Piperaquine and Metabolites

Bioanalysis and Pharmacokinetics

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Gothenburg, Sweden, 2017
Knowledge has always been important, of course. The ancient Egyptians did not raise the stones for the pyramids relying on the incantations of their gods. The waters in the irrigation canals of the great Indus civilization did not flow according to the laws of ignorance. Knowledge has always been power and wealth.

Mahathir Mohammad
Piperaquine and Metabolites

– Bioanalysis and Pharmacokinetics

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Abstract

Antimalarial piperaquine (PQ) is currently used as a partner drug with dihydroartemisinin (DHA), exhibiting high cure rates (>95%) for *P. falciparum*. Despite its raising usage worldwide with DHA, PQ is synthetically developed outside of big pharma pipelines. Thus, there is potentially some scientific gap in the information regarding disposition of the drug not being systematically established. This thesis comprised studies on bioanalysis- (Paper I), CYP3A4/5 inhibitory potential- (Paper II), protein binding- (Paper III) and pharmacokinetics (PK) of piperaquine and its metabolites (Paper IV) with intention of filling these scientific gaps.

PQ in earlier studies metabolized to two main urinary metabolites, M1 which is a carboxylic acid cleavage product and M2, the mono N-oxide of PQ. PQ and M2 were found as potent CYP3A inhibitors whereby M2 showed greater inhibition *in vitro*. Simulation of PQ inhibitory effect, predicted the drug-drug interaction (DDI) between PQ and co-administered midazolam in healthy subjects during antimalarial PQ treatment.

Bioanalytical method was developed using a highly sensitive analytical instrument, LC-MS/MS to determine PQ and its metabolites in human plasma. The simultaneous quantitation
method of PQ and metabolites was developed and validated for the first time based on the FDA guidelines. The method was applied for PK studies of PQ and metabolites after oral administration of single and escalating dose regimen of Artekin® (DHA-PQ) in Vietnamese healthy subjects. PQ exhibited dose- and time independent kinetics. M2 was found to be circulating metabolites in plasma while M1 was hardly detected.

Plasma protein binding of PQ and its metabolites were studied in vitro whereby PQ was extensively bound to plasma proteins with higher affinity towards AGP protein than to the albumin while metabolites, exhibited a much lower degree of binding. Unbound fractions of PQ and metabolites were successfully determined in human plasma by ultrafiltration.

Generally, the utmost contribution of this thesis is the application of bioanalysis method to quantitate the antimalarial PQ and its metabolites for pharmacokinetics including CYPs- and protein binding studies. As other antimalarials, PQ nowadays should be carefully evaluated for its treatment benefit and risk potential considering the challenge of increasing antimalarial resistance. Furthermore, DHA-PQ is suggested for mass-drug-administration (MDA) to eliminate malaria in Sub-Saharan Africa.

Keywords

*Piperaquine, LC-MS/MS, pharmacokinetics, CYP3A inhibition, protein binding*

ISBN 978-91-629-0252-0
List of papers

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

I. **Mohd Yusmaidie Aziz**, