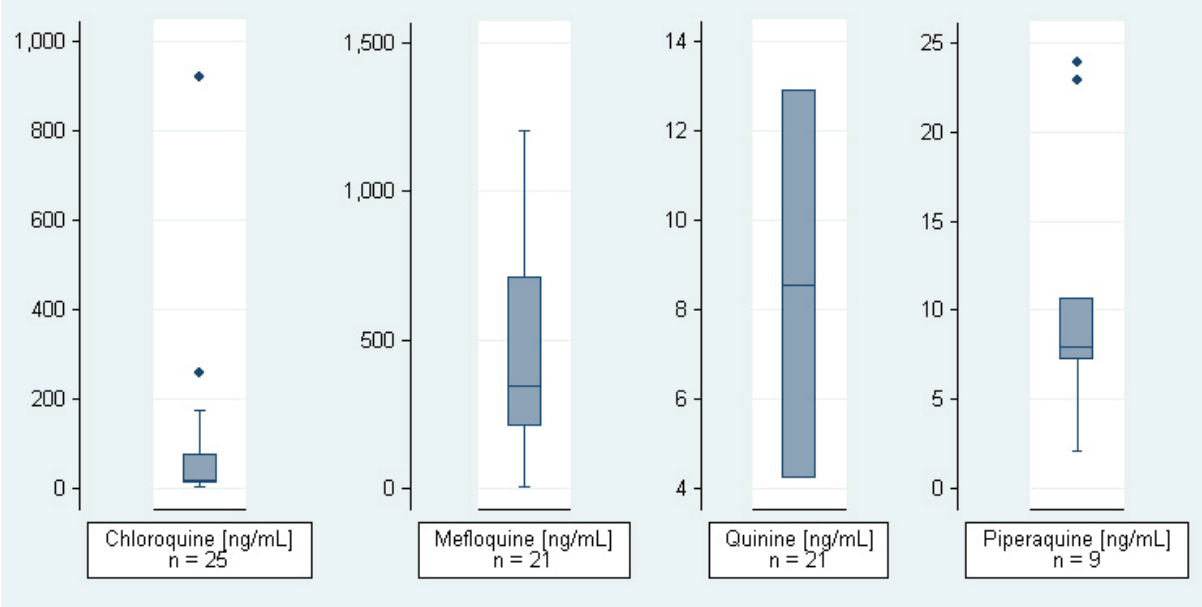

Figure 2. Residual plasma concentrations of antimalarials found prior to treatment in 61 malaria patients from Pursat. Number of patients (n), median, 25th and 75th percentile, lower and upper adjacent values, and outside values are shown for chloroquine mefloquine, quinine and piperaquine [ng/mL].

Table 2. Residual plasma concentrations of antimalarials found prior to treatment in 61 malaria patients from Pursat [ng/mL].

Antimalarial	Patients	Mean	Median	Minimum	Maximum
Chloroquine	25	80.8	16.8	2.6	919.5
Mefloquine	21	431.0	346.2	2.9	1202.4
Piperaquine	9	10.7	7.9	2.1	23.9
Quinine	2	8.6	8.6	4.2	12.9

No artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, desbutyl-lumefantrine, lumefantrine, pyronaridine, sulfadoxine or pyrimethamine was found.

Discussion

This is the first study investigating the presence of a broad range of antimalarials in the plasma of South-East Asian malaria patients prior to treatment. The measurement of 14 antimalarial drugs currently in-use allowed a comprehensive assessment of all circulating drugs in the studied communities in a region with high levels of antimalarial drug resistance,

i.e. Pursat province, and a region with moderate levels of drug resistance, i.e. Preah Vihear province.

Residuals of former first-line treatments

Although Q is not recommended as first-line treatment in uncomplicated falciparum malaria, it was found in 4.7% and 3.3% of patients, respectively. One patient showed a Q plasma concentration of 7.6 mg/L, which corresponds to plasma concentrations during acute oral treatment with ~10 mg/kg Q every 8 hours [245,246]. Furthermore, we could detect MQ in one third of the study patients from Pursat, one patient showing a plasma concentration above the MIC. This shows that people often buy their antimalarials from the private sector as in 2008 the government changed the first-line treatment along the border with Thailand from AS–MQ to DHA–PPQ and thus AS–MQ was no longer available at government health facilities in Western Cambodia. Effective case management including prompt diagnosis and treatment with an appropriate antimalarial are not guaranteed in the private sector. However, this is a key elements of the global strategy to eliminate malaria on a long-term bases [247]. Comprehensive behavioural change of the population is urgently needed to encourage them to seek appropriate management, i.e. laboratory diagnosis and ACT for malaria. The detection of drugs with long residence time does not allow saying neither whether patients have taken it as a monotherapy or as an ACT nor whether the complete dose has been taken. The removal of monotherapies from the market in Cambodia must be enforced as people with poor access to health facilities tend to buy the cheapest antimalarial available in the private sector regardless of its efficacy against the disease.

Residuals of current first-line treatments

We found that more than half of the patients carried residual antimalarials at inclusion into our study. Although CQ has been banned as first-line treatment against *P. falciparum*, it could be detected in the plasma of 25.0% of the patients in the Preah Vihear province and 41.0% of the patients from the Pursat province. This might be explained by the fact that CQ is the recommended first-line treatment against *P. vivax* in Cambodia. We also detected residuals of current first-line treatments in the study patients: 26.6% with MQ in Preah Vihear, where the first-line treatment in 2007 was AS–MQ, and 14.7% with PPQ in Pursat, where the first-line treatment in 2008 was DHA–PPQ. Assuming that the patients have taken MQ (in Preah Vihear) or PPQ (in Pursat) respectively according to the three-day treatment regimen, most

patients must have taken the regional first-line regimen more than 28 days prior to treatment in our study. It is also possible that patients might have taken a sub-therapeutic dose of these antimalarials more recently. However, as indicated by the large variability in elimination half-life, these values represent only rough estimates. The mean Day 7 values observed in this study for the 7 patients with residual DHA–PPQ at inclusion [i.e. mean 49.8 ng/mL (95% CI: 30.9 to 68.7 ng/ml)] were comparable with those of a study in 196 patients from Papua, Indonesia [mean 46.6 ng/ml (95% CI: 43.3 to 49.8 ng/ml) [248]]

Artemisinins

In our study it was not possible to detect any of the artemisinins in the patients' plasma due to the short half-life of these compounds [226]. However, it cannot be excluded that some of the patients might have taken a monotherapy of one of the artemisinins. Considering the high number of patients who did consume antimalarials that should not be used for the therapy of uncomplicated falciparum malaria, it is possible that some might have taken also an artemisinin as either combination or monotherapies but this cannot be verified.

Potential bias in drug safety and efficacy assessment

Previous antimalarial intake may interfere with the outcome of the treatment under investigation. This study shows that only baseline drug concentration measurement in the blood can reliably be used for the purpose. Our LC-MS/MS assay covers 14 antimalarials in a single run. We can confidently exclude a lack of specificity and false positives as we included blank plasma samples as negative controls and systematically repeated the measurement on a new chromatographic column. Previous drug intake may affect the current treatment in several ways. Higher drug exposure resulting from cumulative levels may lead to better efficacy or more toxicity. The parasites causing the disease at the time of enrolment may be less sensitive a population selected by the previous treatment and patients might be considered as treatments failures already at inclusion.

High drug pressure

The likelihood of selecting for drug resistant parasites is highest with sub-therapeutic levels of a single drug. Thus, it is worrying that patients with low residual concentrations of the long-lived antimalarials (i.e. MQ and PPQ) report with a new episode of malaria before they have completely cleared the antimalarial. Price *et al*. reported that the mean plasma PPQ concentration was 16.8 ng/mL (95% CI: 15.1 to 18.6 ng/mL) on Day 28 [248]. In our study, the mean plasma PPQ concentration on Day 0 was 10.7 ng/mL. It is therefore likely that most patients have taken their PPQ treatment as symptomatic treatment of a fever episode or as treatment of a confirmed malaria episode more than one month prior to inclusion. The persisting high drug pressure of MQ facilitates the spread of multi-drug resistant parasites from Western to Northern and Eastern Cambodia where parasites are still susceptible to MQ. The loss of AS–MQ as treatment option against malaria in resource poor areas such as rural Cambodia would not only lead to a higher burden of disease due to treatment failure. The efforts of the ministry of health to restrict the use of DHA–PPQ only to areas of high drug resistance seems to be erased by the non-adherence of the susceptible population to bed net use recommendations and no appropriate seeking behaviour for first-line antimalarial combination therapy in the private and informal sector.

In conclusion this study shows that regardless of the efforts to improve diagnosis and treatment of malaria in government health facilities in Cambodia, many still seek treatment in the private and informal sector which do not necessarily follow national treatment policies. The emergences and spread of parasite resistance against antimalarials along the Thai-Cambodian border can only be contained by comprehensive behavioural change, communication, community-based mobilization and advocacy.

CHAPTER 6

Effects of pharmacogenetics on population pharmacokinetics of artemisinin-based combination therapy in Cambodian and Tanzanian malaria patients

Working paper.

Abstract

The plasma concentration-time profiles of most antimalarial drugs vary considerably between individuals. Whereas several studies have investigated the influence of non-genetic factor on pharmacokinetics of antimalarial none has addressed the effect of polymorphisms in genes encoding enzymes responsible for antimalarial drug metabolism, such as isoenzymes of the cytochrome P450 superfamily (*CYP*) and *N*-acetyltransferase 2 (*NAT2*).

The present study investigated population pharmacokinetics of artesunate, dihydroartemisinin, mefloquine, and piperaquine in Cambodian patients and artemether and lumefantrine in Tanzanian patients. Inter- and intra-individual variability of the pharmacokinetic parameters were assessed and the contribution of demographic, environmental and pharmacogenetic covariates on the inter-individual variability were quantified using a nonlinear mixed-effects model approach.

For artemether, we found that 9% of the inter-individual variability in clearance could be explained by the genotype of *CYP3A5* (reference allele *versus* variant allele *CYP3A5*3*). Heterozygous carriers showed a reduction in clearance of 34%. The alterations in clearance were less pronounced for lumefantrine (increase in clearance of 12% in homozygous carriers of variant allele *CYP3A4*1B*, explaining 2% of the inter-individual variability in clearance) and mefloquine (decrease in clearance of 14% in carriers of homozygous variant allele *CYP3A5*5*, explaining 1% of the inter-individual variability in clearance).

In conclusion, we were able to show that there is a correlation between the pharmacogenetic profile of the host and the pharmacokinetics of antimalarial drugs administered in malaria patients. These results suggest that pharmacogenetics could be one of the basic mechanisms involved in the pharmacokinetics of antimalarial drugs. The knowledge gained from this study could facilitate the selection process of first-line treatment for malaria and would allow dosing adaption based on the pharmacogenetic profile of the population. Such adaptations are needed especially in the most vulnerable groups, including infants, pregnant women, and those with prevalent co-morbidities, where often therapeutic antimalarial drug concentrations over time are not achieved.

Background

One of the most important control measures in malaria is prompt diagnosis and treatment with an effective and safe artemisinin-based combination therapy (ACT). Both drug efficacy and safety are strongly dependent on the achievement of appropriate circulating concentrations under treatment. Insufficient exposure is associated with a risk of failure and emergence of resistance, and too high levels with a risk of toxicity. It has been shown that there is a fixed fractional reduction in parasite number each asexual cycle (first-order kinetics) when blood concentrations of antimalarial drugs exceed the minimum parasiticidal concentration (MPC) for the infecting parasites. The blood concentration at which the multiplication factor per cycle is 1 can be called the minimum inhibitory concentration (MIC) [249]. In order to eradicate malaria parasites from the body, host immune response on the one hand and circulating antimalarial drug concentrations exceeding MIC for the infecting parasites on the other hand are crucial. Several studies showed an association between Day 7 drug concentrations with treatment response [61,147,220,246,249-251]. As the MPC and thus MIC depend on the infecting parasite, these levels should be expressed as a function of the molecular drug resistance markers a parasite is carrying. Dosing-regimens could then be adapted according to the level of circulating resistant parasites. However, dose-adaption in malaria patients cannot be done using the rule of three. There is a marked inter-individual pharmacokinetic variation of some antimalarials [40] and precise pharmacokinetic determinants of treatment outcome in malaria remain uncertain resulting in sub-optimal dosing in vulnerable populations, particularly pregnant women and young children [220]. It is known that drug response is affected by genetic and non-genetic factors. The latter include e.g. food intake, sex, disease status and concomitant treatment. There is data on the influence of age [30], disease status [31], pre-existing host semi-immunity [32], co-morbidity [33-35], concomitant treatment [36,37], environmental factors (e.g. food intake [38]) and pregnancy [30] on the clinical outcome in malaria. However, there is lack of research about genetic factors influencing drug response in malaria. The analysis of genetic polymorphisms in genes encoding proteins and enzymes involved in drug absorption, distribution, metabolism, elimination and action would be a step towards a broader understanding of inter-individual differences in pharmacokinetic profiles and consequential treatment failures and adverse drug reactions in malaria patients. It has been stated that polymorphism of drug-metabolizing enzymes have by far the highest impact on inter-individual differences in drug response [47,252,253]. Depending on the alleles an individual is carrying, the metabolism can be altered**.** A deeper insight in the inter-population variability in the profile of genes encoding

enzymes responsible for antimalarial drug metabolism, such as isoenzymes of the cytochrome P450 superfamily (*CYP*) and *N*-acetyltransferase 2 (*NAT2*), could facilitate the selection of appropriate first-line treatment for uncomplicated malaria in a specific population.

The study of the pharmacogenetic and pharmacokinetic data in two genetically different populations from malaria endemic countries in Asia and Africa might lead to a better understanding of the different factors influencing pharmacokinetic drug profile, i.e. drug response, in malaria patients. Only few studies have assessed the pharmacogenetic profile of enzymes involved in the metabolism of the drug used for the treatments against *Plasmodium falciparum* malaria in a given population exposed to the disease [254-257]. However, none has investigated the influence of the pharmacogenetic profile on pharmacokinetics of antimalarials. The only way to capture numerous single nucleotide polymorphisms (SNPs) in the genes which are known to be involved in drug metabolism is to use large sample size.

The present study investigated population pharmacokinetics of artesunate (AS), dihydroartemisinin (DHA), mefloquine (MQ), and piperaquine (PPQ) in Cambodian patients and artemether (AM) and lumefantrine (LF) in Tanzanian patients. Inter- and intra-individual variability of the pharmacokinetic parameters were assessed and the contribution of demographic, environmental and pharmacogenetic covariates on the inter-individual variability were quantified using a nonlinear mixed-effects model approach.

