Piperaquine

Bioanalysis, drug metabolism and pharmacokinetics

Joel Tärning
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Institute of Neuroscience and Physiology
Department of Pharmacology
the Sahlgrenska Academy at Göteborg University
Sweden

Cover illustration: Scanning electron micrograph picture showing a malaria parasite in the
                        merozoite stage (the small sphere) attached to an erythrocyte just prior to
                        invasion.
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ABSTRACT

Malaria is one of the most abundant parasitic diseases in the world affecting
many of the poorest economies. The estimated prevalence is 300 to 700 million
clinical episodes each year with up to 3 million deaths. Piperaquine replaced
chloroquine as the first line treatment in China for *Plasmodium falciparum* malaria
in the 1970s and was used as mass prophylaxis until the emergence of resistance
in the 1990s. It has recently been the object of renewed interest as a partner drug
in artemisinin-based combination therapy. Artekin® is a fixed oral combination
of dihydroartemisinin and piperaquine showing excellent efficacy and tolerability
against multi-resistant *Plasmodium falciparum* malaria. Only a limited number of
studies have addressed the clinical pharmacokinetics of piperaquine, none of
which have addressed metabolism. Despite its extensive use no published
information is available about the non-clinical pharmacokinetics or drug
metabolism in animals.

The bioanalytical tools used in this thesis were nuclear magnetic resonance,
liquid chromatography, quantitative and qualitative mass spectrometry. Data
analysis was conducted using conventional statistics, population based
pharmacokinetic modeling, individual pharmacokinetic modeling and non-
compartmental analysis.

The results present a new systematic approach for choosing a regression model
during bioanalytical method validation that can be a useful tool for finding the
optimal regression model (paper I). It incorporates predictability of independent
quality control samples as well as the calibration curve fit. This approach was
used to find the best regression model during the development and validation of
a sensitive and selective bioanalytical method for quantification of piperaquine in
urine by automated solid-phase extraction and isocratic liquid chromatography
(paper II).
Five human urinary metabolites of piperaquine were identified and their
molecular structures characterized in two healthy male volunteers after an oral
single dose of the dihydroartemisinin-piperaquine combination (paper III). The
major metabolites are a carboxyl acid cleavage product and a N-oxidated
piperaquine product.

The rat appears to be a suitable animal model for non-clinical *in vivo* studies since piperaquine pharmacokinetic properties and metabolites are similar to those found in humans (paper IV). The absolute oral bioavailability was estimated to approximately 50%. The low between-animal variability in plasma concentrations after intravenous administration suggests absorption to be critical for between-animal variability in drug exposure. Piperaquine displayed a low biliary clearance with less than 1% of the total dose excreted by this route. Enterohepatic circulation would not contribute significantly to a prolongation of the terminal half-life.

Piperaquine elimination half-life might be underestimated due to inadequate assay sensitivity and/or duration of sampling in published information (paper V). This should be considered when establishing the duration of follow-up and the assessment of relapse in clinical studies. The population pharmacokinetics of piperaquine was characterized in 98 patients in Thailand with uncomplicated *Plasmodium falciparum* malaria, ranging from 3 to 55 years of age (paper VI). The study confirms that piperaquine exhibits considerable inter-individual pharmacokinetic variability, has a very large apparent volume of distribution, and a slow elimination phase. Pharmacokinetic modeling suggests that despite having a smaller central volume of distribution and slower elimination than adults, the children in this study had lower piperaquine concentrations in the therapeutically important period immediately following treatment. If this is confirmed in other malaria affected regions, then consideration should be given to increase the weight adjusted dosage in children. No pharmacokinetic differences could be seen between the two investigated study treatment regimens and further support the use of a simplified, once daily treatment, regimen to improve treatment adherence and efficacy.

Overall, this thesis has contributed to a better understanding of the bioanalysis, drug metabolism and pharmacokinetics of piperaquine which may contribute to its future safe and efficacious clinical use as an antimalarial.

Key words: Piperaquine; malaria; *Plasmodium falciparum*; antimalarials; ACT; pharmacokinetics; NONMEM; population pharmacokinetics; NCA; bioavailability; enterohepatic circulation; metabolism; metabolites; animal models; bioanalysis; regression models; validation; HPLC; LC-MS/MS; H-NMR; FTICR-MS

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I.   Singtoroj T.*, **Tarning J**.*, Annerberg A., Ashton M., Bergqvist Y., White N.J., Lindegardh N., Day N.J.P. A new approach to evaluate regression models during validation of bioanalytical assays.
      Journal of Pharmaceutical and Biomedical Analysis. 2006 Apr 11;41(1):219-27.

II.   **Tarning J**.*, Singtoroj T.*, Annerberg A., Ashton M., Bergqvist Y., White N.J., Day N.J.P., Lindegardh N. Development and validation of an automated solid phase extraction and liquid chromatographic method for the determination of piperaquine in urine.
      Journal of Pharmaceutical and Biomedical Analysis. 2006 Apr 11;41(1):213-8.

III. **Tarning J**., Bergqvist Y., Day N.J.P., Bergquist J., Arvidsson B., White N.J., Ashton M., Lindegardh N. Characterization of human urinary metabolites of the antimalarial piperaquine.
      Drug Metabolism and Disposition. 2006 Dec;34(12):2011-9.

IV.   **Tarning J**., Lindegardh N., Sandberg S., Day N.J.P., White N.J., Ashton M. Pharmacokinetics and metabolism of the antimalarial piperaquine after intravenous and oral single doses to the rat.
      Submitted

V.   **Tarning J**., Lindegardh N., Annerberg A., Singtoroj T., Day N.J.P., Ashton M., White N.J. Pitfalls in estimating piperaquine elimination.
      Antimicrobial Agents and Chemotherapy. 2005 Dec;49(12):5127-8.

VI.   **Tarning J**., Ashley E., Lindegardh N., Stepniewska K., Phaiphun L., Day N.J.P., McGready R., Ashton M., Nosten F., White N.J. Population pharmacokinetics of piperaquine after two different treatment regimens of dihydroartemisinin-piperaquine in patients with *Plasmodium falciparum* malaria in Thailand.
      Submitted

      *Equal contribution

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TABLE OF CONTENT

LIST OF ABBREVIATIONS

PQ	Piperaquine
PQP	Piperaquine phosphate
ACT	Artemisinin-based combination therapy
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
FTICR	Fourier transform ion cyclotron resonance
NMR	Nuclear magnetic resonance
CL	Clearance
V_C	Central volume of distribution
V_P	Peripheral volume of distribution
V_{SS}	Steady state volume of distribution
Q	Inter-compartment clearance
F	Bioavailability
C_{max}	Maximum concentration
T_{max}	Time to reach maximum concentration
$t_{1/2,\alpha}$	Disposition half-life during α-phase
$t_{1/2,\beta}$	Disposition half-life during β-phase
$t_{1/2,\gamma}$	Disposition half-life during γ-phase
$t_{1/2,Z}$	Terminal elimination half-life
AUC	Area under the concentration-time curve
IIV	Inter-individual variability
IOV	Inter-occasion variability
NCA	Non-compartmental analysis
OFV	Objective function value
ED_{90}	Curative dose in 90% of investigated animals
IC_{50}	Concentration required to achieve 50% parasite growth inhibition

INTRODUCTION

Pharmacokinetics

Friedrich Hartmut Dost first introduced the term pharmacokinetics in 1953 in his text, *Der Blütspiegel-Kinetic der Konzentrationsablaüfe* published in *der Frieslaufflüssigkeit* (for review, see Wagner, 1981). Pharmacokinetics literally means the application of kinetics to *pharmakon*, the Greek word for drugs and poisons. Pharmacokinetics uses a mathematical representation of data to model and interpret the time-course of drug and metabolite concentrations in biological fluids. Gibaldi and Levy introduced a similar definition in 1976 (Gibaldi and Levy, 1976a; Gibaldi and Levy, 1976b): "Pharmacokinetics is concerned with the study and characterization of the time course of drug absorption, distribution, metabolism and excretion, and with the relationship of these processes to the intensity and time course of therapeutic and adverse effects of drugs. It involves the application of mathematical and biochemical techniques in a physiologic and pharmacologic context." The pharmacokinetic characterization of a drug is thus important to understand and predict its effects. Such information is often scarce for many of the old drugs used in tropical medicine and malaria for which also dose-optimization frequently is a result of a trial-and-error approach.

Malaria

The disease

Malaria is a major health and developmental challenge for many of the poorest economies in the world with 300 to 700 million estimated clinical episodes in 2002 (Snow *et al.*, 2005). It claims more human lives each year than any other infectious disease except AIDS and tuberculosis. Up to three million deaths occur annually throughout the world, mostly in children below the age of five (Breman *et al.*, 2004). It is estimated that 60% of the clinical malaria cases and over 80% of all deaths occur in sub-Saharan Africa (WHO, 2005). Other highly affected regions are South East Asia and South America.

Malaria is a dual-host hematoprotozoan parasitic infection transmitted by certain species of the female *anopheline* mosquitoes. The four species of *Plasmodium* malaria parasites infecting humans are *P. falciparum, P. vivax, P. ovale* and *P. malariae*.
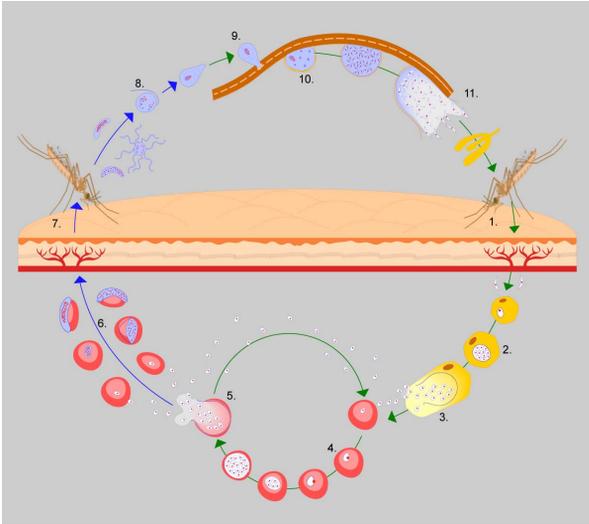
FIGURE 1. Lifecycle of *Plasmodium falciparum*
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The malaria lifecycle of *P. falciparum* begins (Fig.1; #1) with the infected mosquito injecting sporozoits into the host bloodstream during a blood meal (for review, see Griffith *et al.*, 2007). The sporozoites invade human hepatocytes within approximately 45 minutes, where they initiate the multiplication of merozoites. These develop into liver schizonts during their pre-erythrocytic stage of about 5-7 days (Fig. 1; #2). Merozoites are released into the bloodstream following the rupture of the schizonts, ready to invade erythrocytes (Fig. 1; #3). Three morphological stages occur within the invaded erythrocytes, starting with the ring stage that mature to trophozoites and the development of blood schizonts producing new merozoites that consume the cytoplasm of the erythrocytes (Fig. 1; #4). In *P. falciparum,* responsible for the majority of infections and deaths, these blood schizonts rupture in 48 hour synchronized cycles releasing merozoites ready to invade new cells (Fig. 1; #5). It is the rupture of the erythrocytes that is responsible for the characteristic clinical symptoms of malaria with intense fever, nausea, headache and muscular pain. After a few cycles of asexual blood-stages, a number of merozoites enter the sexual reproduction stage of developing male or female gametocytes that are presented in the peripheral circulation within 10 to 14 days after infection (Fig. 1; #6). The gametocytes can re-infect a mosquito during a blood meal (Fig. 1; #7) where fertilization occurs in the mosquito gut (Fig. 1; #8) to produce zygotes (Fig. 1; #9) that develop into oocysts (Fig. 1; #10). These rupture and release thousands of sporozites that migrate to the salivary glands to complete the malaria life cycle (Fig. 1; #11). The life-cycle stage in the mosquito vector takes approximately 6 to 12 days.

Malaria control strategies

Control strategies have traditionally relied on the two arms of vector control and effective case management. All countries in Africa south of the Sahara, the majority of Asian malaria-endemic countries and some American countries have adopted insecticide-treated nets (ITNs) as a key malaria strategy. The long-lasting insecticide-treated nets (LLINs) can be re-treated to generate a lower total price over a duration of 4-5 years compared with ITNs. Indoor residual spraying (IRS) or outdoor insecticide spraying are particularly useful methods for achieving a rapid reduction in transmission during epidemics and other emergency situations. ITNs or LLINs are generally recommended in intense malaria transmission areas because of better sustainability but IRS could have a long-term impact if performed regularly (WHO, 2005). However, a massive logistic and economic effort must be met in order to realize these strategies. It is estimated that an additional 130 million to 264 million ITNs are required in 2007 to achieve a 80% coverage for pregnant women and children under the age of 5 in African risk-areas (Miller *et al.*, 2007).