Materials and methods

Study areas and design

Kibaoni Health Centre, Kilombero district, Morogoro region, Tanzania

The first study was performed from March to May 2008. Suspected malaria cases were screened by rapid diagnostic test (Paracheck, Orchid Biomedical Systems, India). Total number of patients screened was 1672 and out of these 389 (23%) showed a positive test during the recruitment phase. These patients were then seen by the clinical officer who invited them to participate in the study if they did not present with danger signs of complicated malaria or any other severe concomitant illness. After informed consent by the patient, the baseline sample (Day 0, 4.5 mL venous blood collected in an EDTA Vacutainer) was withdrawn, hemoglobin was measured, filter paper sample and a thin and thick smear were

taken. Then the first dose of AM–LF (Coartem, Novartis Pharma, Switzerland) was given according to body weight (see Table 1), time and food intake with drug administration were noted. Patients were then either admitted for three days or asked to come back to the health facility for the following five doses (second dose 8h after first dose, third, fourth, fifth and sixth dose 12, 24, 36, and 48 h after the second dose). Patients were seen by the clinical officer on Days 1, 2, 3, 7, 14, 28, and 42. On Days 1, 2, and 7, 2 mL of venous blood were collected (EDTA Vacutainer) for pharmacokinetics and filter paper sample and a thin and thick smear were taken. The exact time of the blood withdrawal was noted. On Days 3, 14, 28, and 42 only filter paper sample and a thin and thick smear were taken. Hemoglobin was measured on Days 28 and 42. On every visit axillary temperature and respiratory rate were measured. The patients were also asked questions about symptoms such as headache, vomiting and diarrhoea. If patients suffered from concomitant illnesses they were provided with additional treatment (paracetamol, mebendazole, metronidazole, cloxacillin, amoxicillin).

Phnom Dék Health Centre, Rovieng district, Preah Vihear province, Cambodia

The second study was performed from October 2007 to February 2008. Suspected malaria cases were screened by microscopy. Total number of patients screened was 234 and out of these 67 (29%) were infected with *P. falciparum* and 74 (32%) with *P. vivax*. No mixed infections were detected. The patients with a positive slide for *P. falciparum* were then seen by the clinician who invited them to participate in the study if they were older than 6 months, not pregnant or lactating and did not present with danger signs of complicated malaria or any other severe concomitant illness. After informed consent by the patient, the baseline sample (Day 0, 4.5 mL venous blood collected in an EDTA Vacutainer) was withdrawn, hematocrit was measured, filter paper sample and a thin and thick smear were taken. Then the first dose of AS (Arsumax, Sanofi-Aventis, France) and MQ (Eloquine, Medochemie Ltd, Cyprus) was given according to body weight (see Table 1), time and food intake with drug administration were noted. A second blood samples was taken approximately 1 h after the first dose. Patients were then either admitted for three days or asked to come back to the health facility for the following two doses on Day 1 and 2 respectively. Patients were seen by the clinical officer on Days 1, 2, 3, 7, 14, 21, 28, 35 and 42. On Days 1, 2, 7 and 14, 2 mL of venous blood were collected (EDTA Vacutainer) for pharmacokinetics and filter paper sample and a thin and thick smear were taken. The exact time of the blood withdrawal was noted. On Days 3, 14,

21, 28, 35 and 42 only filter paper sample and a thin and thick smear were taken. On every visit axillary temperature and respiratory rate were measured. The patients were also asked questions about history of fever and other symptoms. If patients suffered fever they were provided with paracetamol.

Pramoy Health Centre, Veal Veng district, Pursat province, Cambodia

The third study was performed from July to October 2008. Suspected malaria cases were screened by microscopy. Total number of patients screened was 287 and out of these 82 (29%) were infected with *P. falciparum* and 50 (17%) with *P. vivax*. No mixed infections were detected. The patients with a positive slide for *P. falciparum* were then seen by the clinician who invited them to participate in the study if they were older than 6 years, not pregnant or lactating and did not present with danger signs of complicated malaria or any other severe concomitant illness. The rest of the procedures were the same as those in Phnom Dék except that the drug used was DHA–PPQ (Duo-Cotecxin, Zhejiang Holley Nanhu Pharmaceutical Co., Ltd, China) given according to age (see Table 1).

Table 1. Dosing regimens for the study drugs used

Drug	Weight [kg]	Age [years]	Day 0	Day 1	Day 2
AM – LF	5–14		AM: 2 × 20 mg LF: 2 × 120 mg	AM: 2 × 20 mg LF: 2 × 120 mg	AM: 2 × 20 mg LF: 2 × 120 mg
	15–24		AM: 2 × 40 mg LF: 2 × 240 mg	AM: 2 × 40 mg LF: 2 × 240 mg	AM: 2 × 40 mg LF: 2 × 240 mg
	25–34		AM: 2 × 60 mg LF: 2 × 360 mg	AM: 2 × 60 mg LF: 2 × 360 mg	AM: 2 × 60 mg LF: 2 × 360 mg
	≥35		AM: 2 × 80 mg LF: 2 × 480 mg	AM: 2 × 80 mg LF: 2 × 480 mg	AM: 2 × 80 mg LF: 2 × 480 mg
AS – MQ	10–12.5		AS: 50 mg MQ: 125 mg	AS: 50 mg MQ: 125 mg	AS: 50 mg
	13–15.5		AS: 50 mg MQ: 125 mg	AS: 50 mg MQ: 125 mg	AS: 50 mg MQ: 125 mg
	16–24.5		AS: 100 mg MQ: 250 mg	AS: 100 mg MQ: 250 mg	AS: 100 mg
	25–34.5		AS: 150 mg MQ: 250 mg	AS: 150 mg MQ: 250 mg	AS: 150 mg MQ: 250 mg
	35–37		AS: 200 mg MQ: 250 mg	AS: 200 mg MQ: 250 mg	AS: 200 mg MQ: 250 mg
	38–57		AS: 200 mg MQ: 500 mg	AS: 200 mg MQ: 500 mg	AS: 200 mg MQ: 250 mg
	58–76		AS: 200 mg MQ: 500 mg	AS: 200 mg MQ: 500 mg	AS: 200 mg MQ: 500 mg
DHA – PPQ		6–11	DHA: 60 mg PPQ: 480 mg	DHA: 60 mg PPQ: 480 mg	DHA: 40 mg PPQ: 320 mg
		11–16	DHA: 80 mg PPQ: 640 mg	DHA: 80 mg PPQ: 640 mg	DHA: 80 mg PPQ: 640 mg
		>16	DHA: 120 mg PPQ: 960 mg	DHA: 120 mg PPQ: 960 mg	DHA: 80 mg PPQ: 640 mg

Drugs used: AM: artemether; AS: artesunate; DHA: dihydroartemisinin; LF: lumefantrine; MQ: mefloquine; PPQ: piperaquine

Laboratory techniques

Pharmacokinetics

Blood samples were kept on ice for no longer than 6 h after withdrawal and then aliquoted into whole blood, plasma and pellet and immediately stored in a -80°C freezer. Plasma concentrations of 14 antimalarial drugs and their metabolites, i.e. AM, AS, DHA, amodiaquine, *N*-desethyl-amodiaquine, LF, desbutyl-lumefantrine (DLF), PPQ, pyronaridine, MQ, chloroquine, quinine, pyrimethamine and sulfadoxine, were determined simultaneously using a liquid chromatography–tandem mass spectrometry method (LC–MS/MS) requiring 200 µl of plasma [212]. Plasma was purified by a combination of protein precipitation, evaporation and reconstitution in methanol/ammonium formate 20 mM (pH 4.0) 1:1. Reverse-phase chromatographic separation of antimalarial drugs was obtained using a gradient elution of 20 mM ammonium formate and acetonitrile both containing 0.5% formic acid, followed by rinsing and re-equilibration to the initial solvent composition up to 21 min. Analyte quantification, using matrix-matched calibration samples, was performed by electrospray ionization–triple quadrupole mass spectrometry by selected reaction monitoring detection in the positive mode. The method was validated according to FDA recommendations, including assessment of extraction yield, matrix effect variability, overall process efficiency, standard addition experiments as well as antimalarials short- and long-term stability in plasma. The method is precise (inter-day CV%: 3.1–12.6%) and sensitive (lower limits of quantification 0.15–3.0 for basic/neutral antimalarials and 0.75–5 ng/mL for artemisinin derivatives, respectively). For details see Hodel *et al*. [212]

Pharmacogenetics

DNA was extracted from 200 µl whole blood from the baseline blood samples (Day 0) using the QIAamp 96 DNA Blood Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. Target sequences in cytochrome P450 isoenzymes (*CYP*) and *N*-acetyltransferase 2 (*NAT2*) were amplified by polymerase chain reaction (PCR). Primers were selected to amplify regions containing single nucleotide polymorphisms (SNPs) which are known to alter the function of enzymes involved in the metabolism of antimalarial drugs, namely *CYP2A6*2* (479T>A, L160H), *CYP2B6*5* (1459C>T, R487C), *CYP2B6*6* (only 516G>T, Q172H, also called *CYP2B6*9*), *CYP2C8*3* (only 416G>A, R139K), *CYP2C9*3* (1075A>C, I359L),

*CYP2C9*5* (1080C>G, D360E), *CYP2C19*3* (636G>A, W212X), *CYP2D6*4* (1846G>A, splicing defect), *CYP2D6*10* (100C>T and 4180G>C, P34S and S486T), *CYP2D6*17* (1023C>T and 2859C>T, T107I and R296C), *CYP3A4*1B* (-392A>G), *CYP3A5*3* (6986A>G, splicing defect), *NAT2*5* (341T>C, I114T), *NAT2*6* (590G>A, R197Q), *NAT2*7* (857G>A, G286E), and *NAT2*14* (191G>A, R64Q). SNP selection criteria and the PCR protocol have been described elsewhere (CHAPTER 2). The PCR products were sent to Macrogen, Ltd., Korea for purification and sequencing using the PCR primers. Sequences were analysed using the ABI Prism AutoAssembler version 1.4.0 (Applied Biosystems) for assembly. The genotype of each patient was then assessed visually.

Population pharmacokinetic modelling

Model-based pharmacokinetic analyses

The analysis was performed using the NONMEM computer program [89]. It uses mixed (fixed and random) effects regression to estimate population means and variances of the pharmacokinetic parameters and to identify factors that influence them.

Structural model

One- and two-compartment pharmacokinetic models with elimination from the central compartment and with first-order absorption, with and without absorption lag times, were evaluated. For the antimalarials for which also metabolite concentrations were assessed, i.e. AM, AS and LF, pharmacokinetics of the parent drug and the metabolite were modeled sequentially first and then in a common model. The estimated parameters from the different compartment models (Figure 1) were the systemic clearance (CL), the intercompartmental clearance (Q), the central volume of distribution (V_C), the peripheral volume of distribution (V_P), and the absorption rate constant (k_{12}). Where available, the metabolite was included into the model (i.e. DHA for AM and AS and DLF for LF) and metabolism rate constant (k_{23}) and elimination rate constants for the metabolite (k_{30}) were estimated. The models are described by the following differential equations that express the mass (amount) balance for each of the compartments:

For all drugs

(1) $\frac{dA_D}{dt} = -k_{12} \times A_D$

Artemether and mefloquine

(2) $\frac{dA_C}{dt} = k_{12} \times A_D - k_{20} \times A_C$

Lumefantrine and its metabolite

(3) $\frac{dA_C}{dt} = k_{12} \times A_D - (k_{20}+k_{30}) \times A_C$

(4) $\frac{dA_M}{dt} = k_{23} \times A_C - k_{30} \times A_M$

Piperaquine

(5) $\frac{dA_C}{dt} = k_{12} \times A_D - (k_{23} + k_{20}) \times A_C + k_{32} \times A_P$

(6) $\frac{dA_P}{dt} = k_{23} \times A_C - k_{32} \times A_P$

Where A_D is the amount of the parent antimalarial in the absorption compartment (= depot), A_C and A_M are the amounts derived from the measured concentrations of the parent antimalarial and its metabolite, respectively. A_P is the amount derived from the (unmeasured) concentrations of parent antimalarial in the peripheral compartment. k_{xy} is the rate constant for the transfer from compartment x to y. The amount in a given compartment at a given time t can be derived from the (un)measured concentration C and the estimated volume of distribution V, i.e.:

(7) $A_C(t) = C_C(t) \times V_C$

(8) $A_P(t) = C_P(t) \times V_P$

(9) $A_M(t) = C_M(t) \times V_M$

For PPQ the intracompartmental clearance (Q) was calculated as follows:

(10) $Q = V_C \times k_{23} = V_P \times k_{32}$

Since no intravenous drug concentration data were available, the absolute bioavailability could not be estimated and *CL* and *V* represent apparent values. The V_M for DLF was set to the estimated value for V_C of LF. *CL* was calculated as indicated in Equation 11 for AM, MQ and PQ and Equation 12 for LF:

(11) $CL = k_{20} \times V_C$

(12) $CL = (k_{20} + k_{23}) \times V_C$

Analysis of baseline samples (i.e. Day 0 prior treatment) showed that some patients had residuals of the drug investigated in their plasma from the treatment of the previous malaria episode. To account for this, the residual amount from the previous treatment was estimated as a factor (F_0):

(13) $A_C(0) = F_0 \times e^{\eta_{F_0}}$

Where $A_C(0)$ is the amount of drug present in the central compartment at $t = 0$ and η_{F_0} the inter-individual variability (for details see statistical model).

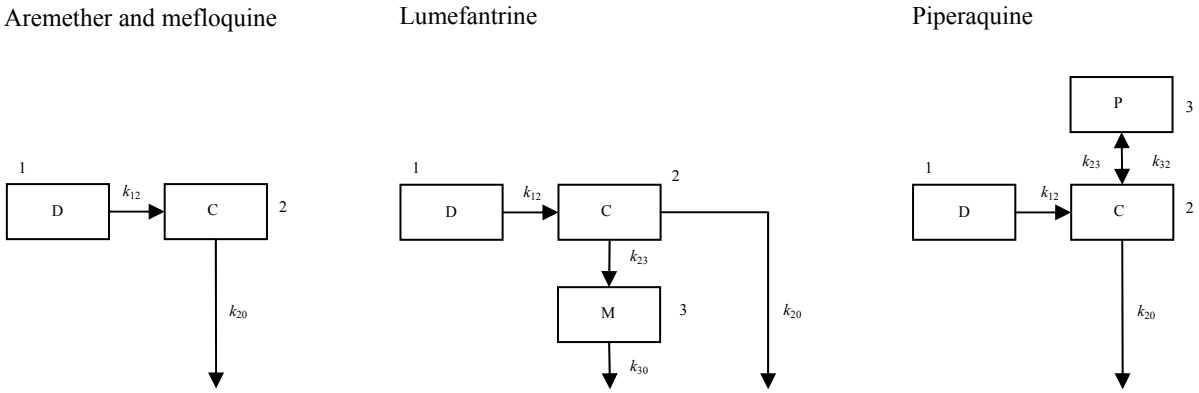

Figure 1**.** Schematic representation of the pharmacokinetic compartmental models. D: Depot, k_a: first-order absorption rate constant, C: central compartment, *CL*: clearance, k_m: metabolism rate constant, M: metabolite compartment, k_e: elimination rate constants for the metabolite, *f*: fraction of the total mass that is not metabolized, P: peripheral compartment, *Q*: intercompartment clearance. See also text.

Statistical model

Exponential errors following a log-normal distribution were assumed for the description of inter-patient variability of the pharmacokinetic parameters and were of the form:

(14) $\theta_i = \theta \times e^{\eta_i}$

Where θ_j is the individual pharmacokinetic parameter value in the *j*th individual, θ is the population parameter estimate, and η_i is the random effect value, which is independently and normally distributed with a mean of 0 and variance ω. Proportional and combined proportional-and-additive error models were compared to describe intra-patient (residual) variability.