A long-hoped-for solution would be an effective malaria vaccine, capable of malaria eradication or host semi-immunity to reduce morbidity and mortality of the disease in endemic areas. About 75 different experimental malaria vaccine formulations have been presented over the last decades of which more than 25 reached or completed the early phases of clinical trials (for reviews, see Aide *et al.*, 2007; Snounou and Renia, 2007). The number of proposed vaccines is likely to increase substantially with the *P. falciparum* genome sequenced, providing a way to identify protective antigens among the ~6000 parasite proteins (Gardner *et al.*, 2002). Two Cochrane reviews of major efficacy trials in endemic areas concluded only one blood-stage vaccine to reduce the density of parasites in the blood, but it did not prevent malaria attacks (Graves and Gelband, 2006a; Graves and Gelband, 2006c). Only one of the four pre-erythrocytic vaccines significantly reduced the number of clinical episodes of malaria and severe malaria in children (Graves and Gelband, 2006b). The reduction of clinical malaria episodes of 26% (95%CI, 13%-37%) and severe malaria by 58% (95%CI, 15%-79%) for up to 18 months in children demonstrate that vaccines are far from providing an absolute resolution of the malaria burden (Alonso *et al.*, 2004; Alonso *et al.*, 2005). Even if promising vaccine candidates and the distribution of ITNs and LLINs are in progress there is an urgent need for effective, safe and cheap antimalarials to treat clinical episodes of malaria in order to reduce morbidity and mortality.

The widening geographic distribution of *P. falciparum* and increasing resistance to monotherapy with conventional antimalarials (*e.g.* chloroquine and sulphadoxine-pyrimethamine) has severely compromised the ability to treat the disease. This emphasizes the urgent need for the development of novel antimalarial drugs (White, 2004). Drug resistance is believed to arise from spontaneous point mutations or gene amplification in infecting parasites. The probability of a parasite spontaneously developing two specific mutations resulting in reduced drug susceptibility to two drugs with different modes of action is very unlikely (White, 1999). This is the rationale of combination therapy to reduce drug resistance and increase the lastingness of active drugs. Artemisinin-based combination therapies (ACTs) are the most highly efficacious treatment regimens now available and recommended as first line *P. falciparum* malaria treatment throughout the world. A promising fixed oral ACT is the dihydroartemisinin-piperaquine combination.

Piperaquine

History and physico-chemical properties

Piperaquine (MW: 535.5 g/mole), 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, is an antimalarial compound belonging to the 4-aminoquinolines (Fig 2). It is a highly liphophilic (LogP value of 6.1) basic molecule with four pKa values of 6.9, 6.2, 5.7, and 5.4 (Warhurst *et al.*, 2007). The base is a pale white to yellow powder with a melting point of 212-213°C and is poorly soluble in water even when fully ionized (Hung *et al.*, 2003). In 1966, the Shanghai Research Institute of Pharmaceutical Industry synthesized this new antimalarial, with a chemical structure identical to 13228 RP earlier synthesized by Rhone Poulence, France. In 1978 it replaced chloroquine as first line monotherapy in China for malaria. It is estimated that approximately 200 metric tons were dispensed between 1978 and 1992 for mass prophylaxis until resistance became too high (for review, see Olliaro and Taylor, 2003). Piperaquine has had a renaissance and recently been the object of renewed interest as a partner drug in ACTs. Like many drugs used to treat infectious diseases primarily affecting individuals in low-income countries, the development of piperaquine has been fragmentary especially regarding non-clinical studies. Despite decades of clinical use, there are limited published non-clinical *in vivo* and *in vitro* information of piperaquine addressing pharmacokinetics, metabolism or toxicity.
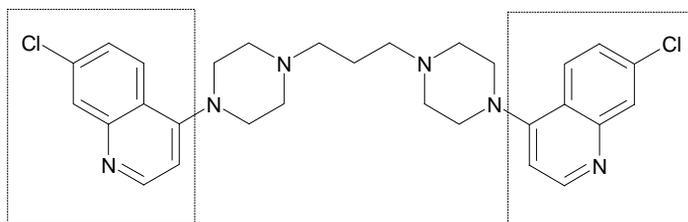
FIGURE 2. Molecular structure of piperaquine (PQ) with the dashed boxes displaying the 7-chloro-4-aminoquinoline structure common to chloroquine.

In vitro properties

Piperaquine is highly active *in vitro* against both chloroquine-sensitive and chloroquine-resistant isolates of *P. falciparum*. Concentrations for 50% parasite growth inhibition (IC_{50}s) *in vitro* range between 7.8 and 78.3 nM (4.2-41.9 ng/mL) (Vennerstrom *et al.*, 1992; Basco and Ringwald, 2003; Akoachere *et al.*, 2005; Fivelman *et al.*, 2007; Snyder *et al.*, 2007). The variation in IC_{50} values is probably a result from differing strain susceptibility and experimental conditions. A low correlation between strain susceptibility to chloroquine and piperaquine could be seen in 103 fresh clinical isolates in Cameron and Central Africa, which would minimize the risk of cross-resistance (Basco and Ringwald, 2003). Piperaquine and dihydroartemisinin showed geometric mean IC_{50} values of 38.9 nM and 1.29 nM, respectively. Chloroquine showed a 50% inhibition at 41.6 nM in chloroquine-sensitive and 201 nM in chloroquine-resistant isolates (Basco and Ringwald, 2003). These results should not be directly extrapolated to clinical *in vivo* concentrations since only the unbound drug is considered to have a pharmacological effect. Also the experimental conditions must be considered (*e.g.* hematocrit and plasma protein content).

The exact mechanism of action of piperaquine is unknown. It is reasonable to assume that the compound has similar targets as chloroquine considering the close structural resemblance (Fig 2). Parasites ingest hemoglobin which is degraded into amino acids, functioning as nutrients for the parasite (Krugliak *et al.*, 2002), and to toxic free heme by-products (*i.e.* haematin) (Goldberg *et al.*, 1990). As a detoxification process, haematin is polymerized to form non-toxic crystal structures known as haemozoin or malaria pigment (Slater *et al.*, 1991; Loria *et al.*, 1999; Pagola *et al.*, 2000). Chloroquine is only active against blood-stage parasites and accumulates in the parasite food vacuole (Yayon *et al.*, 1984; Sullivan *et al.*, 1996; Saliba *et al.*, 1998), where it binds free haematin (Bray *et al.*, 1998; Bray *et al.*, 1999). This binding inhibits the polymerization resulting in accumulation of the toxic free chloroquine-haematin complex and hemoglobin within the food vacuole (Slater, 1993; Egan *et al.*, 1994; Sullivan *et al.*, 1996; Bray *et al.*, 1998; Sullivan *et al.*, 1998; Pagola *et al.*, 2000). The chloroquine-haematin

complex causes a disruption of the vacuole membranes and interferes with enzymatic processes in the parasite (Fitch *et al.*, 1982; Sugioka *et al.*, 1987; Surolia and Padmanaban, 1991; Surolia, 2000). Recent studies have shown the parasite food vacuole to be the site of action also for piperaquine were it accumulates and inhibits the polymerization of haematin (Warhurst *et al.*, 2007). Piperaquine and chloroquine are multi-protic weak bases and highly liphophilic at pH 7.4. Such molecules can diffuse readily through membranes in their unprotonated form and accumulate over the pH gradient into the acidic (pH 4.5-4.9) parasite food vacuole (Yayon *et al.*, 1984; Yayon *et al.*, 1985; Hayward *et al.*, 2006). The ion trapping does not account for the several thousand-fold accumulation at the site of action. Both saturable binding and non-saturable components have been shown to be involved in the accumulation of chloroquine (Fitch, 1970). The high liphophilicity of piperaquine results in an extremely high distribution into parasite lipid membranes (Warhurst *et al.*, 2007). This might be important in order to understand the mechanism of action since recent studies indicate that the polymerization of haematin may take place in or closely associated with lipid droplets in the food vacuole (Egan *et al.*, 2006; Pisciotta *et al.*, 2007).

Chloroquine is very poorly accumulated (~40-fold less accumulation) in chloroquine-resistant parasite strains (Fitch, 1970; Krogstad *et al.*, 1987). This suggest that chloroquine resistance may result from exclusion of the drug from the site of action or an alteration in the chloroquine target (Fitch, 1970; Bray *et al.*, 1998; Bray *et al.*, 1999). Recent studies also show no pH difference in the parasite food vacuole between chloroquine sensitive and resistant strains (Hayward *et al.*, 2006). Lack of accumulation in chloroquine resistant strains is therefore suggested not to be primarily an effect of an altered pH. The exact mechanism of resistance has been heavily debated. Studies have shown a single mutation in the pfcrt gene (*i.e.* K76T), encoding the trans-membrane PfCRT protein in the food vacuole, to be both necessary and sufficient for chloroquine resistance (Fidock *et al.*, 2000; Warhurst *et al.*, 2002; Wootton *et al.*, 2002; Lakshmanan *et al.*, 2005). It has been suggested that mutated PfCRT act as an energy dependent efflux channel responsible for expelling chloroquine from the food vacuole in chloroquine-resistant *P. falciparum* strains (Naude *et al.*, 2005). This mechanism was shown to be a chloroquine-selective efflux since the mutation did not reduce piperaquine accumulation within the food vacuole. These results suggest no cross-resistance by this mechanism. The efflux of chloroquine has recently been shown not to be directly coupled to the energy supply (Bray *et al.*, 2006). PfCRT might instead act as a gated aqueous channel that allows the protonated drug to passively leak out from the food vacuole to reduce the concentration of chloroquine at the site of action. The function of the P glycoprotein (P-gp) homologue 1 (Pgh1; the pfmdr1 gene product) is unknown but it is suggested to also modulate parasite drug susceptibility (Basco *et al.*, 1995; Gavigan *et al.*, 2007). This might be of minor importance since piperaquine is

shown not to be transported by the human P-gp system *in vitro* (Crowe *et al.*, 2006). These findings imply that the complex resistance phenotype may result from the composite effects of the products of one, two or possibly more genes.

The combination piperaquine-dihydroartemisinin exhibits a weak antagonistic effect *in vitro* with a competitive erythrocyte uptake of labeled dihydroartemisinin in the presence of piperaquine (Davis *et al.*, 2006; Fivelman *et al.*, 2007). Borderline antagonism could be shown *in vitro* with the combination piperaquine-artemether both in chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains (Snyder *et al.*, 2007). No *in vitro* interaction could be detected between piperaquine and the new trioxolane antimalarial RBx11160 (Snyder *et al.*, 2007). It is important to understand that the effects/interactions *in vitro* might not be directly comparable to *in vivo* situations and that such extrapolation can be misleading. It should also be emphasized that the observed antagonism is relatively weak and does not necessarily translate into slower clinical parasite clearance compared to when individual compounds are used alone (White, 1999).

Non-clinical in vivo properties

Information on drug disposition and metabolism in a species of investigation is required to interpret and evaluate for example toxicity studies. There are several aspects regarding the emerging knowledge on piperaquine clinical pharmacokinetics that have not yet been investigated or explained mechanistically such as erratic absorption (Roshammar *et al.*, 2006), possible enterohepatic circulation (Hung *et al.*, 2004; Sim *et al.*, 2005), routes of elimination, bioavailability, parenteral administration, dose-linearity, large inter-individual variability, extreme volume of distribution (Hung *et al.*, 2004; Sim *et al.*, 2005; Roshammar *et al.*, 2006), or long terminal half-life. Similar pharmacokinetic properties and metabolism in an animal model as found in humans would indicate it to be a suitable species for further non-clinical piperaquine studies regarding such aspects. Only three original papers can be found in the international literature describing non-clinical *in vivo* studies of piperaquine, none dealing with pharmacokinetics or metabolism (Li *et al.*, 1989; Chen *et al.*, 2001; Snyder *et al.*, 2007).

Piperaquine was administered subcutaneously for 5 days and showed a non-significant cross-resistance in mice inoculated with a chloroquine-resistant (n=10) and chloroquine-sensitive (n=10) *P. Berghi* ANKA strain (Chen *et al.*, 2001). No cross-resistance could be seen for the hydroxyl derivative of piperaquine, 7-OH piperaquine. One Macaca mulatta monkey infected with *P. knowlesi* was cured with piperaquine phosphate but died later from trauma and necrosis of the forelimb (Li *et al.*, 1989). No information was presented about the route of administration, drug dosage or if mortality had a casual relationship to

the administered piperaquine. Snyder *et al* (2007), showed 2.5 mg piperaquine phosphate/kg (*i.e.* 1.3 mg piperaquine/kg) to result in a 90% effective dose (ED_{90}) after a single oral dose of piperaquine phosphate in mice (n=5) infected with a *P. berghi* ANKA strain. No information is available if the parasite strain used was chloroquine-resistant or sensitive. This value can be compared to these for other antimalarials such as pyrimethamine (ED_{90}: 2.0 mg/kg) and artemether (ED_{90}: 19 mg/kg) presented in the same study. The combination piperaquine-artemether was reported to be borderline antagonistic in mice (n=2). No dynamic interactions could be detected with the combination piperaquine-RBx11160 indicating an additive action of the combined drugs. The tested combinations did not show any signs of toxicity at the doses used (Snyder *et al.*, 2007).

Piperaquine is suggested to be less toxic than chloroquine with a putative therapeutic index for piperaquine being over five times greater than for chloroquine (Davis *et al.*, 2005). Similar side-effects could be seen with an acute cardiovascular toxicity manifesting a fall in systolic blood pressure and a prolongation of electrocardiographic PR interval and QRS duration after intravenous administration in rabbits (Davis *et al.*, 2005). Similar to chloroquine, morphological changes could be seen by piperaquine treatment in intra-erythrocytic trophozoits and gametocytes. The food vacuole membranes and mitochondria became swollen and abnormal pigment grains were seen within an hour of drug exposure in mice infected with a *P. Berghi* ANKA strain (Davis *et al.*, 2005). However, all the articles referred to by Davis *et al* (2005) on non-clinical *in vivo* toxicity, safety and efficacy are in the Chinese language, which complicates their scientific evaluation.