Covariate model

Covariates (*X*) evaluated for inclusion during the model building process were body weight, height, age, sex, smoking status, pregnancy (only for Tanzanians as pregnancy was an exclusion criteria in the Cambodian studies), and concomitant medications. Concomitant medications included moderate to strong inhibitors or inducers of the *CYP* responsible for the metabolism of the antimalarial administered to the respective patient (Table 2). Information was based on report of self-medication prior inclusion and prescription during the study. In the analyses of AM and LF, 30 patients included were considered having taken a moderate or strong inhibitor of *CYP2C9* (for AM only) and 14 patients included were considered having taken an inhibitor of *CYP3A4* (AM and LF). In the analyses of AS and MQ, 5 patients were considered having taken an inhibitor of *CYP3A4* and 2 an inducer of *CYP3A4*. 27 patients included in the analysis of PPQ, had taken an inhibitor of at least *CYP2C8*, *CYP2C9* or *CYP2D6*.

The covariate analysis was performed by using a stepwise insertion/deletion approach. Visual inspection of the correlation between post hoc individual parameter estimates and the available covariates (demographic characteristics, concomitant medications) was first conducted by graphical exploration in Microsoft Excel 2000 (Microsoft Corporation). Potentially influential covariates were then incorporated sequentially into the pharmacokinetic model. The typical value of a given parameter θ (e.g., *CL*) was modeled to depend on *X* either

proportionally (Equation 15), linearly (Equation 16) or as an allometric power function (Equation 17):

(15) $\theta = \theta_a \times [1 + \theta_b \times X]$
(16) $\theta = \theta_a \times X$
(17) $\theta = \theta_a \times X^{\theta_b}$

Where θ_a is the estimate of the basal value and θ_b is the contribution of the factor X. For categorical covariates X was set to 0 or 1. For continuous covariates X was tested non-centered and centered on the mean value. In the allometric power models, θ_b was tested fixed to values from literature, i.e. 0.75 on CL and 1 on V_C [258], or estimated.

Table 2. Concomitant medications included in the models

Drug	**Metabolism**	**Concomitant medications taken by study patients**	**Effect†**	***n***
Artemether	*CYP2B6* ‡ [259,260]			0
	CYP2C9‡ [259,260]	Ibuprofen, pyrimethamine, quinine	Strong to moderate inhibitor	30
	CYP2C19‡ [259]			0
	CYP3A4 [37,61,259,260]	Caffeine, doxycycline, erythromycine and metronidazole	Moderate inhibitors	14
	CYP3A5 [61,259]			0
Artesunate	*CYP2A6* [62,64]			0
	CYP2B6 [62,63]			0
	CYP3A4 [62,63]	Clarithromycine, caffeine, metronidazole and tetracycline	Strong to moderate inhibitor	5
		Dexamethasone	Strong inducer	2
Dihydroartemisinin	Glucuronidation [261]	N.A.		
Lumefantrine	*CYP3A4* [37,61]	See artemether *CYP3A4*		
Mefloquine	*CYP3A4* [69-71]	See artesunate *CYP3A4*		
	CYP3A5 [69]			0
Piperaquine	Unknown [58]	Chloroquine, ibuprofen, quinine	Strong to moderate inhibitor of *CYP2C8*, *CYP2C9* and / or *CYP2D6*	27

n: number of patients included receiving the concomitant treatment, N.A.: not applicable because we did not include polymorphisms of UDP-glucuronosyltransferases (UGT) and hence inhibitors of UGT [262,263] as covariates in the population pharmacokinetic models

† From UpToDate Online 17.1 (http://www.uptodateonline.com/online/index.do)

‡ Only *in vitro*, not seen in healthy volunteers [264]

At the end of the analysis, all patient characteristics showing an influence on the parameters were again confirmed by comparing the full model (with all factors included) to models from which each of the factors was removed sequentially.

In the pharmacogenetic analyses SNP information of the enzymes responsible for the metabolism of the respective antimalarial (Table 2) was included. In these analyses, each genotype was entered solo into the model, i.e. homozygous for the reference allele, Heterozygous or homozygous for the mutated allele. A separate fixed effect was assigned to each genotype as follows:

(18) $TV.CL = \theta_1 \times X_1 + \theta_2 \times X_2 + \theta_3 \times X_3$

Where *TV.CL* is the typical value of *CL*. X_i is an indicator variable that takes the value of 1 if an individual carries the *i*th genotypic score (i.e. X_1: homozygous for reference allele Hom-REF, X_2: Heterozygous Het, X_3: homozygous for the allele variant Hom-VAR) and 0 otherwise. The genotypes that yielded a similar fixed effect were then taken together in a reduced model in which the same genotyping group was assigned to Hom-REF compared to Het / Hom-VAR and Hom-REF / Het compared to Hom-VAR:

(19) $TV.CL = \theta_1 \times X_1 + \theta_2 \times X_2$

For PPQ no data on the metabolic pathways was available and thus all available SNPs were tested if their allele frequency was unequal 0% or 100% among the study population, i.e. *CYP2B6*5*, *CYP2B6*6*, *CYP2C9*3*, *CYP2C19*3*, *CYP2D6*10* (100C>T and 4180G>C), *CYP2D6*17* (2859C>T), *CYP3A4*1B*, *CYP3A5*3*, *NAT2*5*, *NAT2*6*, and *NAT2*7*.

Model selection and parameter estimation

The models were fitted by use of the first-order conditional method (and three significant digits) with the subroutines ADVAN2, TRANS 2 (for AM and MQ), ADVAN 4, TRANS 4 (for PPQ) or ADVAN5 (for LF its corresponding metabolite DLF). Goodness-of-fit statistics and graphical displays were used to compare models on each step of model building. As goodness-of-fit statistics, NONMEM uses the objective function, which is approximately equal to minus twice the logarithm of the maximum likelihood. The likelihood ratio test,

based on the reduction in objective function (ΔOF), was used to carry out comparisons between any two models. A ΔOF (−2 log likelihood, approximate χ^2 distribution) of 3.84 points for 1 additional parameter was used for determining statistical significance ($P < 0.05$) of the difference between two models. The figures were generated with GraphPad Prism (version 4.03).

Results

Data

Pharmacokinetic data was obtained for three different ACTs. Table 3 lists the number of samples per time point and Table 4 the patients' characteristics.

Table 3. Number of samples per time point

Day	AM / DHA†	LF / DLF†	AS / DHA†	MQ	DHA	PPQ
Pre-treatment		78 / 7		16		9
0	1 / 1	1 / 0	58 / 62	62	56	57
1	103 / 120	138 / 123	1 / 1	64		59
2	91 / 129	137 / 133		62		60
3	0 / 1	1 / 1				
6				4		6
7		125 / 123		50		48
8		5 / 5		4		5
9		1 / 1		1		
13				6		3
14				46		49
15				4		6
16				2		

Drugs used: AM: artemether; AS: artesunate; DHA: dihydroartemisinin; LF: lumefantrine; MQ: mefloquine; PPQ: piperaquine.

† Parent drug / metabolite

Table 4. Patients' characteristics at inclusion

Characteristic	**AM / DHA†**	**LF / DLF†**	**AS / DHA† and MQ**	**DHA**	**PPQ**
Total patients	135	143	63	56	60
Demographic					
Sex male (%) / female (%)	56 (41) / 79 (59)	62 (43) / 81 (57)	37 (59) / 26 (41)	34 (61) / 22 (39)	38 (63) / 22 (37)
Median age (range) [years]	10 (1–78)	9 (1–78)	18 (2–57)	18 (7–53)	18 (7–53)
Median body weight (range) [kg]	20 (6.5–150)	20 (6.5–150)	43 (10.5–66)	42 (15–67)	42 (15–67)
Median height (range) [cm]	126 (52–181)	126 (52–181)	153 (73–172)	151 (105–171)	152 (105–171)
Clinical					
Pregnant (%)	3 (2)	3 (2)	N.A.	N.A.	N.A.
Smoker (%)	1 (1)	1 (1)	17 (27)	12 (21)	14 (23)
Median time sick (range) [days]	3 (1–14)	3 (1–14)	2 (2–3)	2 (1–3)	2 (1–3)
Median body temperature (range) [°C]	37.5 (35.2–40.4)	37.6 (35.2–40.4)	38.6 (37.9–40.4)	38.4 (37.8–39.8)	38.4 (37.8–39.8)
Median asexual parasites (range) [μL^{-1}]	15,360 (120–>399,960)[a]	15,360 (120–>399,960)[a]	19,600 (1,200–160,000)	16,858 (1038–219,333)	17,229 (1038–219,333)
Median respitarory rate (range) [min^{-1}]	24 (16–38)	24 (16–38)	28 (20–38)	28 (20–40)	28 (20–40)
Median hematrocrit (range) [%]	N.A.	N.A.	30 (24–37)	41 (30–50)	41 (30–50)
Median haemoglobin (range) [g/dL]	10.5 (5.1–16.3)	10.4 (5.1–16.3)	N.A.	N.A.	N.A.

Table 4. Continued.

Characteristic	AM / DHA†	LF / DLF†	AS / DHA† and MQ	DHA	PPQ
Pharmacogenetics allele frequency [%] (n[b])					
*CYP2A6*2*	0.00 (134)	0.00 (142)	0.00 (59)	0.00 (55)	0.00 (59)
*CYP2B6*5*	1.87 (134)	1.76 (142)	5.17 (58)	1.79 (56)	1.67 (60)
*CYP2B6*6*	33.64 (110)	33.62 (116)	41.86 (43)	32.95 (44)	32.61 (46)
*CYP2C8*3*	0.00 (62)	0.00 (66)	0.00 (36)	0.00 (33)	0.00 (37)
*CYP2C9*3*	0.00 (118)	0.00 (125)	8.47 (59)	4.46 (56)	4.17 (60)
*CYP2C9*5*	0.85 (118)	0.80 (125)	0.00 (59)	0.00 (56)	0.00 (60)
*CYP2C19*3*	0.78 (128)	0.74 (136)	3.33 (60)	2.68 (56)	2.50 (60)
*CYP2D6*4*	4.10 (122)	4.30 (128)	0.00 (50)	0.00 (20)	0.00 (22)
*CYP2D6*10* (100C>T)	6.40 (125)	6.92 (130)	65.38 (52)	53.13 (32)	51.43 (35)
*CYP2D6*10* (4180G>C)	74.61 (128)	74.26 (136)	72.41 (58)	68.75 (56)	70.00 (60)
*CYP2D6*17* (1023C>T)	22.38 (105)	20.98 (112)	0.00 (50)	0.00 (13)	0.00 (14)
*CYP2D6*17* (2859C>T)	65.28 (36)	61.84 (38)	10.53 (19)	7.14 (28)	6.45 (31)
*CYP3A4*1B*	72.73 (121)	72.87 (129)	4.72 (53)	1.28 (39)	2.44 (41)
*CYP3A5*3*	19.23 (130)	18.84 (138)	69.49 (59)	58.93 (56)	60.83 (60)
*NAT2*5*	36.43 (129)	36.50 (137)	5.93 (59)	8.04 (56)	7.5 (60)
*NAT2*6*	26.49 (134)	27.11 (142)	35.34 (58)	45.54 (56)	44.17 (60)
*NAT2*7*	2.24 (134)	2.46 (142)	22.88 (59)	19.09 (55)	19.49 (59)
*NAT2*14*	14.17 (127)	14.07 (135)	5.08 (59)	0.00 (56)	0.00 (60)

Drugs used: AM: artemether; AS: artesunate; DHA: dihydroartemisinin; LF: lumefantrine; MQ: mefloquine; PPQ: piperaquine.

† Parent drug / metabolite

[a] Asexual parasites were counted against 200 white blood cells and converted to parasites/µL by assuming a density of 8,000 white blood cells/µL blood

[b] Number of patients from whom SNP data was available

A total of 150 patients was recruited for the study on AM–LF in Tanzania and 7 had to be excluded from the study and the population pharmacokinetic analysis (2 presented with a hemoglobin <5.0 g/dL, 1 patient was unable to swallow the drug, 2 patients withdrew consent, in 1 patient blood withdrawal was not possible and 1 patient presented with >9,999 parasites per 200 white blood cells), leaving 143 patients included in the analysis of LF. For AM, 8 more patients had to be excluded due to undetectable levels of neither AM nor DHA. The median (range) of samples available per subject was 3 (2–4) for LF, 3 (1–4) for DLF, 2 (1–3) for AM and 2 (1–3) for DHA.

A total of 64 patients was recruited for the study on AS–MQ in Cambodia. One had to be excluded from the study due to withdrawal of consent, leaving 63 patients included in the analysis of MQ. In 4 patients only DHA but no AS could be detected. The median (range) of samples available per subject was 5 (3–6) for MQ, 1 (1) for AS and 1 (1) for DHA.

A total of 61 patients was recruited for the study on DHA–PPQ in Cambodia. One had to be excluded from the study and the population pharmacokinetic analysis; the patient withdrew consent, leaving 60 patients included in the analysis of PPQ. For DHA, 4 more patients had to be excluded due to undetectable levels of DHA. The median (range) of samples available per subject was 5 (4–6) for PPQ and 1 (1) DHA.

Population pharmacokinetic analyses

In all three study sites patients with residual concentrations of the study drugs were included. The residual dose from previous treatments was estimated for every patient population. For LF the residual dose was 1.62 mg which corresponds to 0.3–1.4% of the initial dose administered in the study (120–480 mg). Much higher values were estimated for MQ, i.e. 33.4 mg which corresponds to 6.7–26.7% of an initial dose of 125–500 mg, and PPQ, i.e. 123 mg which corresponds to 12.8–25.6% of an initial dose of 480–960 mg.

For AM, a one-compartment model with first-order absorption from the gastrointestinal tract and described appropriately the data. A two-compartment model did not improve the model fit (ΔOFV = 0.015). DHA could not be included into the model because standard errors (S.E.) of the parameters could not be estimated. Furthermore, k_{12} had to be fixed for the calculations of the S.E. of CL and V_C. A better fit was obtained by assigning an inter-patient variability not only to CL but also to V_C (ΔOFV = −20.596). The use of a proportional error models for the

residual intra-patient variability was the most satisfactory. A fixed allometric power function of body weight on *CL* and V_C significantly improved model fit and was kept in the final model ($\Delta OFV \geq -38.816$). Addition of sex, smoking status, pregnancy and concomitant medications on *CL* and V_C did not improve the model significantly ($\Delta OFV \geq -0.577$). However, inclusion of genotype of *CYP3A5* significantly improved the fit, showing a reduction in *CL* of 34% in Het / Hom-VAR compared to Hom-REF ($\Delta OFV = -6.527$). The parameter estimates for the final model and derived parameters are given in Table 5. Figure 2 shows the overall goodness-of-fits plots and the concentration-time plot of AM in the 135 patients included in the analysis.

For LF, a one-compartment model with first-order absorption from the gastrointestinal tract and metabolism into DHA described appropriately the data. A two-compartment model did not improve the model fit ($\Delta OFV = 0.005$). For DLF a one-compartment model was adequately as well. A better fit was obtained by assigning an inter-patient variability not only on *CL* but also on V_C ($\Delta OFV = -75.351$), F_0 ($\Delta OFV = -17.565$) and k_{23} ($\Delta OFV = -198.974$). A proportional error model for the residual intra-patient variability for LF and an additive error model for DLF were the most satisfactory ones. Inclusion of age, height and body weight improved the fit ($\Delta OFV \geq -90.042$). A fixed allometric power function of body weight on *CL* and V_C compared to a simple linear or proportional model was kept in the final model as the linear and proportional models did not show significantly better fits ($\Delta OFV = -0.215$). Addition of sex, smoking status, pregnancy and concomitant medications on *CL* and V_C did not improve the model significantly ($\Delta OFV \geq -0.192$). However, inclusion of genotype of *CYP3A4* significantly improved the fit, showing a modest increase in *CL* of 14% in Hom-VAR compared to Het / Hom-REF ($\Delta OFV = -4.315$). The parameter estimates for the final model and derived parameters are given in Table 5. Figure 3 and 4 shows the overall goodness-of-fits plots and the concentration-time plot of LF and DLF in the 143 patients included in the analysis.