Clinical efficacy and safety

Dihydroartemisinin-piperaquine is available as a fixed oral combination [Artekin®; each tablet containing 320 mg piperaquine phosphate and 40 mg dihydroartemisinin] that is increasingly deployed in Southeast Asia. It is considered a highly promising combination and is already part of national treatment recommendations in Cambodia and Vietnam. The standard treatment regimen is a highly efficacious and safe treatment and comprises 4 doses given over 3 days (*i.e.* an adult dose of two tablets given orally at 0, 8, 24 and 48 hours). Ashley *et al* (2005), recently proposed a simpler regimen of an approximately equivalent total dose divided equally for once daily treatment given over the same period (*i.e.* three tablets given orally at 0, 24 and 48 hours). In patients studied on the northwest border of Thailand, both regimens were well tolerated and there were no differences in PCR genotyped-adjusted cure rates assessed at day 63 (Ashley *et al.*, 2005).

In several studies the fixed combination dihydroartemisinin-piperaquine has resulted in high cure rates (>95%) with excellent tolerability in the treatment of adults and children with *P. falciparum* malaria (Denis *et al.*, 2002; Ashley *et al.*, 2004; Karunajeewa *et al.*, 2004; Tran *et al.*, 2004; Ashley *et al.*, 2005; Tangpukdee *et al.*, 2005; Karema *et al.*, 2006; Mayxay *et al.*, 2006; Smithuis *et al.*, 2006; Hasugian *et al.*, 2007; Janssens *et al.*, 2007; Kamya *et al.*, 2007; Ratcliff *et al.*, 2007).

The fixed dihydroartemisinin-piperaquine combination has been shown to be more effective than the commonly used antimalarial combinations of artesunate-amodiaquine and artemether-lumefantrine in patients with multidrug-resistant *P. falciparum* and *P. vivax* (Hasugian *et al.*, 2007; Ratcliff *et al.*, 2007). Overall risk of treatment failure at day 42 was 45% (n=375; 95%CI, 36%-53%) for artesunate-amodiaquine and 43% (n=166; 95%CI, 38%-48%) for artemether-lumefantrine compared with 19% (n=379; 95%CI, 14%-23%) and 13% (n=168; 95%CI, 7.2%-19%), respectively for the dihydroartemisinin-piperaquine comparative arm when investigated in two large patient studies in Southern Papua, Indonesia (Hasugian *et al.*, 2007; Ratcliff *et al.*, 2007). No significant difference in true recrudescence could be detected between dihydroartemisinin-piperaquine and artemether-lumefantrine with a mean value of 4.7% and 4.1%, respectively. Both treatments were well tolerated with mild and transient adverse advents. The combination dihydroartemisinin-piperaquine reduced true recrudescence significantly (4.8%) compared with artesunate-amodiaquine (16%) and was better tolerated. Almost all patients (97%) were parasite free within 48 hours by dihydroartemisinin-piperaquine treatment demonstrating the rapid parasite clearance of dihydroartemisinin. The increased duration of post-treatment prophylactic effect of the piperaquine combination displays the benefit of piperaquine as a partner drug in ACTs. However, the appropriateness of dihydroartemisinin as a partner drug in ACT combinations has recently been questioned due to poor thermal stability in fixed formulations (Haynes *et al.*, 2007).

Clinical pharmacokinetics

Despite decades of extensive use, the clinical pharmacokinetics of piperaquine has only been assessed in a few studies during the last three years. Total exposure of piperaquine ($AUC_{0\to\infty}$) increased by two-fold when piperaquine was administered with fatty food compared with the fasting state in 8 healthy Caucasian subjects (Sim *et al.*, 2005). The oral bioavailability of piperaquine and other lipid-soluble antimalarial drugs, such as mefloquine (Crevoisier *et al.*, 1997), atovaquone (Rolan *et al.*, 1994), and halofantrine (Milton *et al.*, 1989) is limited by low water solubility and is therefore increased by administration with fats. This could account for some of the inter-individual variability seen in piperaquine pharmacokinetics.

Population pharmacokinetic modeling was employed to characterize piperaquine kinetics after repeated oral administration with two different fixed ACT combinations in Cambodian patients (Hung *et al.*, 2004) and Vietnamese healthy subjects (Roshammar *et al.*, 2006). Piperaquine was characterized by biphasic disposition kinetics with a large steady-state volume of distribution and a low clearance, resulting in a long terminal elimination half-life of about 2-3 weeks. Hung *et al* (2004), suggested children to have a somewhat different pharmacokinetics with a two-fold higher body-weight normalized oral clearance compared with adults. The absorption of piperaquine in fasting healthy subjects was erratic resulting in multiple peaks (Roshammar *et al.*, 2006). This might be a consequence of enterohepatic circulation, food-drug interactions, gastric emptying or non-identical piperaquine release from tablets. Neither study identified any covariates influencing piperaquine kinetics. Published clinical pharmacokinetic parameters and study designs are summarized in table I.

TABLE I. Pharmacokinetics of piperaquine

Subjects	Age Mean ± SD or range (years)	N	Mean total piperaquine treatment (mg/kg)	Total number of samples	Duration of sampling time (days)	Food intake during drug administration	Pharmacokinetic modeling	CL/F (L/h/kg)	V_{SS}/F (L/kg)	$t_{1/2, z}$ (days)	Reference
Patients	30 ± 13	38	32	213 [b]	35	Fasting	Mixed effects [d]	0.90	574	23	*(Hung et al., 2004)*
Patients	2-10	47	35	132 [b]	35	Fasting	Mixed effects [d]	1.85	614	14	*(Hung et al., 2004)*
Healthy volunteers	31 ± 3.5	12	25 [a]	468	29	Fasting	Mixed effects [e]	1.0 [g]	103 [g]	12	*(Roshammar et al., 2006)*
Healthy volunteers	19-42	8	4.2	152 [c]	42	Fasting	NCA [f]	1.14	716	20	*(Sim et al., 2005)*
Healthy volunteers	19-42	8	4.2	152 [c]	42	High-fat	NCA [f]	0.60	365	21	*(Sim et al., 2005)*

[a] These subjects received CV8 compared to all other subjects receiving Artekin®, [b] Total number of samples above LLOQ and included in analysis according to graph (Figure 3. page 260, Hung et al (2003)), [c] Based on duration of sampling since number of samples included in analysis was not reported, [d] using modeling software Kinetica™ version 4.1, [e] using modeling software NONMEM version V level 1.1, [f] using modeling software Kinetica™ version 4.3, and [g] parameter estimates are dose-normalized based on published population mean values divided by mean weight of the subjects.

Mixed effects non-linear mixed effects population modeling, *NCA* non-compartmental analysis, *CL/F* mean oral clearance, *V_{SS}/F* mean oral steady-state volume of distribution and *$t_{1/2, z}$* mean terminal half-life

AIMS OF THE THESIS

With an overall aim of contributing to a better understanding of the bioanalysis, drug metabolism and pharmacokinetics of piperaquine, the specific objectives were to

- develop and evaluate a new approach to choose an optimal regression model during bioanalytical method validation (papers I and II)

- validate a novel and sensitive method suitable for quantification of piperaquine in human urine and demonstrate its clinical applicability (paper II)

- isolate and characterize the main piperaquine metabolites in human urine collected after a single oral administration of the fixed ACT combination dihydroartemisinin-piperaquine (paper III)

- evaluate the pharmacokinetic properties of piperaquine in the rat after intravenous and oral administration and to identify and characterize the main piperaquine metabolites in rat plasma, urine, faeces and bile after intravenous administration (paper IV)

- evaluate if the reported terminal half-life might be underestimated and clinical pharmacokinetics oversimplified by insufficient assay sensitivity and duration of sampling (paper V)

- investigate the pharmacokinetic properties of piperaquine with a population based modeling approach in adults and children with uncomplicated *P. falciparum* malaria treated with the ACT combination dihydroartemisinin-piperaquine once daily for three days or with the standard four dose regimen (paper VI)

MATERIALS AND METHODS

Bioanalysis

Bioanalytical methods

Accurate and reliable quantification of drugs in biological fluids is required to attain pharmacokinetic data. One of the most popular techniques for drug quantification is liquid chromatography (LC) with UV or mass spectrometric (MS) detection.

Quantitative and qualitative LC with UV detection was used throughout this thesis (papers I-VI). This technique is based on a physical separation of the compounds in a mixture as the different compounds have different affinity to the moving liquid phase and the stationary solid phase (*i.e.* chromatographic column). The UV detector will give a response when a compound, which can absorb light at the selected wavelength, passes through the detector cell. The signal is proportional to the concentration resulting in a linear concentration-response signal relationship of the measured compounds.

LC with MS detection was used quantitatively in paper IV and qualitatively for metabolite identification in papers III and IV. MS detection is based on the molecular masses of the separated compounds. The compound needs to be positively or negatively charged in order to be detected. If the compound carries a charge, the MS detector will produce a response at the specific mass/charge ratio for the selected compound. The defined compound can be fragmented by collision with gas molecules (normally nitrogen) inside the MS creating a fragmentation pattern of the molecule. This fragmentation pattern is specific for the molecule and can be used to elucidate the molecular structure of an unknown compound.

Fourier transform ion cyclotron resonance (FTICR)-MS was used in paper III and is a high resolution MS technique able to obtain a very precise estimate of a compound molecular mass or fragments. An accurate molecular mass determination with several significant digits generates only a limited number of possible atomic combinations of the unknown compound. This technique can be helpful in for example identifying the molecular structure of metabolites.

Nuclear magnetic resonance (NMR) was used qualitatively in paper III for structural determination of metabolites. This is a very sensitive method that

provides a map of the carbon-hydrogen framework of a molecule. The nucleus in an atom is shielded by electrons and different nuclei results in tiny differences in the magnetic fields. Thus, a distinct NMR signal for each chemically distinct carbon or hydrogen nucleus can be seen when applying a magnetic field and one can thereby derive structural information about the unknown molecule.

Sample pre-treatment – solid phase extraction

Piperaquine, putative metabolites and internal standards were extracted from biological fluids by solid phase extraction on an automated ASPEC XL robot (Gilson Inc., Middletown, U.S.A.) using MPC-SD columns (4 mm, 1 mL) (papers I-III) or manually using MPC-SD solid phase extraction deep 96-well plates (3M Empore, Bracknell, UK) (papers III-VI). The mixed phase columns (MPC) hold liphophilic binding sites (*i.e.* octylsilica) together with strong cation binding sites (*i.e.* sulphonic acid). Salts and other hydrophilic ions compete with the drug for the ionic binding sites and could cause a partial loss of drug in the absence of a lipophilic binding site. The ability of binding through both liphophilic and ionic interactions increases the recovery of piperaquine and offers an excellent retention mechanism when extracting a drug in a matrix containing an excess of salts. The use of larger urine volumes to increase sensitivity and more concentrated urine samples was investigated in paper II. The presented assay was more or less independent of urine dilution factor and the internal standard compensated fully for any loss of piperaquine at higher urine loads validating that up to 9 mL of urine could be used in the assay to increase sensitivity.

Separation and quantification

Totally there are five previously published methods for quantification of piperaquine in biological fluids (Table II). The LC system used for piperaquine quantification in papers I, II, III, V and VI was a LaChrom Elite system (Hitachi, Tokyo, Japan). The detector was set at 345-347 nm and data acquisition was performed using LaChrom Elite software (VWR International, Darmstadt, Germany). Chromatographic separation was achieved on a Chromolith Performance RP-18e (100×4.6 mm I.D.) column (VWR) protected by a Chromolith Guard Cartridge RP-18e (10×4.6 mm I.D.). The monolith columns minimize the back-pressure by porous macropores, lined with micropores providing the surface area required for separation. This enables LC analysis at very high flow rates. Sufficient separation and efficiency was achieved at 3 mL/min using an isocratic mobile phase containing phosphate buffer (pH 2.5; 0.1 mole/L):acetonitrile (92:8, *v/v*). Previously published methods were used for analyzing piperaquine in plasma (Lindegardh *et al.*, 2005) and whole blood (Lindegardh *et al.*, 2003b).

The LC system used for piperaquine quantification in rat plasma, urine, and bile (paper IV) was an Agilent Technologies 1200 series (Agilent Technologies, Waldbronn, Germany). The LC system was coupled on-line to an API 5000 LC-MS/MS system (Applied Biosystems/MDS SCIEX, Ontario, Canada). The compounds (piperaquine and D6-piperaquine) were analyzed on a Gemini (50×2.0 mm) column protected by a security guard column with a Gemini (4.0×2.0 mm) guard cartridge (Phenomenex, Torrance, USA). An isocratic mobile phase containing ammonium bicarbonate (2.5 mM; pH10):acetonitrile (15:85, *v/v*) was used at a flow rate of 0.5 mL/min. Quantification was performed using selected reaction monitoring (SRM) for the transitions m/z 535–288 for piperaquine and 541–294 for D6-piperaquine. Data were processed using Analyst 1.4 (Applied Biosystems/MDS SCIEX, Foster City, USA). Calibrators and quality control samples were prepared in human plasma and urine for quantification of piperaquine in rat plasma and urine/bile, respectively. Ion suppression and possible matrix differences between calibrators and samples were compensated for by the use of a stable isotope-labeled internal standard (D6-piperaquine). The lower limit of quantification was set to 1.5 ng/mL.