For MQ, a one-compartment model with first-order absorption from the gastrointestinal tract described appropriately the data. A two-compartment model did not improve the model fit ($\Delta OFV = 0.005$). A better fit was obtained by assigning an inter-patient variability not only on *CL* but also on V_C ($\Delta OFV = -171.612$) and F_0 ($\Delta OFV = -210.717$). The use of proportional error models for the residual intra-patient variability was the most satisfactory. Inclusion of age, height and body weight improved the fit ($\Delta OFV \geq -136.107$). A fixed allometric power

function of body weight on *CL* and V_C compared to a simple linear or proportional model was kept in the final model as the linear and proportional models did not show highly significant better fits (ΔOFV = −8.567). Sex as covariate on *CL* was found to increase fit (ΔOFV = −4.049). However, due to the very moderate improve of fit the covariate was not included in the final model. Addition of smoking status and concomitant medications on *CL* and V_C did not improve the model significantly (ΔOFV ≥ −1.216) whereas inclusion of genotype of *CYP3A5* significantly improved the fit, showing a modest reduction in *CL* of 14% in Hom-VAR compared to Het / Hom-REF (ΔOFV = −4.591). The parameter estimates for the final model and derived parameters are given in Table 5. Figure 5 shows the overall goodness-of-fits plots and the concentration-time plot of MQ in the 63 patients included in the analysis.

For PPQ, a two-compartment model with first-order absorption from the gastrointestinal tract described appropriately the data (ΔOFV = −97.234 two- compared to one-compartment model). A better fit was obtained by assigning an inter-patient variability not only to *CL* but also to V_C (ΔOFV = −129.316) and V_P (ΔOFV = −17.290). The use of proportional error model for the residual intra-patient variability was the most satisfactory. Inclusion of a fixed allometric power function of body weight on *CL* (ΔOFV = −6.962) significantly improved the model. The same was found for an allometric power function of body weight on V_C (ΔOFV = −25.777). Although the model with the estimated power was slightly better than the model with power fixed at 1 (ΔOFV = −2.414) the difference was not significant and thus the power was fixed at 1 in accordance with literature [258] and the models of the other antimalarials presented in this study. Addition of sex or smoking status as covariates of *CL* did not improve the model significantly (ΔOFV ≥ −2.014). As the metabolizing cytochromes of PPQ are not known, concomitant treatment was not included in the model. Inclusion of genotype of *NAT2* (variant 590G>A) pharmacogenetics significantly improved the fit, showing a reduction in *CL* of 32% in carriers of Hom-VAR compared to Het / Hom-REF (ΔOFV = −4.460). However, pharmacogenetics was not included into the final model due to reasons discussed later. The introduction of a correlation between *CL* and V_C significantly improved goodness-of fit (ΔOFV = −9.091). The parameter estimates for the final model and derived parameters are given in Table 5. Figure 6 shows the overall goodness-of-fits plots and the concentration-time plot of PPQ in the 60 patients included in the analysis.

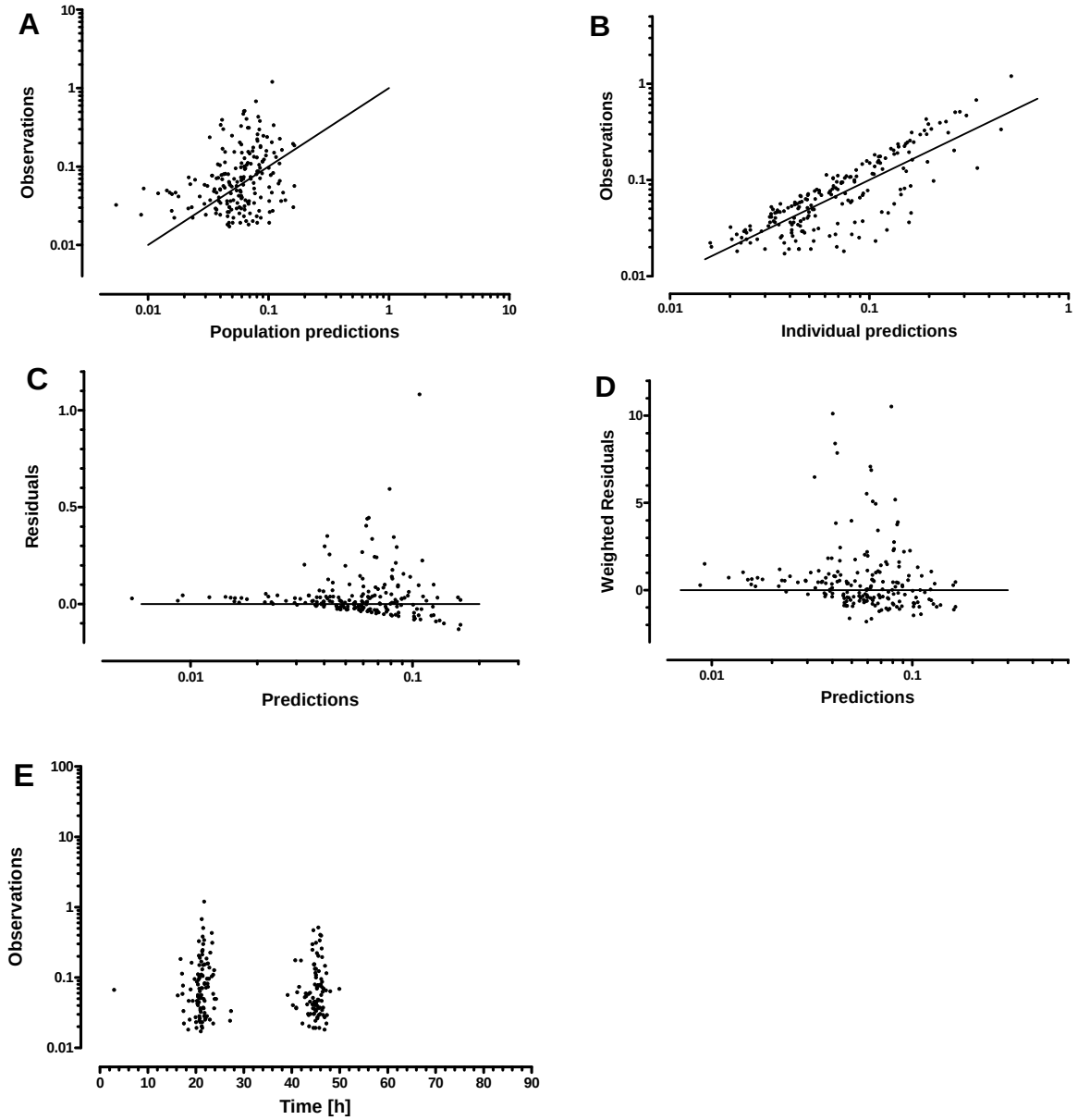

Figure 2. Overall goodness-of-fits plots and the concentration-time plot of AM in 135 patients included in the analysis. Concentrations are in μmol/L. (A) log-log plot of observed concentrations versus population predictions, the line is the line of identity. (B) log-log plot of observations versus individual predictions, the line is the line of identity. (C) Population residuals versus population predictions, the line is at ordinate value zero. (D) Population weighted residuals versus population predictions, the line is at ordinate value zero. (E) Plasma concentration-time plot.

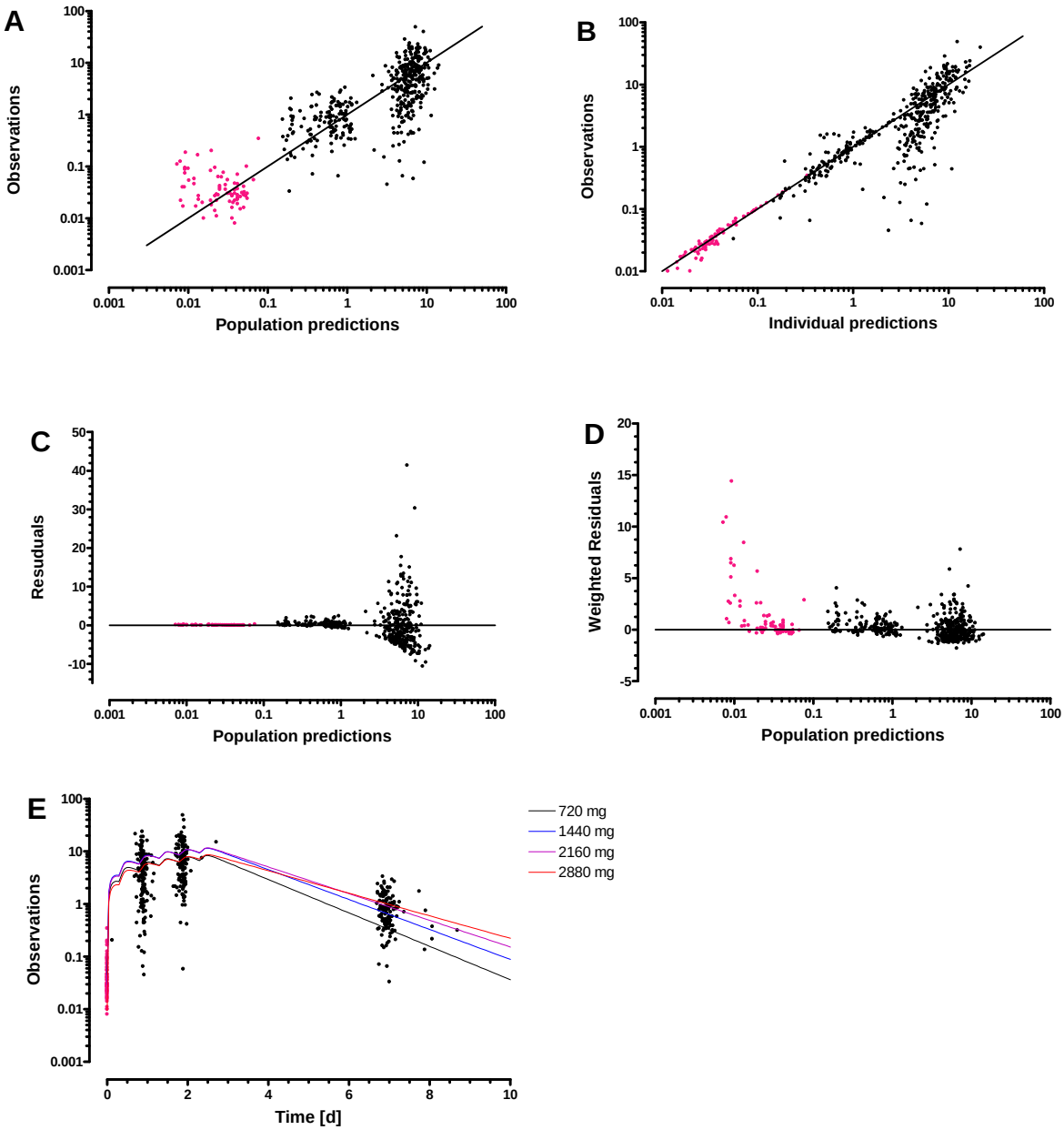

Figure 3. Overall goodness-of-fits plots and the concentration-time plot of LF in 143 patients included in the analysis. Pink dots represent residual plasma concentrations of LF found prior treatment initiation. Concentrations are in µmol/L. (A) log-log plot of observed concentrations versus population predictions, the line is the line of identity. (B) log-log plot of observations versus individual predictions, the line is the line of identity. (C) Population residuals versus population predictions, the line is at ordinate value zero. (D) Population weighted residuals versus population predictions, the line is at ordinate value zero. (E) Plasma concentration-time plot, the lines represent simulated concentrations after different total doses of LF administered to a carrier of two reference alleles of *CYP3A4* with body weight equal to the mean body weight of the study participants who received the same total dose.

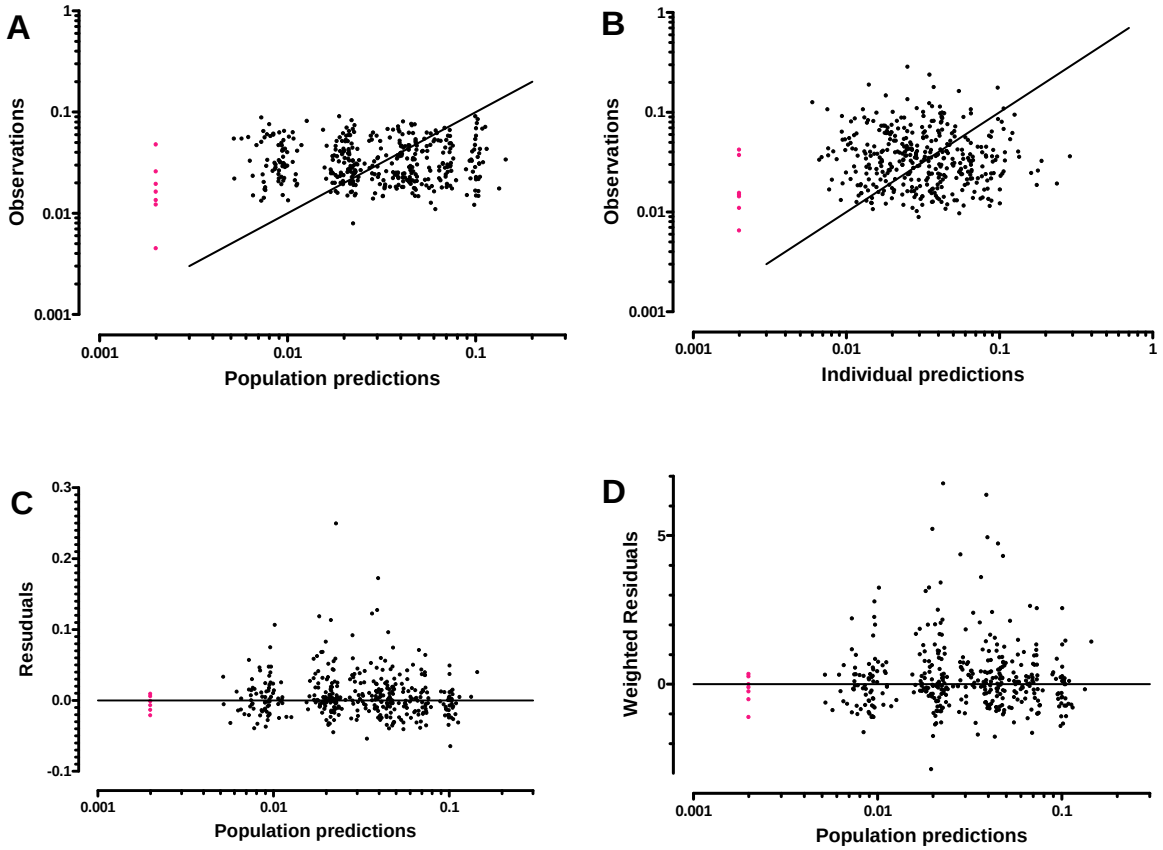

Figure 4. Overall goodness-of-fits plots and the concentration-time plot of DLF in 143 patients included in the analysis. Pink dots represent residual plasma concentrations of DLF found prior treatment initiation. Concentrations are in µmol/L. (A) log-log plot of observed concentrations versus population predictions, the line is the line of identity. (B) log-log plot of observations versus individual predictions, the line is the line of identity. (C) Population residuals versus population predictions, the line is at ordinate value zero. (D) Population weighted residuals versus population predictions, the line is at ordinate value zero.