To ensure satisfactory method performance, triplicates of quality control samples at three levels were analyzed in every analytical run during routine quantification of piperaquine in all assays.

TABLE II. Previously published HPLC methods for quantification of piperaquine

Matrix	Sample volume (μL)	Extraction	Detection	Analysis time (min)	Regression model	LLOQ (ng/mL)	Reference
Whole Blood	1000/500	SPE	UV	2	OLR [a]	27/54	(Lindegardh *et al.*, 2003b)
Capillary blood on filter paper	100	SPE	UV	2	OLR	27	(Malm *et al.*, 2004)
Plasma	500	SPE	UV	8	OLR [a]	13	(Lindegardh *et al.*, 2003a)
Plasma	1000	w/oil/w	UV	14	OLR	5	(Hung *et al.*, 2003)
Plasma	1000/250	SPE [b]	UV	2	OLR - $1/x^2$	2.5/10	(Lindegardh *et al.*, 2005)

[a] Split standard curve for low and high concentration range; [b] 96-wellplate format
LLOQ lower limit of quantification, *OLR* ordinary non-weighted linear regression; *w/oil/w* water-oil liquid extraction and oil-water liquid back-extraction; *OLR-$1/x^2$* ordinary linear regression with $1/x^2$ weighting

Regression analysis and method validation

The quality of bioanalytical data is highly dependent on using an appropriate regression model for calibration curves. Non-weighted ordinary linear regression has traditionally been used but is not necessarily the optimal model. During method development, the regression model is conventionally chosen in the pre-validation phase by evaluating 3-5 series of calibration curves in one analytical

run. Different regression models are fitted to the measured data and judged by the total sum of residuals for each model (Lang and Bolton, 1991; Wieling *et al.*, 1996; Shah *et al.*, 2000; Almeida *et al.*, 2002). Two approaches to evaluate the choice of regression models were compared in paper I. The traditional approach of 5 evaluated calibration curves was compared to the novel approach that uses calibration curve fit and calibration curve predictability. Average relative residuals (ΔRR) at each calibration level served as indices for calibration curve fit [Eq 1]. Accuracy [Eg 2] and precision (*i.e.* coefficient of variation) for the three independent quality control levels provided indices for predictability.

$$RR = 100 \times \frac{x_{predicted} - x_{nominal}}{x_{nominal}} \quad (1)$$

$$Accuracy = 100 \times \frac{\Delta x_{predicted} - x_{nominal}}{x_{nominal}} \quad (2)$$

The evaluated regression models were initially ranked for calibration curve fit and predictability separately. The rank sum of these two indices generated a final ranking for each regression model and the model with lowest rank sum represented the optimal model amongst those evaluated. Nineteen different regression models were evaluated, with respect to the two above approaches, using a validation data set for piperaquine quantification in urine (paper II).

Animal models

Animals

Paper IV used male Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden) that were acclimatized for at least 5 days before the experimental procedure. Rats were housed under controlled environmental conditions (12-h light-dark cycle, 26±1°C, 60-65% humidity), four to five rats in each cage until surgery and thereafter separately during recovery. Food (B&K Feeds) and tap water were available *ad libitum* and experiments were performed during the light phase. The experimental procedures were approved by the Ethics Committee for Animal Experiments, Göteborg, Sweden (82-2004).

Surgery

The rats were anesthetized by inhalation of isoflurane (Forene®, Abbot Scandinavia AB, Solna, Sweden) and the left jugular vein was catheterized. The catheter was tunneled subcutaneously to emerge at the back of the neck.

Catheters were kept patent with heparinized saline solution (20 IE/mL) before and between sampling to prevent blood clotting. All animals were allowed to recover for at least 12 hours after surgery. The bile duct was catheterized in four rats, which were kept under anesthesia during surgery and bile collection (8 hours).

Drug formulation and study design

Formulations used were either 5 or 10 mg piperaquine phosphate per mL emulsion consisting of saline solution:peanut oil:Tween® 80 (65:25:10, *v/v/v*). A total of 28 rats were administered piperaquine emulsions orally (n=12) or intravenously (n=16) with the intention to serially sample blood for up to 90 hours post-dose. The oral doses (10 mL/kg) were administered by gavage and the intravenous doses (1.25 mL/kg) were administered as a short-term infusion (approximately 20 minutes) via the jugular vein catheter. Intravenous drug administration and repeated blood sampling were achieved through the same catheter. Blood (1 mL) was withdrawn before the infusion and re-administered directly after drug administration. This was shown in an *ex vivo* pilot study prior to experiments to prevent residual drug concentrations in subsequent blood samples. Urine, bile and faeces were collected after an intravenous infusion in rats kept in metabolic cages or in bile-canulated anesthetized rats.

Drug metabolism

Study design

In paper III, two healthy male volunteers received a single oral dose of dihydroartemisinin-piperaquine (3 tablets of Artekin®) together with a fatty meal to maximize bioavailability (Sim *et al.*, 2005). In one subject, urine was collected for 16 hours following the dose and one venous blood sample drawn at 3 hours post dose for metabolite structural characterization by LC-UV, qualitative MS and H-NMR. In the other subject, 24 hours urine samples collected at 3, 4, 5, 8, 11, 15, 22, 31, 46, 64, 79, 93 and 123 days after drug administration were analyzed by LC-UV and evaluated with respect to the time-profile of metabolites.

Analytical procedure

A Varian pump (Walnut Creek, CA, USA) and a manual injector (VICI, Schenkon, Switzerland) were used to analyze the 16 hours urine sample. Separation was performed on a Chromolith Flash (25×4.6 mm) column protected by a Chromolith guard column (5×4.6 mm I.D) (VWR) at a flow rate

of 0.5 mL/min. Piperaquine and metabolites were eluted with 1% formic acid in acetonitrile and 1% aqueous formic acid using a 10 minutes gradient program to achieve sufficient separation between the structurally related molecules. The LC-system was coupled on-line to a quadrupole-iontrap mass spectrometer, QTRAP, (Applied Biosystems/MDS Sciex, Concord, Canada). The QTRAP was operated in positive ion mode and the collision energy varied to achieve more information about the fragmentation pattern. Data acquisition was performed using Analyst 1.3 software (Applied Biosystems/MDS Sciex).

Piperaquine and its metabolites were also extracted and metabolite fractions (M1, M2, M3&4 and M5) collected manually using the above described gradient program at a flow rate of 1 mL/min on a LC-UV system as described for piperaquine quantification. The pure metabolite 1 (M1) fraction was analyzed on a Bruker Daltonics (Billerica, MA, USA) BioAPEX-94e superconducting 9.4 T FTICR-MS to obtain a precise estimate of its molecular mass. The FTICR-MS was calibrated with piperaquine as reference and the molecular mass obtained for M1 was corrected using the internal error observed for piperaquine.

Piperaquine powder and collected metabolite fractions were reconstituted in D_2O and analyzed on a Varian Discovery 900 MHz NMR spectrometer to confirm the proposed molecular structures of piperaquine metabolites. Chemical shifts (δ) were reported relative to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) at 25°C.

Fischer esterification converts acids into their corresponding esters using alcohol and acid catalysis. All metabolite fractions were exposed to a Fischer esterification in order to confirm the presence or absence of a carboxylic acid. LC-collected fractions of the metabolites were exposed to Fischer esterification using acidic methanol and ethanol under the influence of heat (65°C) and analyzed with LC-UV.

The LC system used in paper IV to analyze pooled plasma, urine, faeces and bile samples from four animals was a LaChrom Elite system as described previously for piperaquine quantification. The LC column eluate was split so that approximately 0.75 mL/min entered the UV detector and 0.25 mL/min into an Esquire 4000 iontrap mass spectrometer equipped with an ESI interface (Bruker Daltonics, Bremen, Germany). Piperaquine and metabolites were eluted over 20 minutes and the MS was operated in a positive mode with a scan range varied between 150–900 m/z and fragmentation amplitude between 0.4-1 (arbitrary units). Data acquisition was performed using Hystar™ and DataAnalysis™ (Bruker Daltonics GmbH, Bremen, Germany).

Clinical pharmacokinetics

Study design

In paper V, a healthy Caucasian male volunteer received a single oral dose of Artekin® (3 tablets), together with a fatty meal to facilitate absorption (Sim *et al.*, 2005). Blood was taken at pre-specified time points up to 93 days after administration. Piperaquine was quantified in both blood and plasma with previously described methods.

The population pharmacokinetic study in paper VI was conducted along the Thai-Myanmar border, which is an area of unstable low and seasonal transmission of multidrug-resistant *P. falciparum* malaria (Luxemburger *et al.*, 1996). Written informed consent was obtained and approval for the study was granted by the Faculty of Tropical Medicine Ethical Committee, Mahidol University, Bangkok, Thailand and the Oxford Tropical Research Ethics Committee (OXTREC), UK. Ninety-eight Burmese or Karen patients aged 3-55 years, with symptomatic uncomplicated *P. falciparum* infections were enrolled. Patients were randomly allocated into one of the two treatment arms to generate similar demographic characteristics. Dihydroartemisinin-piperaquine (DP) was administered to achieve a total dose of 7 mg/kg body-weight of dihydroartemisinin and 55 mg/kg body-weight of piperaquine phosphate. The dose was divided into 4 (DP-4) occasions at 0, 8, 24 and 48 hours or 3 (DP-3) occasions at 0, 24 and 48 hours. Each treatment was supervised. All patients provided a pre-treatment sample and 2-4 additional samples randomly drawn from the following time windows after administration: 0-4, 8-12, 24-28, 48-52 hours (DP-3) or 4-8, 12-24, 28-48, 52-72 hours (DP-4) plus one additional sample on either day 7, 14, 21, 28, 35, 42, 49, 56 or 63.

Pharmacokinetic data analysis

Non-compartmental analysis

Piperaquine pharmacokinetic characterization after oral and intravenous administration in paper IV was performed by non-compartmental analysis as implemented in WinNonlin Version 5 (Pharsight Corporation, Ca., USA). The log-linear trapezoidal method with extrapolation to time infinity by C_{PRED}/λ_Z was used for each individual animal. The terminal elimination half-life was estimated by log-linear regression of 4 to 6 observed concentrations. Maximal piperaquine plasma concentration (C_{max}) and time to maximal concentration (T_{max}) were taken directly from the observed data. An estimate of oral bioavailability at each dose level was obtained by dividing AUC_{ORAL} by AUC_{IV} for low and high doses,

respectively, using dose-adjusted geometric mean values. Pharmacokinetic parameters for the two doses were compared using the Mann-Whitney U test with a significance level of 0.05. Non-compartmental analysis might underestimate the AUCs after intravenous administration of drugs displaying a rapid initial distribution with incomplete individual data during the α-phase. However, non-compartmental analysis has the advantage of not assuming a specific compartment model for the analyzed data. This approach allow pharmacokinetic parameter estimates to be compared for different routes of administration displaying different disposition kinetics, such as two- and three-compartment for oral and intravenous administration, respectively.

Pharmacokinetic modeling

In paper IV, naïve pooled concentration-time data from both intravenous dose levels were modeled simultaneously using WinNonlin (Pharsight Corporation, Ca, USA). Residual plots were used to choose between different weightings. The F-test was used to compare the weighted sum of squared residuals for the fitted one-, two-, and three-compartment models with identical weighting. The naïve pooled data approach incorporate more data when fitting the model and will, for the given data, probably achieve more reliable parameter estimates than individual non-compartmental analysis. However, this assumes that the same pharmacokinetic model and its primary parameter values are applicable for both doses (*i.e.* dose-proportional kinetics).

Population pharmacokinetic modeling

In paper VI, only sparse concentration-time data was available and a population based modeling approach was employed with nonlinear mixed effects modeling using NONMEM version V level 1.1 (Icon Development Solutions, Maryland, USA). Mixed effects modeling comprise both random effects (inter-individual-, inter-occasion- and residual variability) and fixed effects (covariates, time, dose and pharmacokinetic parameters such as clearance). Piperaquine plasma concentrations were transformed into their natural logarithms. Compartment models were fitted to the concentration-time profile for all patients using the first-order estimation (FO) and the first-order conditional estimation (FOCE) method (Beal and Sheiner, 1982; Beal. *et al.*, 1992).

Two- and three-compartment pharmacokinetic models with first order absorption, with and without absorption lag-time and with first order elimination from the central compartment were evaluated. The models were parameterized as oral clearance (CL/F), central volume of distribution (V_C/F), absorption rate constant (k_a), inter-compartmental clearance(s) (Q/F) and peripheral volume of distribution(s) (V_P/F). F is the fraction of drug available in the systemic

circulation after oral administration. Inter-individual random variability in all parameters was modeled exponentially as illustrated for clearance [Eq 3].

$$(CL/F)_i = CL/F \times \exp(\eta_i^{CL/F}) \tag{3}$$

where $(CL/F)_i$ is the individually estimated oral clearance value for the ith patient, CL/F is the typical clearance value for the modeled population, and $\eta_i^{CL/F}$ is the between-patient random variability assumed to be normally distributed (zero mean, variance ω^2) (Karlsson and Sheiner, 1993). Potentially influential continuous covariates (age, weight, height, initial hematocrit and parasitemia) and categorical covariates (gender and treatment group) were investigated using the stepwise general additive method as implemented in the S-PLUS based program Xpose 3.1 (Jonsson and Karlsson, 1999). Influential continuous covariates were evaluated by including them in the model as linear, allometric and hyperbolic Emax functions centered on the median value explaining some of the parameter variability. Treatment group was incorporated as a dichotomous covariate on all pharmacokinetic parameters to evaluate potential differences based on different treatment regimens.

Discrimination between models was assessed by a likelihood ratio test using the objective function values computed by NONMEM. The objective function value is equal to -2×log of the likelihood and the difference in objective function value between nested models is assumed to be χ^2-distributed (Beal and Sheiner, 1982). A 3.84 difference in objective function value is considered to be significant ($p<0.05$) with one degree of freedom when comparing two nested models. The recently proposed method of internal evaluation by the visual predictive check was used as a diagnostic tool for both the fixed and random effects in the model (Holford, 2005). A symmetrical distribution of observed data on the 50th percentile profile with approximately 10% of the data distributed outside the 5th–95th percentile boundaries should reflect that expected clinical variability is representatively described.

RESULTS & DISCUSSION

Bioanalysis (papers I-II)

Regression analysis

Nine out of the 19 evaluated regression models met the FDA requirements with respect to accuracy and precision and could theoretically be selected as the final model (paper I). The traditionally used ordinary linear regression displayed an impaired accuracy at the lower concentration range compared to weighted or transformed models (Fig 3). This stresses the importance of weighting or data transformation to achieve high-quality data when using a wide concentration range.
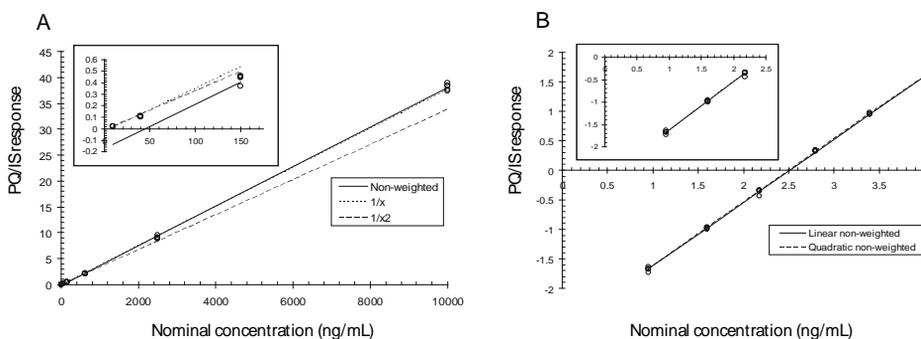

FIGURE 3. Selected regression models fitted to mean of 5 replicates at each calibration level. Graphs display piperaquine/internal standard height ratios *versus* nominal concentration for (*panel A*) linear regression models with different weights (*i.e.* non-weighted, $1/x$ and $1/x^2$) and (*panel B*) log-log transformed non-weighted regression models. The inserted panels shows the calibration curve fit at the three lowest concentrations.

The final ranking of the models according to the proposed new approach was based on a combination of calibration curve fit and calibration curve predictability to discriminate between the eligible models (Table III). The best model for the piperaquine validation data set with respect to overall characteristics was the linear log-log transformed model. This choice was based on 12 different parameters covering the whole calibration range. The data were generated over 4 different days and should reflect the actual performance during routine use of the assay. The FDA guidelines state that the simplest model that adequately describes the data should be used and that more complex models need justification (FDA, 2001). This originates from the concern of over-fitting the calibration curve when using a more complex model. The proposed new

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approach will provide enough support for the analyst to justify the choice of a more complex regression model and minimize the likelihood of over-fitting the calibration curve as it incorporates curve-independent quality control samples.

TABLE III. Final ranking for the regression models (range 9 to 10000 ng/mL)

Regression model				Ranking of calibration standards and QC samples			
Fitting	Transformation	Weight	Forced through origo	Ranking of calibration standards	Ranking of QC samples	Rank sum	Final ranking
Linear	No	No	No	18	17	35	18
Linear	No	1/x	No	15	7	22	12
Linear	No	1/x²	No	14	11	25	14
Linear	No	No	Yes	18	19	37	19
Linear	No	1/x	Yes	17	14	31	17
Linear	No	1/x²	Yes	16	14	30	16
Linear	log-log	No	No	1	3	4	1
Linear	log-log	1/x	No	2	9	11	5
Linear	log-log	1/x²	No	2	11	13	6
Linear	Box-Cox	No	No	13	2	15	7
Linear	Square root	No	No	5	4	9	3
Linear	Square root	1/x	No	7	1	8	2
Linear	Square root	1/x²	No	11	6	17	8
Quadratic	No	No	No	9	9	18	9
Quadratic	No	1/x	No	12	8	20	11
Quadratic	No	1/x²	No	8	14	22	12
Quadratic	log-log	No	No	4	5	9	3
Quadratic	log-log	1/x	No	6	13	19	10
Quadratic	log-log	1/x²	No	9	18	27	15

The applied data transformations significantly reduce the absolute range of calibrators compared with ordinary linear regression. The decreased distance between high and low data in the calibration range stabilizes the variance over the concentration range and equalizes the influence of each point in the regression (Armitage and Berry, 1994). Even if log-log transformation was the optimal model for the generated data, the square root transformation might be a better choice if a more narrow calibration range is used considering that the reduction is less drastic. Weighting factors had an important effect on non-transformed models and resulted in a considerable improvement in predictability, especially in the low concentration region.

Method validation

This new systematic approach of choosing an optimal regression model was evaluated during the development and validation of a drug quantification assay of piperaquine in urine (paper II). The accuracy and precision of the assay using a log-log transformed linear regression were well within the stipulated FDA limits for bioanalytical method validation (Table IV) (FDA, 2001). Also, the precision and accuracy of the method was not affected by using 1, 3 or 9 mL of urine and the limit of quantification could be lowered to 0.33 ng/mL if needed for increased sensitivity.

TABLE IV. Validation performances of the final piperaquine assay

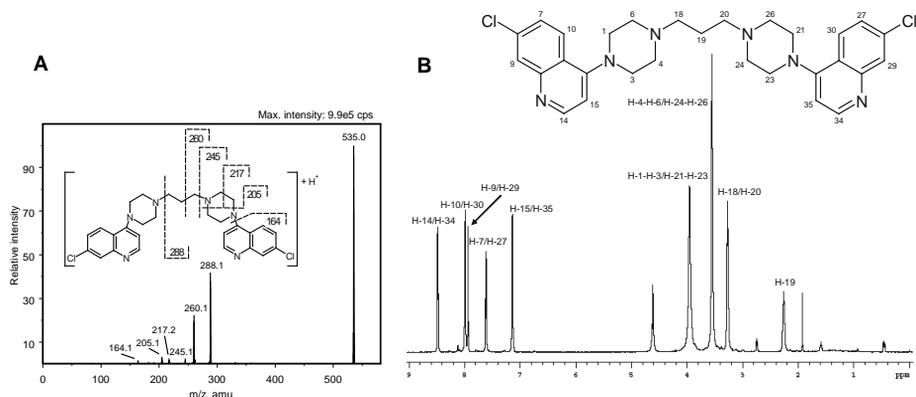|	QC 1 (50 ng/ml)	QC 2 (500 ng/ml)	QC 3 (5000 ng/ml)
Predicted concentration (mean)	49.9	535	5510
Within-day precision (%), n=20	5.9	5.3	1.4
Between-day precision (%), n=4×5	8.9	5.1	4.3
Total-assay precision (%)	9.2	5.6	3.9
Accuracy (%)	0.3	7.0	10.14
Recovery (mean %)	88	80	89

Clinical applicability

Urine sampling has the benefit of almost unlimited sample volumes. Larger volumes for an increased sensitivity will not entail any additional suffering or risk for the patient. This sampling approach is non-invasive and can be performed in home environment, which has practical and safety advantages compared to blood sampling. Clinical applicability and routine drug assay performance were demonstrated analyzing urine samples from one healthy volunteer after a single oral dose of dihydroartemisinin-piperaquine (3 tablets of Artekin®). Total precision for all quality controls (triplicates at each level) were below 15% as required for routine analysis.

Piperaquine could be quantified up to 123 days after a single oral dose, which is considerably longer than previously described in plasma (Hung *et al.*, 2004; Sim *et al.*, 2005; Roshammar *et al.*, 2006). This matrix presents an alternative approach for estimating the terminal elimination half-life for piperaquine. Regressing the exponential decline of piperaquine renal excretion rates against the midpoint of the collection intervals suggested a terminal elimination half-life of approximately 88 days (based on the 7 last data points). When fitted simultaneously, urine excretion data can support plasma concentration data

when describing the rather extreme pharmacokinetic profile of piperaquine. The assay is not only a valuable tool for pharmacokinetic studies of piperaquine but can be of benefit when studying the development of resistance since the duration of sub-therapeutic concentrations can be quantified for a longer period of time. Large amounts of two unidentified molecules, probably metabolites were also observed using the reported assay settings.

Drug metabolism (paper III)

Two metabolites (M1 and M2) were present in human urine and only small amounts in serum when analyzed isocratically by LC-UV (papers II and III). Eluting with a mobile phase resembling physiological pH suggested all metabolites to be more polar than piperaquine, as would be expected for most metabolites. Five human urinary metabolites were recognized using LC-MS/MS with gradient elution (M1:320 m/z, PQ:535 m/z, M2:M3:M4:551 m/z [PQ+16 m/z], and M5:567 m/z [PQ+32 m/z]). The different metabolite fractions were analyzed with H-NMR and supported the proposed structures for M1 and M2 and gave inconclusive results for the other three metabolites. The NMR-spectra of piperaquine were compared to that of chloroquine, which has an identical aromatic structure (*i.e.* 7-chloro-4-aminoquinoline seen in figure 2). This provided the relative order of aromatic hydrogen signals. Based on the H-NMR spectra for chloroquine (Maschke *et al.*, 1997) and the J-couplings detected for piperaquine, all hydrogens in piperaquine could be assigned. The proposed piperaquine fragmentation pattern detected with LC-MS/MS and hydrogen NMR assignment for piperaquine are shown in figure 4A and 4B, respectively.

FIGURE 4. Fragmentation pattern detected by LC-MS/MS (*panel A*) and H-NMR spectra and assignment of protons (*panel B*) for piperaquine in 16-h urine after a single oral administration of the dihydroartemisinin-piperaquine combination in a healthy male subject.

Carboxylic acid metabolite

The carboxylic acid metabolite (M1: 320 m/z) contained only one chloride atom indicating a cleavage in the aliphatic bridge of piperaquine. This structure was further supported by H-NMR, where no change in chemical shift could be seen in the aromatic part of the molecule compared to piperaquine. The proposed carboxylation would not affect the aromatic signals but created a downfield chemical shift in the aliphatic region of the molecule as expected from carboxylation in the proposed position. The Fischer esterification supported this metabolite to be a carboxylic acid since almost all metabolite was transformed into a more lipophilic substance. The proposed structure was further supported by its theoretical mass being in agreement with the precise mass determination by FTICR-MS. The proposed fragmentation pattern with LC-MS/MS and hydrogen NMR assignment for the carboxylic acid metabolite are shown in figure 5A and 5B, respectively.
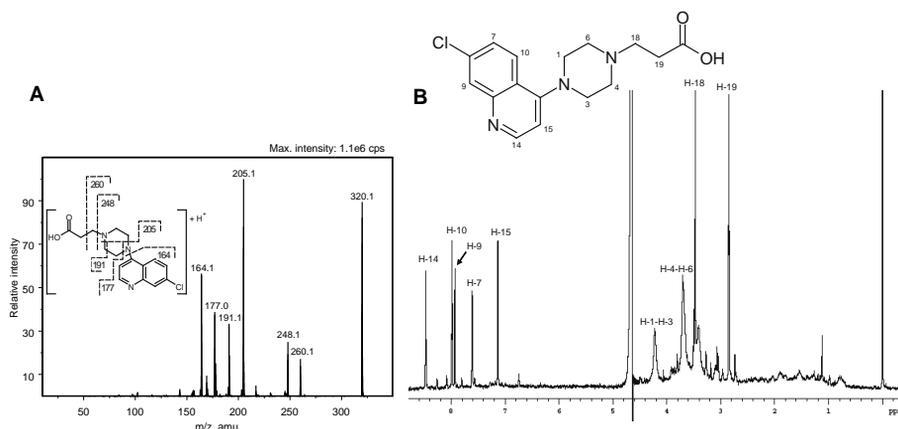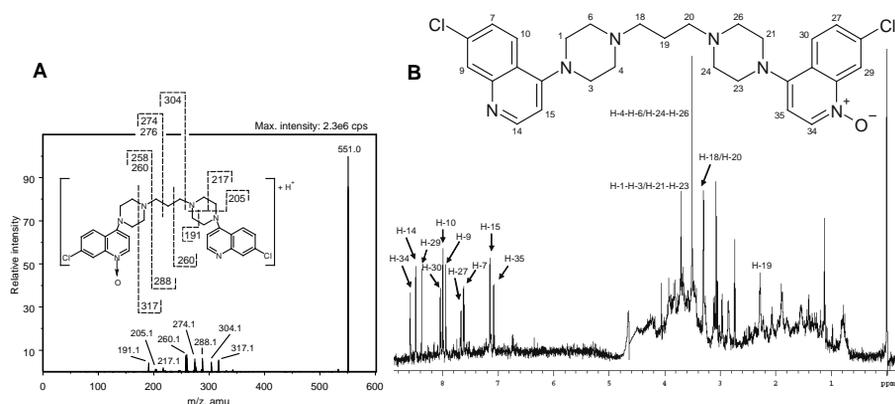

FIGURE 5. Fragmentation pattern detected by LC-MS/MS (*panel A*) and H-NMR spectra and assignment of protons (*panel B*) for the piperaquine carboxylic acid metabolite in 16-h urine after a single oral administration of the dihydroartemisinin-piperaquine combination in a healthy male subject.