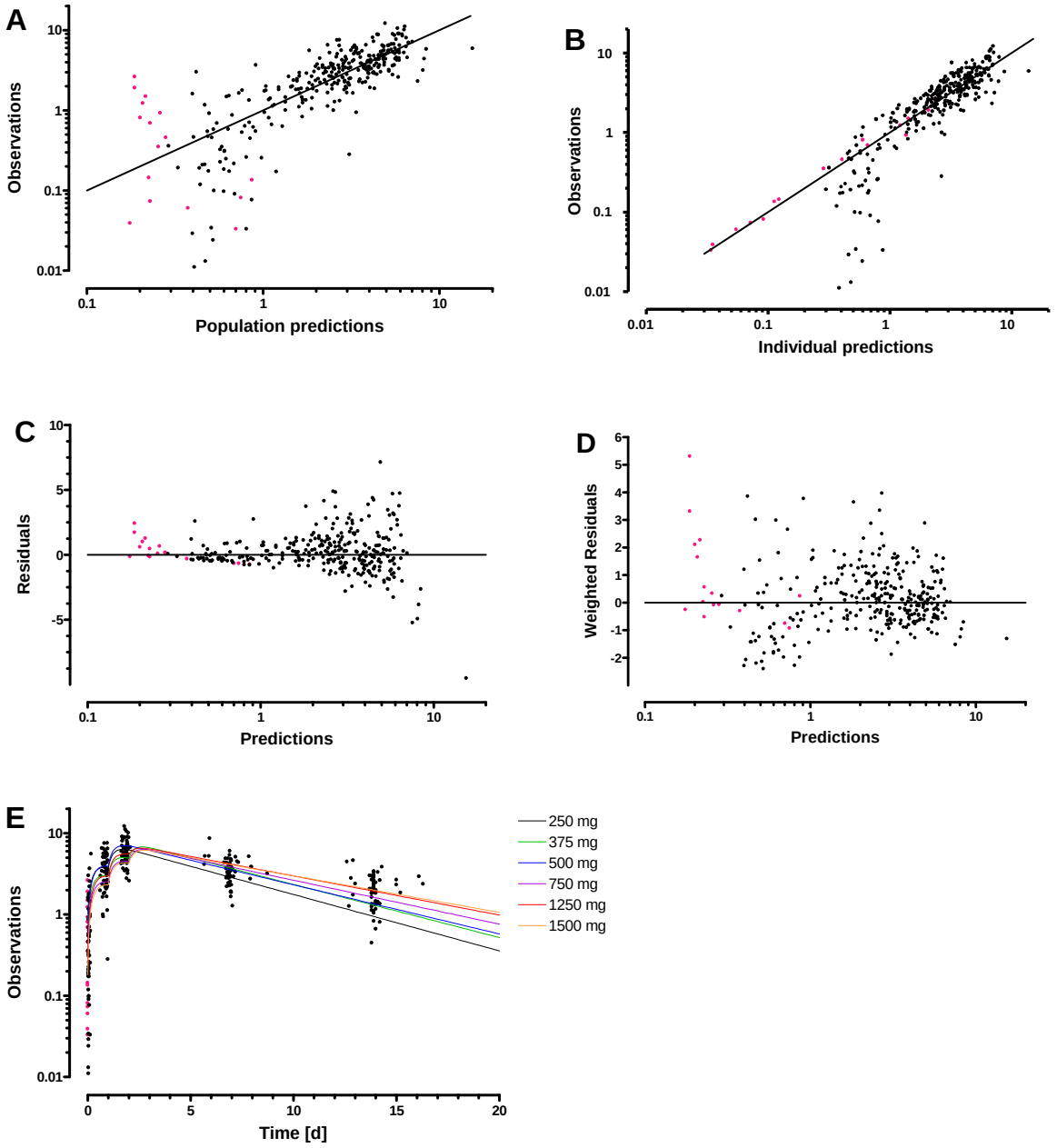

Figure 5. Overall goodness-of-fits plots and the concentration-time plot of MQ in 63 patients included in the analysis. Pink dots represent residual plasma concentrations of MQ found prior treatment initiation. Concentrations are in µmol/L. (A) log-log plot of observed concentrations versus population predictions, the line is the line of identity. (B) log-log plot of observations versus individual predictions, the line is the line of identity. (C) Population residuals versus population predictions, the line is at ordinate value zero. (D) Population weighted residuals versus population predictions, the line is at ordinate value zero. (E) Plasma concentration-time plot, the lines represent simulated concentrations after different total doses of MQ administered to a carrier of two reference alleles of *CYP3A5* with body weight equal to the mean body weight of the study participants who received the same total dose.

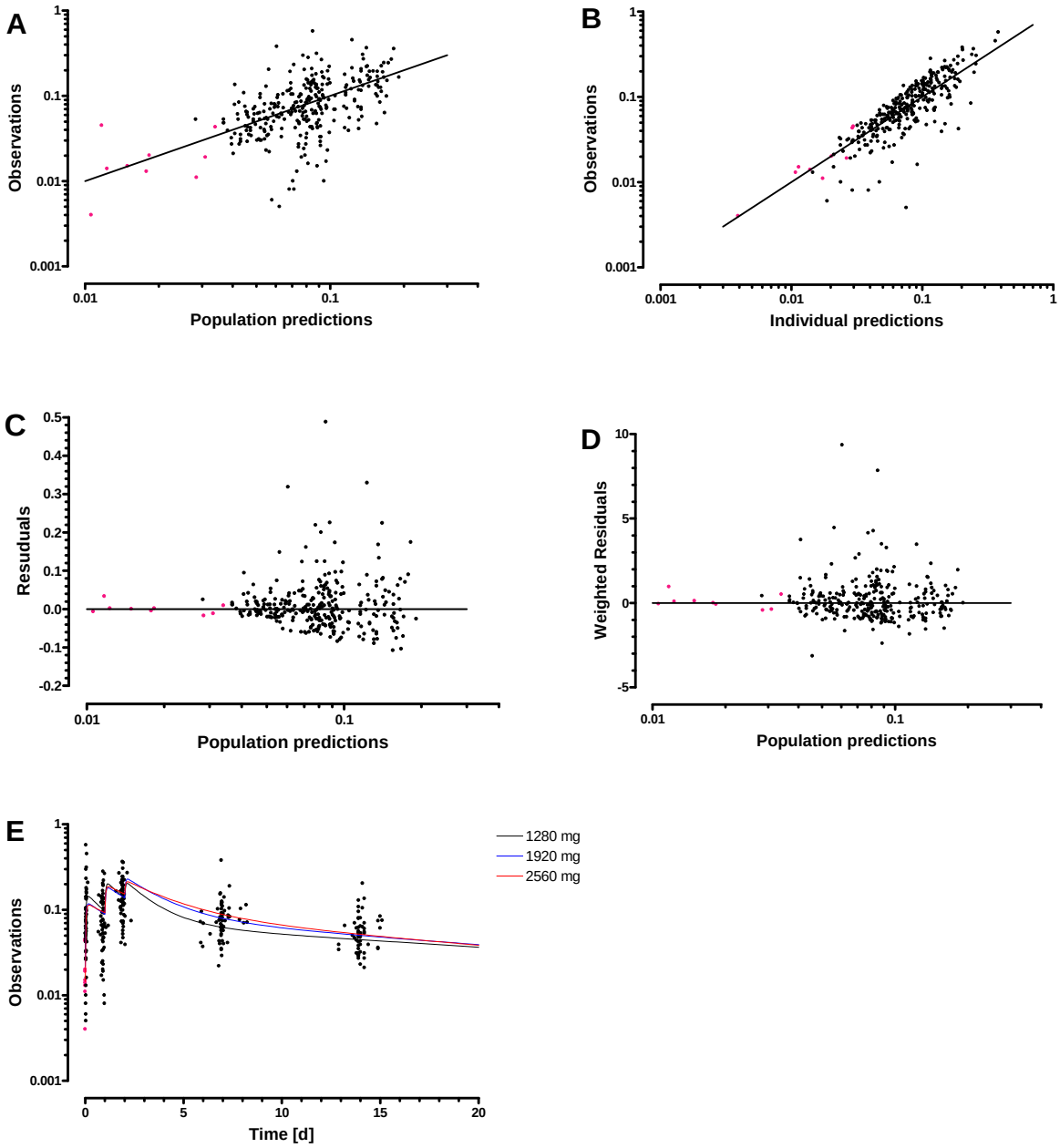

Figure 6. Overall goodness-of-fits plots and the concentration-time plot of PPQ in 60 patients included in the analysis. Pink dots represent residual plasma concentrations of PPQ found prior treatment initiation. Concentrations are in µmol/L. (A) log-log plot of observed concentrations versus population predictions, the line is the line of identity. (B) log-log plot of observations versus individual predictions, the line is the line of identity. (C) Population residuals versus population predictions, the line is at ordinate value zero. (D) Population weighted residuals versus population predictions, the line is at ordinate value zero. (E) Plasma concentration-time plot, the lines represent simulated concentrations after different total doses of PPQ administered to a person with body weight equal to the mean body weight of the study participants who received the same total dose.

Table 5. Parameters estimated for the final models describing population pharmacokinetics of artemether, lumefantrine, mefloquine and piperaquine

Parameter	Artemether		Lumefantrine		Mefloquine		Piperaquine	
	Estimate (S.E.[a])	IIV[b] (S.E.[c])	Estimate (S.E.[a])	IIV[b] (S.E.[c])	Estimate (S.E.[a])	IIV[b] (S.E.[c])	Estimate (S.E.[a])	IIV[b] (S.E.[c])
CL [L/h/kg]	25.0 (7%) × $BW^{0.75}$	27% (71%)	0.36 (10%) × $BW^{0.75}$	36% (43%)	0.11 (7%) × $BW^{0.75}$	10% (98%)	4.50 (13%) × $BW^{0.75}$	28% (61%)
CL [L/h/kg] for *CYP3A4*1B/*1B*			0.41 (13%) × $BW^{0.75}$	36% (43%)				
CL [L/h/kg] for *CYP3A5*1/*3* or **3/*3*	17.5 (13%) × $BW^{0.75}$	27% (71%)						
CL [L/h/kg] for *CYP3A5*3/*3*					0.09 (6%) × $BW^{0.75}$	10% (98%)		
V_C [L/kg]	50.8 (12%)× BW	137% (43%)	6.49 (9%) × BW	46% (38%)	8.91 (6%) × BW	17% (95%)	346 (12%) × BW	32% (48%)
Q [L/h]							122 (13%)	
V_P [L]			Fixed to V_C				18’600 (22%)	39% (77%)
k_{12} [h^{-1}]	0.92		0.54 (89%)		0.15 (14%)		0.93 (28%)	
F_0 [mg]			1.62 (6%)	111% (20%)	33.4 (38%)	82% (47%)	123 (18%)	
k_{23} [h^{-1}]			0.0003 (12%)	32% (70%)				
k_{30} [h^{-1}]			0.03 (7%)					
σ_C [µmolL^{-1}]	0.27 (10%)		0.48 (26%)		0.18 (33%)		0.17 (31%)	
σ_M [µmolL^{-1}]			0.0002 (31%)					

CL: clearance, V_C: central volume of distribution, Q: intercompartment clearance, V_P: peripheral volume of distribution, k_a: first-order absorption rate constant, F_0: residual amount from the previous treatment, k_{23}: metabolism rate constant, k_{30}: elimination rate constants for the metabolite, σ_C: exponential residual error for the central compartment, σ_M: exponential residual error for the metabolite compartment

[a] Standard error (S.E.) of the estimate θ_i defined as (S.E.$_\theta$/θ)*100%

[b] Inter-individual variability defined as √(η)*100%

[c] Standard error (S.E.) of the coefficient of variation defined as √(S.E.$_\eta$ /η)*100%

Owing to the limited number of samples and inadequate sampling time the data collected for AS and its metabolite DHA from the combination treatment with MQ and the data collected for DHA from the combination treatment with PPQ did not allow the estimation of population pharmacokinetic parameters. Thus, the results for AS and DHA are not presented.

Discussion

This study allowed analyses of population pharmacokinetics of several antimalarials currently used as first-line treatments in Cambodia and Tanzania. Congruence of the pharmacokinetic estimates from this study with estimates from previously published population pharmacokinetic models was assessed. The estimated values for *CL* and *VC* for AM, LF, MQ and PPQ in this study are in line with the previously published estimates (see Table 6 for review). However, no literature data is available for DLF. All estimates from this study are within the range of the published data, except for the V_C of PPQ which was found to be smaller in this study than reported by other authors. This could be explained by the number of compartments used in the reference studies, i.e. steady-state volume of distribution instead of V_C and V_P.

In this study we were able to quantify the effect of different covariates on the pharmacokinetic parameters of ACTs. Inter-individual variability in pharmacokinetic parameters of antimalarials depends on many non-genetic factors such as age, disease status, co-morbidity, concomitant treatment, environmental factors (e.g. food intake), pregnancy, and adherence. Whereas in our study body weight accounted for 10–30% of the inter-individual variability in *CL*, other covariates such as sex, smoking, pregnancy and concomitant treatment did not show a significant contribution to the inter-individual variability of the estimated pharmacokinetic parameters. As expected, an allometric power function of body weight on *CL* and V_C improved model fit for all antimalarials investigated. This is in line with the applied dosing regimens of antimalarials according to body weight or age as surrogate for body weight. According to literature, 0.75 for θ_b in *CL* and 1 for θ_b in V_C were selected [258] as the models with the estimated θ_b did not show a significant change in the OFV. Concomitant treatment did not show a significant effect on clearance for any of the drugs. This might be explained by the fact that the information included into the model was based on self-reporting by patients or guardians, and administered drugs, dose, last intake and duration of treatment were thus difficult to assess. Polycyclic aromatic hydrocarbons in tobacco smoke are believed to be responsible for the induction of cytochrome *CYP1A2* and possibly *CYP2E1* [271]. Most

of the antimalarials investigated are not metabolized by any of these enzymes (Table 2), and thus it was not expected that smoking would be a significant covariate in our model. Tarning *et al*. speculated that *CYP1A2* might be involved in the metabolism of PPQ [58]. However, smokers did not show a significantly altered PPQ metabolism compared to non-smokers. This can be because *CYP1A2* is not the (main) enzyme metabolizing PPQ and/or because of high exposure of the whole study population to soot (e.g. cooking at fireplace, slash and burn agriculture, production of wood charcoal) and a resulting bias. Although it is well known that fatty meals increases bioavailability of antimalarials [259,272-274], the information on food intake was not tested as covariate in the population pharmacokinetic models. Patients who were not admitted to the health centre were encouraged to take at least a light meal at home. However, assessment of meals taken with the drugs (i.e. none, light, heavy) may not be as accurate as in inpatients. In a study on adherence to treatment regimens in Tanzania, only 0.4% of patients took the antimalarials with food (Kabanywanyi *et al.*, in preparation). Thus, the data seemed too unreliable and was not included as covariate. This might explain some of the misfit in the goodness-of-fit plots (Figures 2 to 6), i.e. fasting might have resulted in lower observed concentrations than predicted. As small children were not able swallow pills, AM–LF had to be crushed, mixed with a few milliliters of water and given to the children on a spoon. Often some of the drug got spilled by the person preparing and/or administering the drug. Likewise, this might have led to a non-quantifiable loss of administered dose and possibly to lower observed concentrations than predicted.