N-oxidated metabolite

Hydroxylated and N-oxidated metabolites have the same molecular mass and it would be difficult to differentiate them by LC-MS/MS analysis. The 551 m/z metabolite 2 (M2) had a distinct difference in both polarity and fragmentation pattern compared to the other 551 m/z metabolites, piperaquine and 7-OH piperaquine. The suggested N-oxidation would influence the fragmentation

pattern and polarity without affecting the molecular mass weight of 551 m/z. N-oxidation was supported by H-NMR since all hydrogen signals were still present and the J-couplings unaltered. There was a change in chemical shift in one of the two aromatic parts producing a different electron density in one of the aromatic parts of the molecule. The only possible oxidation site in the aromatic part of the molecule is the nitrogen and it is likely that this metabolite is a nitrogen-oxide piperaquine product. The proposed fragmentation pattern with LC-MS/MS and hydrogen NMR assignment for the N-oxidated metabolite are shown in figure 6A and 6B, respectively.

FIGURE 6. Fragmentation pattern detected by LC-MS/MS (*panel A*) and H-NMR spectra and assignment of protons (*panel B*) for the piperaquine N-oxidated metabolite in 16-h urine after a single oral administration of the dihydroartemisinin-piperaquine combination in a healthy male subject.

Metabolite pharmacokinetics

As seen in papers II and III, the carboxylic acid metabolite was the most abundant metabolite and was present with chromatographic peaks larger than those of piperaquine. No reference compound was available at that time. For this reason a piperaquine/internal standard calibration curve was used to approximate the urine concentrations of the carboxylic acid metabolite (*i.e.* piperaquine equivalents). Assuming formation rate limited kinetics, regressing excretion rates against midpoint of collection intervals suggested a terminal piperaquine elimination half-life of 53 days. This data originates from one healthy volunteer but supports that the previously reported half-life of piperaquine might be underestimated (Hung *et al.*, 2004; Sim *et al.*, 2005; Roshammar *et al.*, 2006). It is also possible that this metabolite displays elimination rate limited kinetics which would indicate a longer terminal half-life than the parent drug. No information is published addressing non-clinical *in vivo* piperaquine pharmacokinetics or metabolism.

Animal models (paper IV)

Pharmacokinetics

Piperaquine displayed similar pharmacokinetics in the rat as in humans, with multiphasic disposition, low clearance, and a large steady-state volume of distribution resulting in a long terminal elimination half-life (Fig 7; Table V). Six out of ten animals receiving the high intravenous dose died suddenly from unknown cause during the experiment. There was no mortality in any other group. The results presented are for the 22 surviving animals out of the 28 dosed.

Substantially higher between-animal variability in plasma concentrations could be seen after oral administration compared with intravenous administration. This is in agreement with high inter-individual variability in the clinical pharmacokinetics of piperaquine after oral administration (Hung *et al.*, 2004; Roshammar *et al.*, 2006). This suggests that some variability in drug exposure might be explained by inter-individual variability in drug absorption. Piperaquine is not a substrate for P-gp and no major efflux by this common transporter is to be expected during absorption. Thus, P-gp would not explain the present variability in absorption (Crowe *et al.*, 2006). Piperaquine clearance was significantly lower after the higher intravenous dose suggesting piperaquine kinetics to be dose-dependent with saturable elimination. This might be artefactual considering the small sample size. Possible dose-dependent exposure needs to be confirmed in larger groups of animals at a minimum of three dose-levels with a broad range.
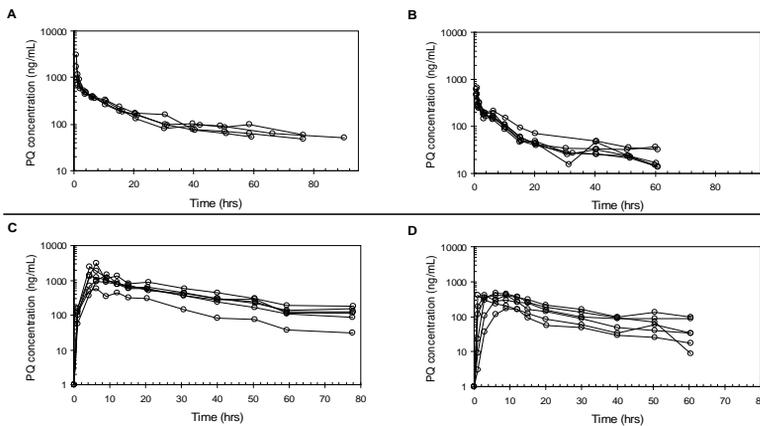

FIGURE 7. Piperaquine (PQ) plasma concentrations *versus* time after an intravenous 17 minute infusion of 26 mg PQ phosphate/kg (*panel A;* n=4) or 13 mg PQ phosphate/kg (*panel B;* n=6). PQ plasma concentration *versus* time after an oral single dose of 100 mg PQ phosphate/kg (*panel C;* n=6) or 50 mg PQ phosphate/kg (*panel D;* n=6). The drug was formulated as a saline solution:peanut oil:Tween® 80 (65:25:10, *v/v/v*) emulsion and administered to male Sprague-Dawley rats. Concentrations are on the logarithmic scale.

TABLE V. Non-compartmental analysis of piperaquine pharmacokinetics after a single oral dose and intravenous short-term infusion

	Intravenous administration		Oral administration	
	Low dose (13 mg PQP/kg; n=6)	High dose (26 mg PQP/kg; n=4)	Low dose (50 mg PQP/kg; n=6)	High dose (100 mg PQP/kg; n=6)
Body-weight (kg)	0.32 ± 0.02	0.34 ± 0.02	0.32 ± 0.01	0.34 ± 0.02
C_{max} (μg/mL)	0.48 ± 0.13*	1.4 ± 0.94*	0.41 ± 0.10*	1.4 ± 0.95*
T_{max} (hr)	N/A	N/A	7.5 ± 1.6	6.3 ± 1.8
CL (L/hr/kg)	1.6 ± 0.33*	0.95 ± 0.15*	N/A	N/A
V_{SS} (L/kg)	52 ± 19	41 ± 9.7	N/A	N/A
$T_{1/2,Z}$ (hr)	34 ± 19	38 ± 16	23 ± 8.0	25 ± 2.9
$AUC_{0-\infty}$ (hr×μg/mL)	4.6 ± 1.3*	15 ± 2.3*	11 ± 4.5*	34 ± 13*
$AUC_{0-\infty}$/dose (hr/L)	N/C	N/C	1.2 ± 0.50	1.8 ± 0.64
F (%)	N/A	N/A	50	52

Estimates are presented as median values ± standard deviation (SD).

* Statistically significant difference between dose levels using the Mann-Whitney U test (p<0.05).

PQP piperaquine phosphate, C_{MAX} maximum observed piperaquine plasma concentration, T_{MAX} observed time to reach C_{MAX}, *CL* clearance, V_{SS} steady-state volume of distribution, $T_{1/2,Z}$ terminal elimination half-life, $AUC_{0-\infty}$ area under the plasma concentration time curve, *F* oral bioavailability, *N/A* not applicable, and *N/C* not calculated. The percentage (*mean [min-max]*) of total AUC extrapolated to infinity was 23% [14-53] and 19% [15-26] for intravenous low and high dose and 18% [9-34] and 10% [6-12] for oral low and high dose, respectively.

Pharmacokinetic modeling showed that a first order elimination three-compartment mammilary model with iteratively re-weighting ($1/C_{PRED}^2$) provided the best fit (p<0.01) to the observed pooled plasma concentrations combining both intravenous dose levels (Fig 8). Model-based pharmacokinetic piperaquine parameters after intravenous administration are summarized in table VI. Each dose level was also modeled separately with an estimated mean relative contribution of each phase to the total AUC of 51% ($t_{1/2,\alpha}$ = 0.11 hrs), 15% ($t_{1/2,\beta}$ = 5.0 hrs) and 34% ($t_{1/2,\gamma}$ = 58 hrs), respectively.
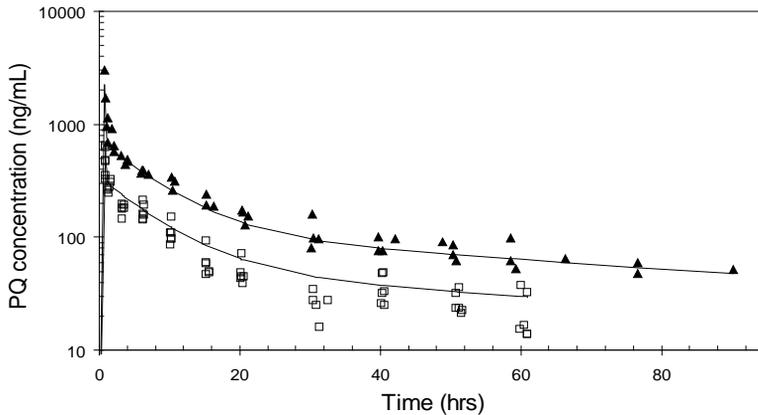
FIGURE 8. Piperaquine (PQ) plasma concentrations *versus* time on a semi-logarithmic scale after an intravenous 17 minute infusion of 26 mg PQ phosphate/kg (▲; n=4) or 13 mg PQ phosphate/kg (□; n=6). The solid lines (━) represents the three-compartment concentration-time profiles for the two doses simultaneously modeled on naïve pooled data. The drug was formulated as a saline solution:peanut oil:Tween® 80 (65:25:10, *v/v/v*) emulsion and administered to male Sprague-Dawley rats.

TABLE VI. Parameter estimates of the final three-compartment model describing piperaquine pharmacokinetics in the rat after an intravenous infusion

	Parameter estimate	CV %
CL (L/hr/kg)	0.53	8.6
V_C (L/kg)	0.18	18
Q_1 (L/hr/kg)	0.35	24
V_{P1} (L/kg)	1.9	21
Q_2 (L/hr/kg)	0.35	13
V_{P2} (L/kg)	21	23
V_{SS} (L/kg)	24	-
$t_{1/2,\gamma}$ (hr)	69	-

CL clearance, V_C central volume of distribution, Q inter-compartmental clearance V_P peripheral volume of distribution, V_{SS} steady-state volume of distribution estimated as $V_{SS}=V_C+V_{P1}+V_{P2}$, $t_{1/2,\gamma}$ terminal elimination half-life estimated from the terminal slope of concentration-time profile, and *CV %* coefficient of variation.

Piperaquine displays a rapid initial disposition phase, reminiscent of chloroquine pharmacokinetics after intravenous administration in 12 healthy volunteers (Looareesuwan *et al.*, 1986). The cardiovascular toxicity with a significant fall in systolic blood pressure of parenteral chloroquine was related to the high plasma concentrations occurring early in the distribution phase. The intravenous high-dose of piperaquine was associated with high mortality which might be connected to the same cardiovascular response as seen for chloroquine. The initial distribution phase of piperaquine contributed significantly to the total exposure of the drug and is therefore likely to contribute to the acute toxicity. The slow absorption seen after oral administration would attenuate the initial concentrations of piperaquine and could explain why severe side-effects were not seen in these animals. An intravenous formulation for clinical use is unlikely to be developed considering the poor solubility of piperaquine. Should an intravenous formulation of piperaquine be developed these results and previous results for chloroquine suggest extreme caution in its administration.

All animals reached plateau levels of the cumulative excretion of unchanged piperaquine in urine by 50 hours after intravenous administration. Less than 1% of the administered dose was excreted unchanged in the urine indicating this route of elimination to be negligible. Cumulative biliary excretion did not plateau during the 8 hour collection period. Regressing biliary excretion rates against midpoint plasma concentrations estimated the biliary clearance of piperaquine in the three rats to be 3.8, 3.1, and 4.2 mL/h/kg, respectively. This should not contribute to the total elimination of piperaquine in the rats. Contrarily to what have been speculated regarding piperaquine elimination in humans, these results suggest that any enterohepatic circulation would not contribute significantly to a prolongation of the terminal half-life (Hung *et al.*, 2004; Sim *et al.*, 2005).

Metabolism

The human carboxylic acid metabolite found in plasma and urine could be detected in all rat matrices analyzed but only at considerable levels in urine. The molecular structure was further supported by comparison with the synthesized metabolite. The carboxylic acid metabolite thus appears to be the major piperaquine metabolite in both the rat and human beings (paper III & IV). Surprisingly, no glucuronide metabolites could be detected even though both piperaquine and metabolites have functional groups amenable for phase II metabolism. Taken together these results indicate the rat to be a suitable animal model for non-clinical *in vivo* studies for future pharmacokinetic, toxicokinetic and metabolism studies.

Clinical pharmacokinetics (papers V-VI)

Single dose pilot study in a healthy volunteer

The single dose study in one healthy volunteer presented in paper V indicated the estimated terminal half-life to be correlated to sampling duration with decreasing slopes (*i.e.* lengthening half-lives) with increasing sampling duration. Plasma sampling up to 21, 64 and 93 days gave estimated half-lives ($t_{1/2}$ based on all measurements from day 10 and forward) of 14, 30 and 33 days, respectively as a result of multiphasic elimination. Quantification of piperaquine in blood was achieved over a period of 64 days and suggests a terminal half-life of 36 days. These are preliminary observations from only one healthy volunteer and further studies in a larger series will be necessary to determine the elimination profile and the true population terminal half-life of piperaquine. The blood-plasma ratio was constant over the plasma concentration range (*i.e.* 4.6-360 ng/mL) with a mean value of 1.3 (95%CI, 1.2-1.4). This is similar to what is previously reported in a Cambodian patient (Hung *et al.*, 2003).

Population pharmacokinetics in malaria patients

The population pharmacokinetic study presented in paper VI is to date the largest pharmacokinetic study of piperaquine conducted in patients with malaria. This study displays no significant pharmacokinetic differences for piperaquine between the older standard four dose regimen and the newer simplified three dose regimen. The two regimens resulted in similar mean drug exposure (*i.e.* $AUC_{\text{day 0-63}}$ of 19.4 and 20.7 h×μg/mL; $AUC_{\text{day 3-20}}$ of 8.65 and 9.07 h×μg/mL). This further supports the use of this once daily treatment regimen to improve treatment adherence and efficacy. The pharmacokinetic properties of piperaquine in these patients were characterized by a two-compartment disposition model with first-order absorption and an additive error model. The effective random residual error should be considered proportional since the modeled data was log-transformed. When superimposed on simulations, the measured piperaquine concentrations were symmetrically distributed on the 50th percentile profile with approximately 9% of the data distributed outside the 5th–95th percentile boundaries, reflecting that expected clinical variability was representatively described (Fig 9).
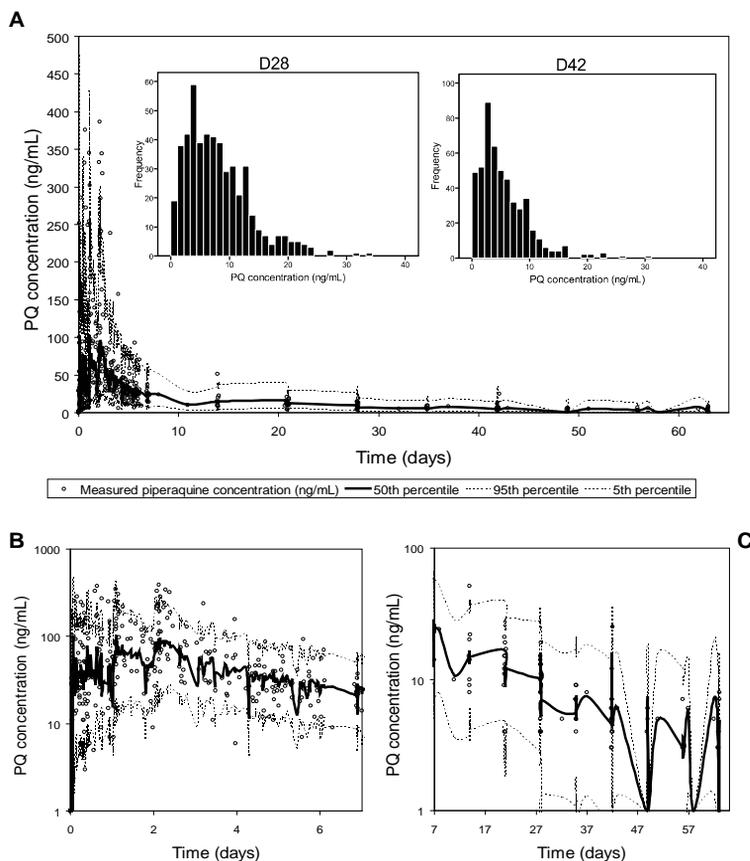
FIGURE 9. Measured piperaquine (PQ) concentrations are superimposed over population median concentration (50th percentile), 5th percentile and 95th percentile from simulating from the final model 500 concentrations at each of the individual sampling times after treatment initiation (*panel A*). Inserted figures indicate the simulated distribution of piperaquine concentrations at the common follow-up days 28 and 42. *Panel B* and *C* show data on a semi-logarithmic scale for days 1-7 and days 7-63, respectively.

The derived population estimates of pharmacokinetic parameters were generally similar to those previously reported (Tables I and VII). This study confirms that piperaquine exhibits considerable inter-individual pharmacokinetic variability, a very large mean steady-state volume of distribution (V/F = 874 L/kg), and a long estimated terminal half-life ($t_{1/2,\beta}$) of 28 days. This half-life might still be underestimated since the sparsely collected data were distributed over a long sampling period with no more than one measured concentration beyond seven days after treatment initiation for each patient. Even though the two-compartment model employed under-predicted the highest concentrations as well as those at the last sampling time point, the available data did not support a three-compartment disposition model.

TABLE VII. Parameter estimates of the final two-compartment model describing piperaquine population pharmacokinetics in patients with uncomplicated *Plasmodium falciparum* malaria

	Population estimate [RSE %]	Inter-individual variability (CV % [RSE %])
CL/F (L/h)	66.0 [6.9] × (1+0.0262 [2.9] × (WT-48))	42 [44]
V_C/F (L)	8660 [14] × (1+0.0273 [11] × (WT-48))	101 [17]
Q/F (L/h)	131 [13]	85 [18]
V_P/F (L)	24000 [13]	50 [76]
V_{SS}/F (L) [a]	38100 (min: 9980 – max: 115500)	
k_a (h^{-1})	0.717 [25]	168 [38]
σ (CV %)	31.4 [29]	
$t_{1/2, Z}$ (days) [a]	27.8 (min: 10.2 – max: 216)	

F oral bioavailability, *CL* clearance, V_C central volume of distribution, *Q* inter-compartment clearance, V_P peripheral volume of distribution, V_{SS} steady-state volume of distribution, k_a first-order absorption rate constant, σ additive residual error, $t_{1/2, Z}$ terminal half-life, *RSE* relative standard error [(SE/mean)×100%], *WT* body-weight with a median value of 48 kg
[a] mean population value

The best fit to the data was seen with body-weight linearly centered on the median value to explain the inter-individual random variability in clearance and central volume of distribution, *i.e.* 2.6% and 2.7% increase in oral clearance and central volume of distribution, respectively, per kg body-weight increase from median weight. Body-weight normalized oral clearance was lower in children compared to adults and the resulting derived terminal half-life showed a marked prolongation in small children (Fig 10). One may speculate whether this might provide some indication of the drug metabolizing enzymes involved.
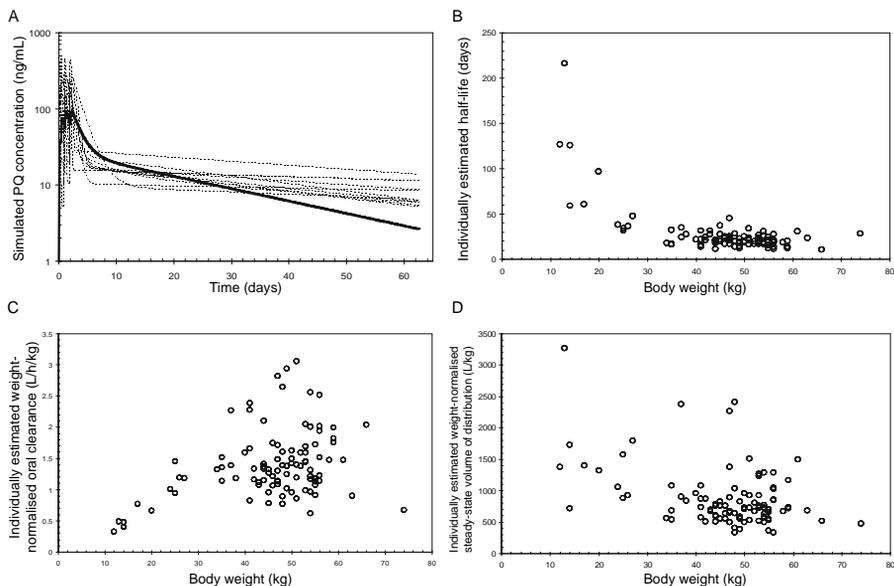
FIGURE 10. Simulated piperaquine (PQ) concentrations plotted on a semi-logarithmic scale against time for population mean (▬) and patients below 30 kg of body-weight (---) (*panel A*). Individually estimated terminal half-lives (*panel B*), individually estimated body-weight normalized oral clearance (*panel C*) and individually estimated body-weight normalized oral steady-state volume of distribution (*panel D*) plotted *vs* body-weight for all patients.

Plasma concentrations were not different in re-infected patients compared with the population mean and there were no major differences in pharmacokinetic parameters compared to the population mean estimates (Fig 11). The re-infected patients showed simulated piperaquine plasma concentrations of between 6-13 ng/mL and 1-8 ng/mL at the time of presentation with re-infections of *P. falciparum* and *P. vivax*, respectively. The study was conducted in a low transmission setting so these values must represent concentrations below the *in vivo* minimal inhibitory concentration (MIC) for prevalent parasites. Considering a blood stage incubation period of not less than 7 days, then the concentrations one week before re-infection of *P. falciparum* emerged were between 8-14 ng/mL which suggests this as a lower limit for the *in vivo* MIC in this region. Population mean plasma concentrations of 12 and 13 ng/mL could be seen at day 20 after treatment initiation of DP3 and DP4, respectively. This suggests a mean post-treatment prophylactic effect of approximately 20 days with the current dosage. Simulated distribution of concentrations indicates most patients to be below 10 ng/mL at day 28 (inserts in Fig 9). Estimation of the *P. vivax* MIC is more complex as, unlike *P. falciparum* re-infections which may be assumed to be random, the emergence of relapses occurs at approximately three week intervals.
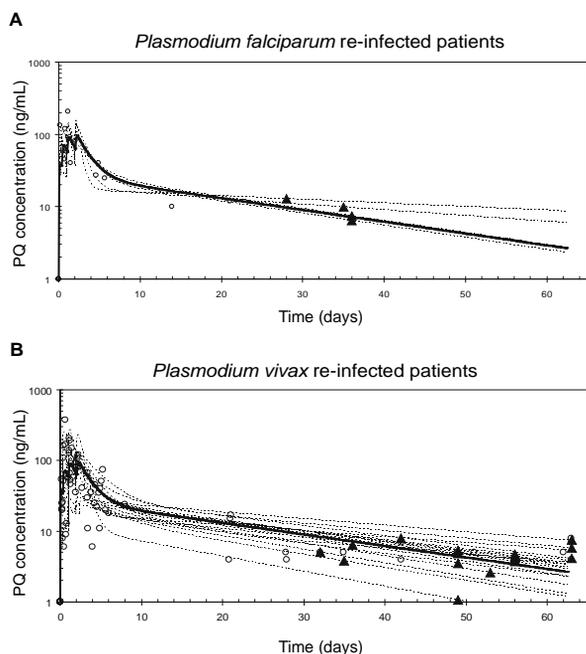
A

Plasmodium falciparum re-infected patients

B

Plasmodium vivax re-infected patients

FIGURE 11. Piperaquine (PQ) plasma concentration-time profiles of *Plasmodium falciparum* (*panel A*) and *Plasmodium vivax* (*panel B*) re-infected patients. The solid line (▬) represents the population concentration-time profile and the dashed lines (---) represent the concentration-time profiles of re-infected patients. The day of re-infection is marked by a solid triangle (▲) and measured piperaquine concentrations by open circles (○). All y-axes are on the logarithmic scale.

Although there were only 11 children in this study, there were therapeutically relevant pharmacokinetic differences compared with adults. They had a smaller central volume of distribution, a shorter distribution half-life ($t_{1/2,\alpha}$), and a more rapid fall in early piperaquine plasma concentrations compared to the population mean profile (Fig 10). The children in this study also displayed lower plasma concentrations during the putative, for efficacy, critical period between 3 and 20 days after starting treatment. These results are somewhat different to those in a previous report in 47 Cambodian children (2-10 years) with malaria which described a shorter 14 day elimination half-life in children (Hung *et al.*, 2004). This difference could result from the relatively small numbers studied here (n=11) and might therefore be non-representative. However, the lower $AUC_{3-20days}$ values in this study are supported by studies in West Papua where day 7 piperaquine concentrations in children were lower than in adults when dosed per kg body-weight (Hasugian *et al.*, 2007; Ratcliff *et al.*, 2007). This might have potentially important therapeutic consequences when the combination dihydroartemisinin-piperaquine is used. If this is confirmed in other malaria affected regions, then consideration should be given to increase the weight adjusted dosage in children.