Apart from non-genetic factors, differences in ethnicity have a profound impact on drug clearance due to pharmacogenetic polymorphism in drug metabolizing enzymes and transporters, or drug targets. Alterations in clearance can have an impact on safety, efficacy and dosing regimen. In the context of tropical regions, the situation may be even more serious due to co-infections and multiple drug therapy, which may affect drug clearance. Up to this paper, there was no information on the influence of pharmacogenetics on pharmacokinetics of ACTs. Thus, the main aim of the present study was to investigate whether specific SNPs in *CYP* and *NAT2* could explain parts of the inter-individual variability in the pharmacokinetic profile of antimalarials in Cambodian and Tanzanian patients.

Table 6. Population estimate of clearance and steady-state volume of distribution of antimalarials for a person with a median body weight of 70 kg from mixed effects models from the present study and in previous studies

Drug	Subjects	Median age (range) [years]	No. of patients	*CL* [L/h]	V_{SS} [L]	Reference
AM	Patients	10 (1–78)	135	605[a]	3556	Present study
	Patients	22 (14–60)	217	180[b]	217[b]	[265]
	Patients	3.0 (0.4–9)	90	125	63	[266]
LF	Patients	9 (1–78)	143	8.7[a]	454	Present study
	Patients	22 (14–60)	217	15.4	684	[265]
	Patients	23 (13–59)	102	7	298	[61]
	Patients	20 (5–66)	309	7.6	361	[61]
	Healthy			29.8	388[c]	[61]
	Pregnant women	24 (15–42)	103	8.7	257	[267]
MQ	Patients	18 (2–57)	63	2.7[a]	623	Present study
	Patients	14.8 (8–61)	128	1.4	574.7	[268]
	Patients	9.3 (4–15)	74	3.71	1089.2	[268]
	Prophylaxis	26[d] (18–55)	1,111	1.75	863	[269]
	Patients	19 (2–55)	50	2.1	767.62	[270]
PPQ	Patients	18 (7–53)	60	109[a]	42’820	Present study
	Patients	3–55	98	98[e]	61,180[e]	[242]
	Patients	30 ± 13[f]	38	63	40,180	[242]
	Healthy volunteers	2–10	47	129.5	42,980	[242]
	Healthy volunteers	31 ± 3.5[f]	12	70[g]	7210[g]	[242]

Drugs used: AM: artemether; AS: artesunate; DHA: dihydroartemisinin; LF: lumefantrine; MQ: mefloquine; PPQ: piperaquine.

[a] Population estimate for a patient with reference allele of the metabolizing cytochrome P450

[b] Fixed parameter at mean value

[c] Body weight not specified and thus estimate for a patient of unknown body weight

[d] Mean

[e] Population estimate for a patient with a median body weight of 48 kg

[f] Mean ± standard deviation

[g] Parameter estimates are weight normalized based on published population mean values divided by the mean weight of subjects

For AM, the effects of *CYP2B6*, *CYP2C9*, *CYP2C19* and *CYP3A4/5* genotypes on *CL* of AM were assessed. Previous studies on AM turnover to DHA showed that the turnover was highest for human recombinant *CYP2B6* [260]. Although, intestinal *CYP3A4* might play a role in the presystemic metabolism of AM [275,276] interaction studies indicated that liver *CYP3A4* is not important in the *in vivo* metabolism of AM [277-279]. This is in line with our finding that there was no difference in *CL* in Het / Hom-VAR compared to Hom-REF of *CYP3A4*. Likewise, no significant difference in the pharmacokinetic profile of AM could be seen in carriers of *CYP2B6*5*, *CYP2B6*6*, *CYP2C9*5* or *CYP2C19*3* compared to carriers of the respective reference allele, indicating that either allele frequencies were too low to show a significant effect on *CL* or that there is indeed no interethnic difference in metabolism of AM on the basis of a genetic polymorphism of these enzymes. However, inclusion of the pharmacogenetic profile of *CYP3A5* significantly improved the fit, showing a reduction in *CL* of 34% in Het / Hom-VAR compared to Hom-REF and explaining 9% of the inter-individual variability. *CYP3A5*3* is the most frequent and functionally important SNP in the *CYP3A5* gene [280]. The mutation confers low *CYP3A5* protein expression as a result of improper mRNA splicing and reduced translation of a functional protein [168]. In our study, about one third of patients were carriers of at least one *CYP3A5*3* allele. In these patients, a slightly prolonged therapeutic effect of AM can be expected. Although AM is generally well tolerated [281], toxicity due to interactions with other drugs could be anticipated. There is considerable geographic overlap between occurrence of HIV/AIDS and malaria [282] and thus many malaria patients are likely to take ARVs. As most interactions with ARVs involve drugs that interact with *CYP* enzymes [283], one could imagine that competition at the binding site of *CYP3A5* is more prominent in carriers of *CYP3A5*3* resulting in a prolonged circulation of ARVs and higher risk of toxicity. Hence, the pharmacogenetic profile of malaria patients might not only affect the pharmacokinetic profiles of the antimalarials administered but also their potential of drug-drug interactions. Considering the high number of malaria patients worldwide, further studies on drug-drug interactions of antimalarials in specific patient populations would therefore help to identify patients at higher risk of toxicity or treatment failure.

For LF, 2% of the inter-individual variability in *CL* could be explained by the genotype of *CYP3A4*. An increase in *CL* of 12% in Hom-VAR was seen in comparison with Het / Hom-REF. The literature about alterations of the metabolism in *CYP3A4*1B* carriers is very inconclusive. Some authors proposed higher doses of tacrolimus or docetaxel, both substrates

of *CYP3A4*, for carriers of *CYP3A4*1B* due to enhanced clearance of these drugs and potential risk of underexposure [158,159]. Amirimani *et al.* suggested that the increased enzyme activity in carriers of the A>G substitution 290 bp upstream of the *CYP3A4* transcription start site (*CYP3A4*1B*) leads to enhanced expression of the enzyme [160,162]. On the other hand, studies in cancer patients reported decreased metabolism of drugs in carriers of *CYP3A4*1B* [156,157]. Many publications even doubt that inter-individual differences in drug metabolism can be attributed to the allelic variant *CYP3A4*1B* [163,164,166-170,284]. Our results support rather the findings of enhanced clearance in carriers of the allelic variant. Although, the phenotypic effect observed in this study was rather moderate it could become more important in the context of reduced parasite susceptibility. The combination of AM–LF achieves its antimalarial effect through an initial rapid reduction in parasite biomass attributable to the short-acting but highly potent AM, with the subsequent removal of the remaining parasites by the intrinsically less-active but more slowly eliminated LF and thus overall cure rates depend on there being sufficient LF to remove the residual parasite biomass left by AM [61,224,229]. Currently, AM–LF shows high efficacy in Tanzania [146]. However, when the emergence of the reduced susceptibility to artemisinins observed in South-East Asia [177-184] spreads to Africa, the protecting effect of LF will become pivotal for cure. In our study, allele frequency *CYP3A4*1B* was above 70% and mean LF plasma concentration observed in Hom-VAR on Day 7 was 447.6 ng/mL compared to 508.6 ng/mL in Het / Hom-REF. The relationship between drug concentrations and therapeutic response depends on the drug susceptibility of the infecting parasites [224]. Hence, quite a large proportion of patients might be at higher risk of treatment failure due to lower protecting levels of LF if doses are not adapted for this population.

Interestingly, we observed a high allelic frequency of the variant allele *CYP3A4*1B* and low frequency of the reference allele *CYP3A5*1* in Tanzania and *vice versa* in Cambodia (Table 4). Whereas studies in Cambodia on efficacy of AM–LF showed cure rates of only 71.1% [147], AM–LF stays highly efficacious in Tanzanian [146]. The high treatment failure rate in Cambodia could not be explained entirely by food intake nor could parasite resistance be demonstrated using molecular markers [147,173]. Thus, one could speculate that host factors might influence treatment outcome. Among the Cambodian subjects in the present study, about 13% were Hom-REF for *CYP3A5* (CHAPTER 2). If we translate the findings from the Tanzanian population to the Cambodian population, we would expect that 13% of Cambodians show a 32% higher *CL* and a resulting lower area under the plasma drug

concentration-time curve (AUC) of AM than the majority of Cambodians. In these 13%, the risk of treatment failure could be increased due to reduced exposure of parasites to the artemisinin component of the ACT. This hypothesis is strengthened by the findings from the above mentioned efficacy study on AM–LF in Cambodia where treatment failure rate was still around 13% even after food supplementation. To confirm the hypothesis that the observed increase in drug *CL* is clinically significant, it will be necessary to do a population pharmacokinetic study of AM–LF in Cambodians. Furthermore, data on therapeutic drug levels of ACTs must be included in the analysis, especially in infections with parasites showing reduced susceptibility to ACTs.

For MQ, only 1% of the inter-individual variability in *CL* could be explained by the genotype of *CYP3A5*. A slight reduction in *CL* of 14% was seen in Hom-VAR compared to Het / Hom-REF. As mentioned above, the mutation confers low *CYP3A5* protein expression [168] and could explain the lower clearance in homozygous carriers of *CYP3A5*3*. Whereas *CL* of AM in Het was similar to *CL* in Hom-VAR in MQ only Hom-VAR showed a significant reduction in *CL*. The drug dependent impact of *CYP3A5* genetic polymorphism on drug disposition might be explained by the substrate specificity and product regioselectivity [168]. Although, the reduction in *CL* of 12% seems rather moderate, the allele frequency of *CYP3A5*3* in the patients studied was nearly 70% and thus a considerable number of patients receiving MQ are at higher risk of neuropsychiatric adverse effects. These findings highlight the importance of better understanding of dose-dependant adverse effects in populations with high frequencies of slow metabolizer alleles.

For PPQ, inclusion of pharmacogenetics significantly improved the fit, showing a reduction in *CL* of 32% in carriers of *NAT2*6*/**6* compared to carriers of at least one reference allele. The reduction in inter-individual variability of *CL* was 3%. However, *NAT2*6* was not included into the final model of PPQ as the chemical structure of PPQ and the described metabolites [58] do not suggest an *N*-acetylation as metabolic pathway. Furthermore, due to lack of data on PPQ metabolism all SNPs available were tested individually. Thus the significant improve of the model fit due to inclusion of the genotype of *NAT2* as covariate in the model at 5% significance level could represent a chance finding. For PPQ none of the 12 SNPs tested lead to a significant difference in OFVs of ≥8.28 (corresponding to Bonferroni corrected *P*-value of ≤0.004).

Conclusion

Inter-individual variability in pharmacokinetic parameters of antimalarials depends on many factors such as age, disease status, co-morbidity, concomitant treatment, environmental factors (e.g. food intake), pregnancy, and adherence. This study assessed the impact of the pharmacogenetic profile on the clearance of several antimalarial drugs currently in use in South-East Asia and Africa. Overall, pharmacokinetics explained up to 10% of the differences in *CL* between subjects. Whereas for AM, the change in clearance between carriers of different allelic variants of the main metabolizing *CYP* was prominent, in LF and MQ it was rather moderate. These might represent isolated findings and more studies, ideally with the same ACTs, in different populations are needed to confirm the influence of the pharmacogenetic profile on the clearance of antimalarials. There are still large gaps of knowledge about (i) the metabolism of antimalarials, e.g. PPQ, (ii) the genotype–phenotype association of SNPs in drug metabolizing enzymes, e.g. *CYP3A4*1B*, and (iii) the correlation of drug concentrations and drug response of antimalarials.

A large population is exposed to malarial and consequently to antimalarials. Thus, even if only a small relative number of people experiences changes in drug disposition leading to increased risk of toxicity or treatment failure as well as altered potential of drug-drug interactions, this might represent a considerable absolute number of patients worldwide. As allele frequencies vary greatly between countries and continents a region-specific degree of the impact of pharmacogenetics on pharmacokinetics may be expected. As a consequence, treatment-policies should incorporate information on the metabolic pathway of the drugs and the frequency of alleles associated with slow, rapid and ultrarapid metabolizing phenotypes in the selection process. Drug safety and efficacy could thereby be improved.

General discussion

Between 2007 and 2008 three *in vivo* studies have been preformed in 125 and 150 malaria patients from Cambodia and Tanzania, respectively, in order to assess the effect of pharmacogenetics on the pharmacokinetic profile of artemisinin-based combinations. Plasma concentrations of the administered antimalarials were measured using a LC–MS/MS method developed specifically for this purpose and the pharmacogenetic profiles of the patients were determined by direct sequencing. The collected data was included in population pharmacokinetic models enabling the quantification of the effect of different covariates on pharmacokinetic parameters of ACTs.

Determination of plasma concentration of ACTs

Among the various determinants of treatment response, the achievement of sufficient circulation drug concentrations is essential for curing malaria. So far, one of the obstacles to obtain this information has been the lack of sensitive, reliable, robust analytical methodologies for the detection of antimalarials in blood or plasma. The methods developed previously mostly aimed at detecting a single or few antimalarials generally belonging to a single chemical class. Most of these assays were used for clinical trials or studies on a limited number of antimalarial compounds [58,94-130]. However, our study design necessitated an assay that allowed extraction and detection of several drugs and their main metabolites simultaneously with no limitation for drug classes. To be used on a large number of samples, an assay with reduced overall analytical time and costs compared to previously described methods was needed.

High performance liquid chromatography (HPLC) is widely used and relatively economical and triple stage mass detection (MS/MS) qualifies for the measurement of arrays of structurally unrelated antimalarial agents and their metabolites in a single analytical run. In collaboration with the laboratory of the Division of Clinical Pharmacology (PCL) of the University Hospital in Lausanne (CHUV), Switzerland, we thus developed a sensitive LC–MS/MS method for the simultaneous detection of the major antimalarial agents currently used as drug combinations (artemether, artesunate, lumefantrine, piperaquine, pyronaridine, amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine and pyrimethamine) as well as some of their metabolites (dihydroartemisinin, desbutyl-lumefantrine, desethyl-amodiaquine) in a small volume of plasma. The assay requires only as little as 200 µl of plasma and is an improvement over previous methods in terms of convenience (a single extraction procedure for 14 major antimalarials and metabolites reducing significantly analytical time), sensitivity,

selectivity and throughput. The method was validated according to well-established recommendations [131], including assessment of extraction yield, matrix effect variability, overall process efficiency, standard addition experiments, as well as short- and long-term stability of antimalarial drugs in plasma.

A major limitation for the use in field studies in malaria endemic countries is investment costs for equipment. However, we could demonstrate that there was no loss of stability in samples collected in the field and stored on ice for a few hours or in liquid nitrogen for several days, respectively, before transfer in a –80°C freezer and subsequent shipment to the laboratory for analysis. Thus, the method is suitable for the analysis of field samples collected in remote areas where no electricity is available but dry ice or liquid nitrogen is available.

The successful performance of the assay has been demonstrated in our three *in vivo* studies. In rural areas of Cambodia and Tanzania, baseline plasma samples from malaria patients reporting no antimalarial drug intake within the last 28 days were screened for the presence of 14 antimalarials prior to the initiation of study ACTs. The analyses of samples revealed residual concentrations of antimalarials from previous episodes in more than half of the patients both in Tanzania and Cambodia. The simultaneous analysis of 14 antimalarials allowed the detection of residuals not only of the study drugs, i.e. recommended national first-line drugs against *falciparum* malaria, but also of first-line treatments previously used and the recommended first-line treatments against *vivax* malaria. Such detection is an important advantage compared to other methods were parallel runs would be necessary to collect the data about all anticipated drugs. The data obtained from the baseline samples enabled us to establish a better assessment of the antimalarial drugs circulating in the local population, and hence of the drug pressure on the parasites in both countries. Due to the high sensitivity of the assay, residual plasma concentrations of antimalarials with long half-life were quantifiable even several weeks after previous intake, allowing us to estimate time between baseline and the previous intake of a standard dose of the detected antimalarial. This time interval indicated either the number of fever episodes or, if a reliable diagnostic test was used, the number of malaria episodes in the study populations. For some drugs the interval was as short as a few days, suggesting that the drug was taken during the current episode (i.e. quinine), and for others it was estimated to be 1 (i.e. lumefantrine), 2 (i.e. mefloquine) or 3 months (i.e. sulfadoxine–pyrimethamine).

The assay was also applied for the measurement of plasma drug concentrations over time during the *in vivo* studies. The high-throughput of the assay allowed the analysis of a large number of samples in a relatively short time. Furthermore, the small volume of plasma needed for analysis was a major advantage in terms of ethical considerations, and permitted the inclusion of very small children (1 year of age) into the study. Although children <5 years belong to the most vulnerable group of malaria patients [285], pharmacokinetic data in these patients are very scarce and hence attempts for pooling data from several small studies were discussed [40,220]. The main limitation in pooling individual patient data is the variability in assay and analysis methodology between pharmacokinetic studies. The assay methodology is a key determinant of the accuracy of the results and should thus meet high quality requirements in terms of recovery, coefficients of variation (for quality control samples), back-calculated concentrations for calibration standards, regression model, stability, lower limits of quantification, sample handling, volume of sample, biological matrix, anticoagulant, duplicate assay, inter- and intra-assay variability, and data handling [40]. Our method met these criteria and could thus be used for future pharmacokinetic studies of antimalarials.

In summary, we conclude that our method is suitable for simultaneously detecting the presence of drug in subjects for screening purposes and quantifying drug exposure after treatment. It may contribute to filling the current knowledge gaps in the pharmacokinetic–pharmacodynamic relationships of antimalarials and better define the therapeutic dose ranges in different patient populations. Future steps could be an extension of the method by including antiretroviral drugs (ARVs) in the same run. The geographical prevalence of HIV/AIDS and malaria overlap because a significant number of HIV-infected individuals live in regions with different levels of malaria transmission. The simultaneous analysis of plasma concentrations of antimalarials and ARVs could contribute to a better understanding of chemotherapeutic interactions during malaria in HIV co-infected individuals.

The data from the two baselines surveys gave information about circulating antimalarial drugs in the communities and the resulting drug pressure. Our findings challenge the usual statement that access to drugs in remote rural places is poor and that stock-outs are frequent [213,214,237,286-288]. In order to investigate whether access to antimalarial treatment depends on the distance from home to health facilities and drug shops, a more formal assessment of the geographical distribution of antimalarial drugs circulating in different

communities should be performed. This would allow quantifying the magnitude of drug pressure according to drug availability.

Assessment of the pharmacogenetic profiles of malaria patients

Genetic polymorphisms in drug metabolizing enzymes can lead to differential enzymatic activity due to altered substrate binding capacity. Resulting phenotypes are slow, rapid or ultrarapid metabolizers. The analysis of these polymorphisms is a step towards a broader understanding of inter-individual differences in pharmacokinetic and pharmacodynamic profiles and consequential treatment failures and adverse drug reactions. The main enzymes involved in the metabolism of antimalarial drugs are the cytochrome P450 isoenzymes (*CYP*) and *N*-acetyltransferase-2 (*NAT2*). Thus, we analysed allele frequencies of single nucleotide polymorphisms (SNPs) in *CYP* and *NAT2* in samples from Cambodia and Tanzania in order to assess whether ethnic differences in the pharmacokinetic profile of these enzymes exist.

DNA microarrays are considered to be cost-effective tools for the high-throughput analysis of known SNPs in a large sample size [188]. Building on the experience of a previously in-house designed DNA microarray for the SNP analysis of drug resistance markers in *P. falciparum* [188], we developed a microarray for the simultaneous analysis of several SNPs in *CYP* and *NAT2*. Fluorescence scanners for the acquisition of microarray data are now available in many laboratories in malaria endemic areas and the technology could be transferred to malaria endemic areas. However, the development of the pharmacogenetics microarray proved to be more difficult than expected. The design of the primers for the PCR and primer extension reactions was compromised by the large sequence homology of the *CYP* genes and their pseudogenes. Comparison of SNP data gathered from the DNA microarray and direct sequencing in 96 patients from Tanzania and Cambodia showed that the performance of the microarray was not satisfactory. Furthermore, whilst the cost of sequencing has decreased considerably since this project was started, the cost for microarray reagents (especially Cy3- and Cy5-labeled ddNTPs) and glass slides for arraying has increased. Therefore, the costs per SNP with the microarray technology are not considerably lower and overall costs of both methods have become comparable. This implies that samples from future studies on SNP in malaria endemic areas might be sent to one of the service laboratories providing sequencing services at competitive prices.

For the analysis of the pharmacogenetic profile of the patients included in our *in vivo* studies, direct sequencing of PCR products was applied. We investigated SNPs in *CYP2A6, CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, *CYP3A5*, and *NAT2*. Most alleles were found to be in Hardy-Weinberg equilibrium, indicating that sampling was unbiased. The comparison of allele frequencies in our study populations with the available data from literature [149-152,152-155] showed high agreement. Alleles showing major inter-ethnic differences in frequency were of *CYP2D6*, *CYP3A4*, *CYP3A5*, and *NAT2*. This is in accordance with previous reports showing that allele frequencies of *CYP2D6* and *NAT2* vary considerably between continents or even countries [149,150], leading to differing distribution patterns of phenotypes, i.e. poor, rapid and ultra-rapid metabolizers.

CYP2D6, *CYP3A4/5* and *NAT2* are not only responsible for the metabolism of antimalarials but also many drugs used for long-term therapy of infectious diseases highly prevalent in malaria endemic areas are substrates, inducers or inhibitors of these enzymes, e.g. ARVs and antituberculosis (anti-TB) drugs. *CYP3A* is highly inducible, extremely polymorphic and metabolizes many of the drugs that are key components of highly active ARV therapy regimens [289]. The anti-TB drug isoniazid is a substrate of *NAT2* [290] and the anti-TB drug rifampicin is an inducer of several cytochromes, including *CYP2D6* and *CYP3A* [291-294]. These are just a few examples illustrating the importance of considering information about the metabolic pathway of drugs when selecting the treatment regimen for patients receiving long-term therapy with different drugs that alter the metabolism of each other. Taken together, these data show that we are facing two different phenomena. On the one hand mutual transcriptional alteration of the metabolizing enzymes can lead to drug-drug interactions. On the other hand pharmacogenetic differences alter the metabolic capacity of *CYP* and *NAT2*. While the former phenomenom is relatively well investigated [295], the latter has only recently begun to receive appropriate attention and clearly illustrates that the data gained from drug-drug interaction studies cannot be transferred directly from one population to another due to differences in the pharmacogenetic profile. While this problem is buffered to some extent in industrialized countries by a trend towards individualized pharmacotherapy according to the genetic profile of a patient [296,297], similar approaches are far from reality in resource poor settings. Consequently, if individual pharmacogenetic information is not available, at least allele frequencies within a given population should be considered during treatment policy making at the local level.

A limitation of this work was the sample selection process. Originally, it was planned to perform a cross-sectional community based survey in Cambodia and Tanzania in addition to the *in vivo* studies in order to get a broader view of the pharmacogenetic profile in these populations. Due to increase in costs of the field work, we were not able to perform these additional studies. Comparison of the data collected from malaria patients shows similar results to other (South-East) Asian and African populations, indicating that malaria patients seem to represent an accurate subsample of the general population. Nevertheless, future studies aiming at collecting pharmacokinetic data in the general population should be based on a cross-sectional approach with random selection of households.

In summary, we were able to assess the pharmacogenetic profiles of the populations in Cambodia and Tanzania regarding genes known to govern drug disposition. We found clear differences in the distribution of genotypes of certain enzymes between the two populations. The resulting differences in phenotypes could explain partially the inter-individual variability in drug metabolism. Hence, pharmacogenetic information should be included in future decisions in first-line treatment selection at the national level.

Effects of pharmacogenetics on the pharmacokinetic profile of ACTs

The present PhD thesis investigated population pharmacokinetics of artesunate–mefloquine and dihydroartemisinin–piperaquine in Cambodian patients and artemether–lumefantrine in Tanzanian patients. Inter- and intra-individual variability of the pharmacokinetic parameters were assessed and the contribution of demographic, environmental and pharmacogenetic covariates to the inter-individual variability were quantified using a nonlinear mixed-effects model approach.

Data on population pharmacokinetics of antimalarials is still scarce. The few studies available often include only a small number of patients, constraining the ability to draw conclusions on optimal dosing. Even fewer studies were conducted in the most vulnerable populations such as pregnant women or children [40]. Owing to the small plasma volume per time point required by our LC–MS/MS assay for the determination of circulating drug concentrations, we were able to include also very small children into our pharmacokinetic analysis.

The pharmacokinetic parameters we estimated in our models were in agreement with those from other population pharmacokinetic studies. As expected, the inclusion of a fixed allometric power function of body weight on clearance and central volume of distribution significantly improved model fit. Allometric scaling accounts for development of body size and function but not for the fact that the drug-metabolizing capacity of the liver is generally low at birth [298]. It could thus be interesting to develop the model further and include information about the stage of maturation of the drug-metabolizing part of the *CYP*. Unfortunately, there is still not enough data on the time point the activity of the applicable *CYP* isoform(s) attains adult levels. Available studies even on the same *CYP* isoform have given very divergent results [298].

In order to account for parts of inter-individual variability in drug-metabolizing capacity of the liver we included pharmacogenetic data as covariate. For artemether, we found that 9% of the inter-individual variability in clearance could be explained by the genotype of *CYP3A5* (reference allele *versus* variant allele *CYP3A5*3*). Carriers of at least one variant allele showed a reduction in clearance of 34%. The alterations in clearance were less pronounced for lumefantrine (increase in clearance of 12% in homozygous carriers of variant allele *CYP3A4*1B*, explaining 2% of the inter-individual variability in clearance) and mefloquine (decrease in clearance of 14% in homozygous carriers of variant allele *CYP3A5*5*, explaining 1% of the inter-individual variability in clearance). Interestingly, we also found a reduction in inter-individual variability of clearance of 3% when including the genotype of *NAT2* (reference allele *versus* variant allele *NAT2*6*) in the model for piperaquine. A reduction in clearance of 32% was observed in homozygous carriers of the variant allele. The literature on piperaquine metabolism is scarce and the involved enzymes are unknown. But data on the metabolites found in urine indicated that *CYP* might play a role [58]. The chemical structure of piperaquine and its metabolites does not favour *N*-acetylation, questioning our finding that *NAT2* genotypes seem to modify the clearance of this drug. We were not able to find a plausible explanation of the results and therefore omitted pharmacogenetics as a covariate in the final model of piperaquine, claiming that it might have been a chance finding due to multiple testing of SNPs. One could imagine that *NAT2*5* is an effect modifier for another factor involved in piperaquine clearance and future studies might reveal a linkage equilibrium with another gene coding for a protein or enzyme involved in the clearance of piperaquine. Considering the relatively moderate number of patients included in the population pharmacokinetic model of piperaquine, more data would be necessary to assess whether the

genotype of *NAT2* has an impact on the inter-individual variability of clearance of piperaquine or not.

In order to place our findings within a broader context, we referred to studies on the efficacy of artemether–lumefantrine in Cambodia and Tanzania (CHAPTERS 2 and 6) [146,147]. Artemether–lumefantrine was very effective against *falciparum* malaria in Tanzania on the one hand and much less effective in Cambodia on the other hand, i.e. up to ~30% treatment failures. Because of the differences observed between Cambodians and Tanzanians in allele frequencies of *CYP3A4/5*, the main enzymes involved in the metabolism of artemether–lumefantrine [61], we hypothesized that pharmacogenetics might play a role in treatment outcome. In our population pharmacokinetic models of the two antimalarials we could confirm the impact of the genetic makeup on metabolism of these antimalarials and the resulting circulating plasma concentrations over time. Among the Cambodian subjects in our study, about 13% were Hom-REF for *CYP3A5* (CHAPTER 2). If we translate the findings from the Tanzanian population treated with artemether to the Cambodian population, we would expect that 13% of Cambodians show a 32% higher clearance and a resulting lower area under the plasma drug concentration-time curve (AUC) of artemether than the majority of Cambodians. In these 13%, the risk of treatment failure could be increased due to reduced exposure of parasites to the artemisinin component of the ACT. This hypothesis is strengthened by the findings from the above mentioned efficacy study on artemether–lumefantrine in Cambodia where treatment failure rate was still around 13% even after food supplementation and correction for drug resistance markers. To confirm the hypothesis that the observed increase in drug clearance is clinically significant and potentially leading to treatment failure, it would be necessary to (i) perform a population pharmacokinetic study of artemether–lumefantrine in Cambodians and (ii) ideally also to assess the pharmacogenetic profile of the patients included in the study on artemether–lumefantrine showing 13% treatment failure rates [147].

In summary, we were able to show that there is a correlation between the pharmacogenetic profile of the host and the pharmacokinetics of antimalarial drugs administered in malaria patients. The results from our study may contribute to achieve one of the major aims of the clinical pharmacology component of the Worldwide Antimalarial Resistance Network (WARN), i.e. to investigate whether therapeutic antimalarial drug concentrations over time are achieved in the majority of all target groups, including infants, pregnant women, and those

with prevalent co-morbid diseases (especially HIV/AIDS, malnutrition) or if there are important sub-groups being under (or over) dosed [40]. A subsequent step should then be the analysis of covariates influencing the pharmacokinetic profile, i.e. circulating drug concentrations over time.

Treatment effectiveness does depend not only on the achievement of appropriate circulating concentrations of the antimalarial drugs but also on host immunity and parasite resistance. Immunity to malaria is very complex and still not fully understood [299]. Therefore, it is difficult to include this data in the assessment of treatment effectiveness. However, appropriate circulating drugs can be easily defined by the therapeutic window of the drug. The upper limit of the therapeutic window is usually known from clinical studies during the development phase of the drug. The lower limit of the interval, i.e. minimal inhibitory concentration (MIC), strongly depends on the parasite resistance and resulting susceptibility to the drug. Optimal dosing can only be ensured through defining the correlation between drug concentration and clinical and parasitological response. Hence, another issue addressed by the WARN is the collection of data on therapeutic drug levels of antimalarial drugs, *in vitro* drug susceptibility of the parasite and molecular markers associated with drugs resistance [93]. Once the data from the WARN database is available, one should try to define therapeutic levels as a function defined by molecular drug resistance markers in the parasite and the half maximal inhibitory concentration (IC50) from *in vitro* assay. In a second step, the function could be expanded by including pharmacogenetic data. Taken together national first-line treatment selection and necessary dosing adaptation could then be based on current data on the level of drug resistance of circulating parasites and the pharmacogenetic profile of the population.

In order to confirm our findings of the influence of the pharmacogenetic profile on the clearance of antimalarials more studies, ideally with the same ACTs, in different populations are needed. The main limitations in pooling population pharmacokinetic data are the differences in study design and the variability in assay and analysis methodology between pharmacokinetic studies. This complicates the meta-analysis of results from different laboratories and studies. Additional studies should thus be conducted using the same study design and methodologies.