GENERAL DISCUSSION

Sensitive and reliable analytical tools are essential for producing high-quality data suitable for pharmacokinetic data analysis. Pharmacokinetic characteristics might be lost in the noise of low-quality data and for example completely miss a putative late redistribution phase due to inadequate method sensitivity. Using an appropriate regression model is vital for generating reliable data, especially at low concentrations. Predictability of unknown samples is the main goal of any analytical method used for quantification and it is reasonable to also include this parameter as one of the criteria when choosing the regression model. The presented new systematic approach of choosing a regression model is a significant change compared with the traditional approach but has the advantage of discriminating between different regression models in order to choose the optimal one. This might be a better approach in the overall aim to produce reliable high-quality data suitable for pharmacokinetic evaluation.

Understanding the pharmacokinetic properties of drugs and active metabolites *in vivo* are important to explain and predict treatment outcome. International regulatory agencies and the pharmaceutical industry have over the last couple of years progressed towards a modeling and simulation approach to describe and predict the intricate relationship between drug exposure and treatment outcome (*i.e.* pharmacokinetic-pharmacodynamic modeling). This is particularly important in vulnerable groups that might display different pharmacokinetic profiles leading to impaired treatment outcome or drug induced side-effects compared to the mean population. Understanding piperaquine pharmacokinetics is the first step in rational dose-optimization when using piperaquine as a combination drug in ACTs (Fig 12).

The development of piperaquine has been fragmentary and there are still many areas that have not yet been evaluated regarding piperaquine pharmacokinetics, drug metabolism or toxicokinetics. Plasma protein binding is not investigated and might be different in patients exhibiting altering plasma protein binding over time as the disease progress. The accumulation of piperaquine in parasites, sequestering in the red blood cells, might affect the blood-plasma ratio and therefore the pharmacokinetics. Altered plasma protein binding and/or blood-plasma ratio would result in time-dependent pharmacokinetics in malaria patients and differences between healthy volunteers and patients. The mechanism and involvement of different metabolizing enzymes should be evaluated to study drug-drug interactions and population differences in the elimination of piperaquine. Drug-drug interactions can potentially be important considering the extreme half-life of piperaquine with detectable levels in plasma for as long as 93

days after a single dose. The effect of concomitant food intake is only studied in healthy Caucasian volunteers and needs to be investigated in the affected population, preferably in patients. A number of on-going projects are on the way in populations especially vulnerable to malaria and will evaluate the pharmacokinetic properties of piperaquine in children and pregnant women. Clinical pharmacokinetic-pharmacodynamic modeling is somewhat more difficult since piperaquine is administered as a combination treatment where both drugs contribute to the parasiticidal effect. Even so, such modeling needs to be developed to understand the relationship between drug exposure and treatment outcome.
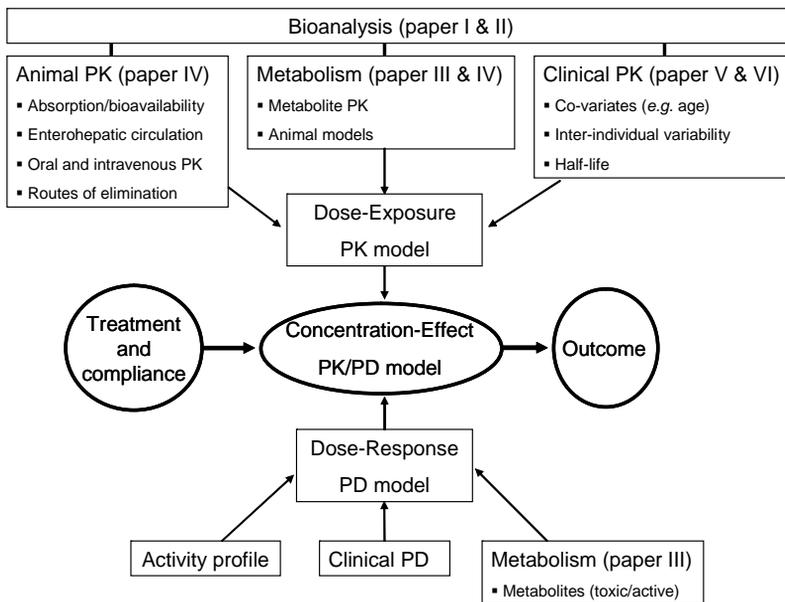
FIGURE 12. The role of bioanalysis, drug metabolism and pharmacokinetics in the development of the antimalarial piperaquine.

The question whether piperaquine is the most appropriate candidate for artemisinin derivatives in ACTs can be debated. The artemisinin compounds used in ACTs have a very short terminal elimination half-life and a suitable partner drug should provide a long-acting cover for at least two asexual erythrocytic life-cycles (>4 days for *P. falciparum*). Piperaquine has a long half-life and display excellent tolerability and efficacy against multi-drug resistant parasites. However, piperaquine exhibits considerable inter-individual variability which makes the treatment outcome more difficult to predict. The pharmacokinetic properties of piperaquine are not yet fully investigated and

piperaquine is somewhat predisposed to the emergence of drug resistance on account of its extreme half-life, as was evidenced by its use as monotherapy in China during the 1980s. The fixed oral combination of dihydroartemisinin-piperaquine has a very rapid paraciticidal effect and quickly reduces the biomass of parasites after treatment initiation. This is mainly attributed to dihydroartemisinin and will decrease the risk of surviving mutant parasites. Long acting drugs, such as piperaquine can prevent recrudescence by killing residual parasites. Reducing the surviving residual mutant parasites will prevent resistance towards either drug. Furthermore, resistance to piperaquine has not yet become a clinical problem when used in combination with artemisinin derivatives.

Overall, the fixed oral combination of dihydroartemisinin-piperaquine appears to be a promising alternative to treat clinical episodes of malaria. However, dihydroartemisinin might not be the most suitable artemisinin derivative in ACTs considering the poor thermal stability in fixed formulations. Artesunate-piperaquine might be a superior alternative with the advantage of an increased thermal stability compared to dihydroartemisinin. The combination should display equivalent efficacy since artesunate is rapidly metabolized into dihydroartemisinin after administration. Results presented in this thesis provide novel information about the pharmacokinetic properties of piperaquine, including both non-clinical and clinical *in vivo* studies and lend further support to the use of piperaquine as a partner drug in ACTs in malaria endemic areas.

CONCLUSIONS

This thesis has contributed to a better understanding of the bioanalysis, drug metabolism and pharmacokinetics of the increasingly important antimalarial piperaquine with respect to method development and regression model evaluation, *in vivo* metabolite characterization in human urine and in the rat, *in vivo* pharmacokinetics in the rat and human beings.

Bioanalysis
- Ordinary linear regression should not be the primary regression method of choice; on the contrary it should generally be avoided especially when a broad concentration range is used.
- A combination of relative residuals at each calibration level together with precision and accuracy of independently spiked quality control samples over four days provide a new systematic approach to select and justify the best regression model during bioanalytical method validation.
- The validated assay of quantifying piperaquine in human urine presents a sensitive, reproducible and non-invasive alternative to quantify piperaquine in the body and can be an important tool for pharmacokinetic evaluation of combined urine and plasma data in clinical studies or to evaluate patient relapses with respect to drug resistance.

Drug metabolism
- Piperaquine displayed 5 human urinary metabolites in two healthy male volunteers after a single oral dose of the dihydroartemisinin-piperaquine combination. The major metabolites are a carboxyl acid cleavage product, the other a mono-*N*-oxidated piperaquine product.

Animal models
- The rat is a suitable animal model for non-clinical *in vivo* studies of piperaquine pharmacokinetics and metabolism. The major piperaquine metabolite found in the rat was the same carboxylic acid product which was also the major metabolite in humans. Similar pharmacokinetic properties could be seen for piperaquine administered in the rat as in humans with multiphasic disposition, large volume of distribution and a long terminal half-life.
- Enterohepatic circulation will not contribute significantly to a prolongation of the terminal half-life in the rat, contrarily to what have been speculated regarding piperaquine elimination in humans.

Clinical pharmacokinetics

- Published estimates of piperaquine terminal elimination half-life are likely to be underestimates resulting from insufficient assay sensitivity and/or duration of sampling with oversimplified fitting of a two compartmental model.
- Piperaquine exhibits considerable inter-individual pharmacokinetic variability, has a very large apparent volume of distribution, and a very slow elimination phase.
- A linear relationship between body-weight and clearance and body-weight and central volume of distribution was shown with a 2.6% and 2.7% increase in oral clearance and central volume of distribution, respectively, per kg body-weight increase from median weight.
- Children had a higher total exposure of piperaquine compared to the population mean value but importantly a decreased exposure from days 3 to 20. It suggests that despite having a smaller central volume of distribution and slower elimination than adults, children have lower piperaquine concentrations in the therapeutically important period immediately following treatment. If this is confirmed in series from other malaria affected regions, then consideration should be given to increase the weight adjusted dosage in children.
- No significant pharmacokinetic differences for piperaquine between the older standard four dose regimen and the newer simplified three dose regimen was presented, and therefore further supports the use of this once daily treatment regimen to improve treatment adherence and efficacy.

SWEDISH SUMMARY

Malaria är en av de vanligaste parasitsjukdomarna i världen och drabbar 300 till 700 miljoner människor varje år. Upp till tre miljoner människor, de flesta barn, dör årligen till följd av malaria. Piperakin är ett malarialäkemedel som använts kliniskt i årtionden men som på senare år fått allt mer uppmärksamhet. En kombinationsbehandling innehållande piperakin är rekommenderad behandling i stora delar av världen där tidigare behandling har blivit verkningslös på grund av resistens. Denna avhandling redogör för piperakins omsättning i råtta och människa med fokus på bioanalys, metabolism och farmakokinetik.

Farmakokinetik är det forskningsområde som beskriver och förklarar de processer där ett läkemedel tas upp, fördelas och elimineras i kroppen. Läkemedel elimineras ofta genom metabolism vilket innebär att läkemedlet bryts ner till mindre beståndsdelar – så kallade metaboliter. För att kunna utreda ett läkemedels farmakokinetik och metabolism behövs känsliga och tillförlitliga bioanalytiska metoder som kan mäta läkemedelskoncentrationer i kroppen. Med kunskap om ett läkemedels farmakokinetik och metabolism kan effekter och biverkningar förutsägas och därigenom också en optimal behandling rekommenderas.

Läkemedelsutveckling är traditionellt en linjär process där ett läkemedel först testas i djurmodeller för att sedan ges till friska frivilliga försökspersoner och slutligen till patienter. Många läkemedel inom tropikmedicin och malaria genomgår inte alltid alla dessa nödvändiga steg. Därför saknas ofta en grundläggande förståelse för dessa läkemedels farmakokinetik och metabolism innan de ges till patienter. Det finns hittills ingen publicerad information där detta har studerats i djurmodeller och endast tre studier har undersökt piperakins farmakokinetik i människa.

Avhandlingen bygger på följande sex delarbeten:

I. Ett *nytt systematiskt tillvägagångssätt* som beskriver hur analysmetoder kan optimeras. Det nya tillvägagångssättet kan hjälpa forskaren att optimera sin analysmetod för att generera så tillförlitliga data som möjligt.

II. En *ny känslig bioanalytisk metod* för att analysera piperakin i urin. Det finns tidigare ingen publicerad metod för att bestämma koncentrationen av piperakin som utsöndras i urin. En sådan metod är värdefull då man kan kombinera data från urin och plasma för att få en fullständig bild över piperakins farmakokinetik.

III. En klinisk pilotstudie som för första gången redogör för piperakins *metabolism* i två friska försökspersoner. De metaboliter som bildas vid nedbrytning i kroppen kan vara inaktiva eller ha en farmakologisk effekt och ge upphov till biverkningar. Att beskriva strukturerna för de metaboliter som bildas är ett första steg i att förstå vad som händer med piperakin i kroppen.

IV. En *djurstudie* som redovisar piperakins farmakokinetik och metabolism. Djurmodeller används ofta för att förklara och utreda orsaken till varför ett läkemedel uppvisar vissa egenskaper. Råtta visas här vara en lämplig djurmodell för att studera piperakins mycket speciella farmakokinetiska egenskaper.

V. En klinisk pilotstudie där piperakins *farmakokinetik* visas i en frisk försöksperson. Tidigare publicerad information underskattar troligen tiden det tar för piperakin att utsöndras från kroppen. Då läkemedlet antagligen finns kvar längre i kroppen än man tidigare trott innebär det framförallt ett längre profylaktiskt skydd mot nya malariainfektioner.

VI. En omfattande *farmakokinetisk* studie på 98 malariapatienter från 3 till 55 år i nordvästra Thailand. Denna studie visade att barn troligen har en skild farmakokinetik från vuxna vad gäller piperakin. Om detta bekräftas i större studier kan det leda till en optimering av malariabehandlingen genom lämplig dosjustering. Studien visade också att piperakins farmakokinetik är oförändrad med en förenklad behandling jämfört med standardbehandlingen. En förenklad behandling förväntas vara mer effektiv då patienter antas följa behandlingsinstruktionerna bättre.

Sammanfattningsvis föreslår denna avhandling ett nytt systematiskt tillvägagångssätt för att optimera analysmetoder, en bioanalytisk metod för att mäta piperakin i urin samt denna substans farmakokinetik och metabolism i både människa och råtta. Det studerade kombinationspreparatet som innehåller piperakin utgör en säker och effektiv behandling mot malaria. Resultaten som presenteras i denna avhandling bidrar med ökad kunskap om piperakin och fortsatt stöd för att använda piperakin i kombinationsbehandling mot malaria.

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