Conclusion and recommendations

We developed a sensitive LC–MS/MS method for the simultaneous analysis of the major antimalarial agents currently used and successfully applied it to samples collected in the field. Furthermore, we were able to detect differences in the pharmacogenetic profile of enzymes responsible for the metabolism of antimalarial drugs in Cambodians and Tanzanians. Plasma concentrations and the data gained from the pharmacogenetic analysis were included in pharmacokinetic models and allowed the estimation of the effect of pharmacogenetics on the pharmacokinetic profile of different ACTs. Pharmacogenetics accounted for up to 9% of the inter-individual variability in total clearance of the ACTs investigated. The knowledge gained from this study could facilitate the selection process of first-line treatment for malaria and would allow dosing adaptation based on the pharmacogenetic profile of the population. In order to exclude that these are isolated findings, we recommend that future studies collect more date on:

(i) The enzymes involved in the metabolism of antimalarials
(ii) The phenotype associated with specific mutations in drug metabolizing enzymes
(iii) The frequencies of allele variants causing metabolic alterations in different populations
(iv) The therapeutic drug levels of antimalarials as a function of parasite susceptibility
(v) The effect of co-medication on plasma levels of antimalarials
(vi) The pharmacokinetic profile of antimalarials in specific vulnerable patient groups
(vii) The association of pharmacogenetics and pharmcodynamics in malaria patients
(viii) The relation of population pharmacokinetics to overall treatment effectiveness

Appendix

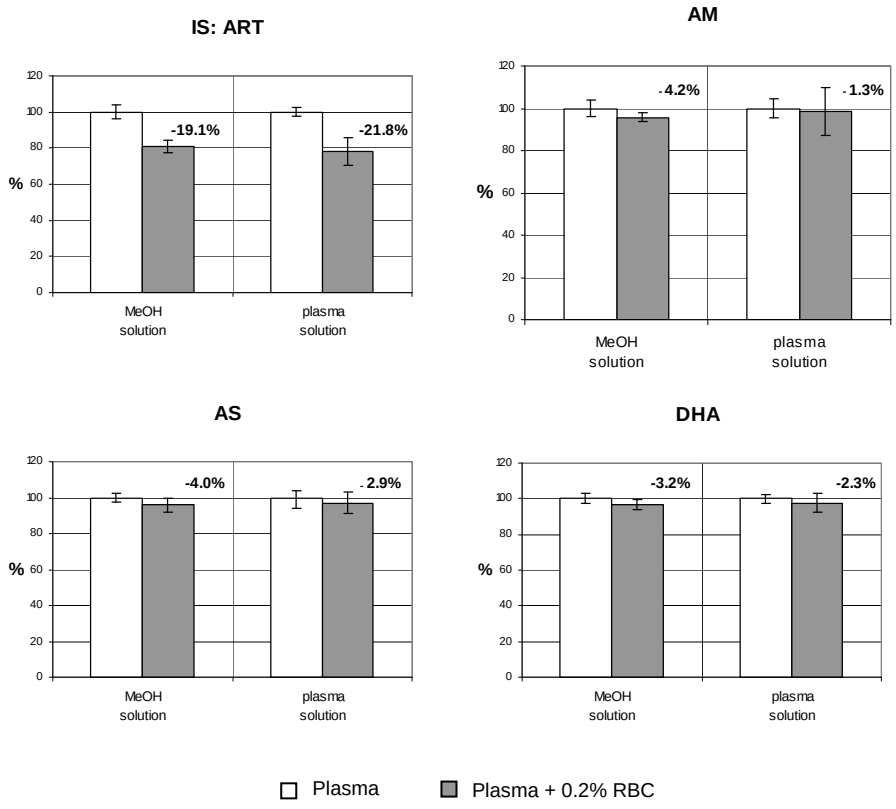

Figure S1. Impact of the presence of MeOH on the assay of endoperoxide antimalarials in hemolysed plasma. Signal intensities for endoperoxide antimalarials are expressed as the mean percentage of calibration samples prepared in plasma with no RBC, used as control (100%).

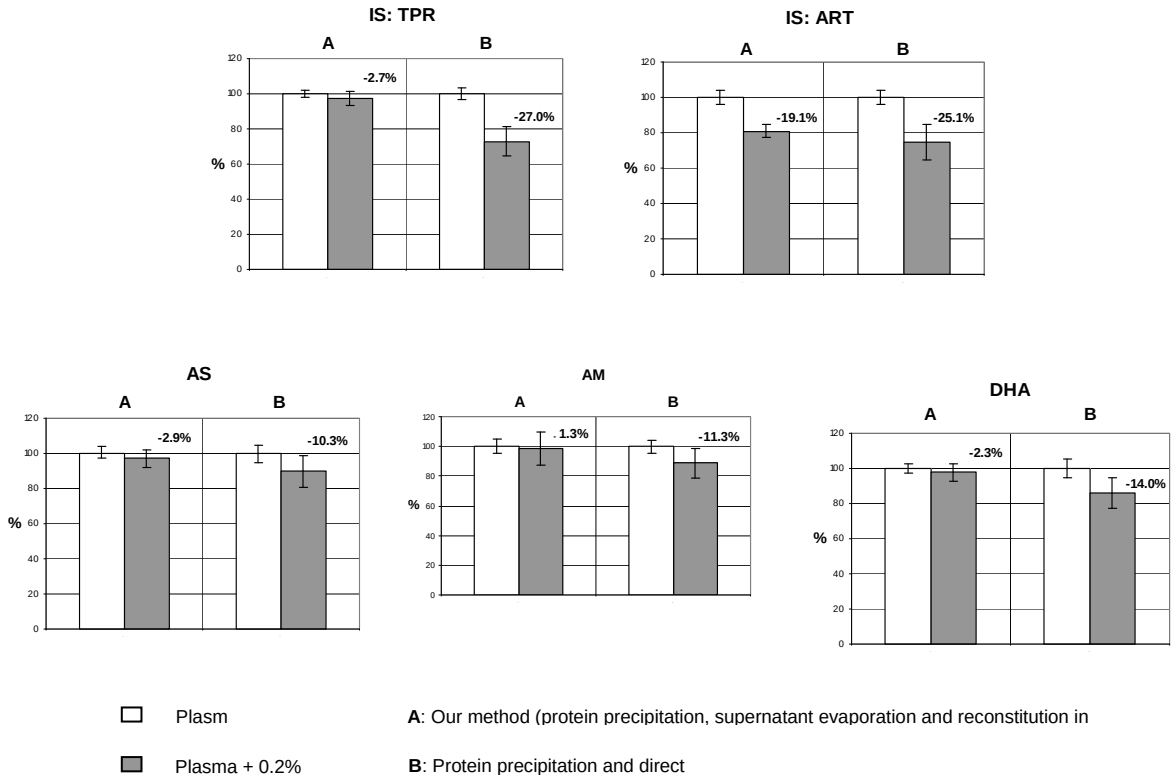

Figure S2. Influence of extraction procedure on the assay of artemisinin derivatives in hemolysed plasma. Signal intensities for endoperoxide antimalarials are expressed as the mean percentage of calibration samples prepared in plasma with no RBC, used as control (100%).

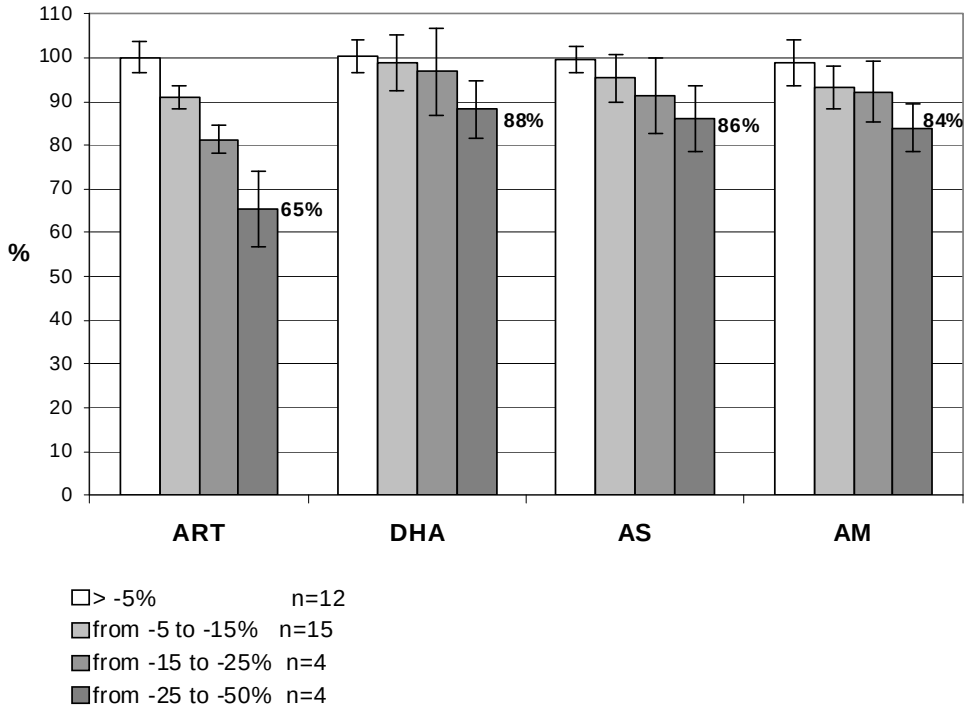

Figure S3. Endoperoxide drugs addition experiments with blank malaria patients plasma samples.

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SHORT COMMUNICATION

Lack of multiple copies of *pfmdr1* gene in Papua New Guinea

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Summary We describe here the results of an analysis of *Plasmodium falciparum* multidrug resistance protein 1 (*pfmdr1*) gene copy number from 440 field isolates from Papua New Guinea. No multiple copies of the gene were found, which corresponds to the lack of usage of mefloquine. These data extend regional knowledge about the distribution of multidrug-resistant *P. falciparum*.

1. Introduction

Recent in vitro and in vivo studies showed an association between artesunate—mefloquine therapy failure and genetic changes in *Plasmodium falciparum* multidrug resistance protein 1 (*pfmdr1*) gene (Price et al., 2004; Sidhu et al., 2006). These findings underline the contribution of *pfmdr1* copy number to the susceptibility of *P. falciparum* to antimalarial drugs. Most malaria-endemic countries have recently adopted artemisinin combination therapies (ACTs) as first-line therapy for *P. falciparum* infections because of increasing resistance to all currently used antimalarial drugs.

As the world's most multidrug-resistant *P. falciparum* parasites are found along the borders of Thailand and are also emerging elsewhere in Southeast Asia, many studies on *pfmdr1* gene copy number have been performed on samples from Southeast Asia. Studies in Africa indicate that *pfmdr1* gene copy number is rare in the field outside Southeast Asia (Ursing et al., 2006). However, no research has been conducted on *pfmdr1* copy number in Papua New Guinea (PNG), where drug pressure of mefloquine on *P. falciparum* has been minimal so far. Until recently, standard first-line

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treatment in PNG for malaria consisted of chloroquine or amodiaquine plus sulfadoxine—pyrimethamine. In order to describe the baseline situation, we determined *pfmdr1* copy numbers in 440 field isolates from PNG. With this analysis we extend the knowledge about the global distribution of multidrug-resistant *P. falciparum*.

2. Materials and methods

Pre-treatment fingerprick samples were collected from 440 patients within several in vivo drug efficacy studies conducted between 2003 and 2005 in three health centres in Simbu, East Sepik and Madang Province in PNG (Marfurt et al., 2007).

The *pfmdr1* gene copy number was assessed as described previously (Price et al., 2004). The assays were run in triplicate on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Rotkreuz, Switzerland) and every run contained two calibrator DNA samples from clones 3D7 and W2-mef, having *pfmdr1* copy numbers of 1 and 3, respectively. The Ct threshold was set manually at 0.05 for *pfmdr1* and at 0.03 for β-*tubulin*, respectively, and the baseline was calculated automatically. Results were analysed by the comparative $\Delta\Delta$Ct method described previously (Price et al., 2004). Assays were repeated if one of the following results was obtained: no Ct value for more than one sample in the triplicate; calibrator copy number $\neq$ 3; $\Delta\Delta$Ct spread >1.5; Ct values >35.

The multiplicity of infection was determined as described previously by fragment-sizing of *P. falciparum* merozoite surface protein 2 (*pfmsp2*) using GeneMapper Software, v3.7 (Applied Biosystems) (Falk et al., 2006). The primary and nested PCR protocol was changed to 2 min at 94 °C followed by 25 cycles (30 s at 94 °C, 45 s at 50 °C and 1 min 30 s at 70 °C) with a final elongation for 10 min at 70 °C each. Of the diluted nested PCR product (1:10 in H_2O), 2.5 μl were combined with 10 μl diluted ROX-500 size standard (1:40 in H_2O; Applied Biosystems). Assays were repeated if one of the following results was obtained: no sizing data; *pfmsp2* PCR artefacts; no *pfmsp2* PCR product.

Samples were excluded from the final analysis if one or more of the following results were obtained after repetition of the assay: no Ct value for more than one sample in triplicate; $\Delta\Delta$Ct spread >1.5; Ct values >35; *pfmsp2* PCR artefacts; no sizing data; no *pfmsp2* PCR product.

3. Results

Of the 440 samples analysed, 35 were excluded from the final analysis (15 no Ct value for more than one sample in triplicate; one Ct value >35; three no Ct value for more than one sample in triplicate and no *pfmsp2* PCR product; six no sizing data; three *pfmsp2* PCR artefacts; seven no *pfmsp2* PCR product). The remaining 405 samples included in the final analysis showed only one copy of the *pfmdr1* gene. The mean multiplicity of infection found in the patients was 1.56 ± 0.76 (mean $\pm$ SD), with 240 patients showing single infections.

4. Discussion

The number of samples excluded from the final analysis might be explained by the fact that field samples sometimes contain very low levels of parasite DNA, thus leading to indeterminable Ct values in the real-time PCR or missing *pfmsp2* PCR products.

Mefloquine use has not been significant yet in PNG, and the occurrence of only one gene copy of *pfmdr1* strengthens the assumption that mefloquine selects for copy number increase. Studies that have tested different *P. falciparum* isolates from PNG for mefloquine resistance showed full in vitro susceptibility (Hombhanje, 1998). By contrast, other authors previously have shown a high frequency of *pfmdr1* sequence polymorphisms, confirming that chloroquine selects for polymorphisms (Nagesha et al., 2001).

The multiplicity of infections found is similar to results in other settings in PNG (Cortes et al., 2004). However, it is possible that a small percentage of the parasites in multiple infections carry more than one copy of *pfmdr1* and will not appear as multiple copies in the analysis due to lack of statistical power. In addition, the fact that within the large number of single infections there was no gene amplification supports the idea that *pfmdr1* is still not amplified in PNG.

In conclusion, we show that drug-resistance-associated *pfmdr1* gene amplifications have not yet been selected in PNG. Therefore mefloquine and artesunate might still be highly effective first-line treatment options against *P. falciparum* infections.

Authors' contributions: HPB conceived the study; JM, SB, IM, JCR, PS and BG designed the study; JM collected samples; EMH, DM and AR carried out the laboratory work; EMH and HPB analysed the data; SB, IM, JCR, PS and BG interpreted the data; EMH and HPB wrote the manuscript. All authors read and approved the final manuscript. HPB is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The study was approved by the Papua New Guinea Medical Research Advisory Committee.

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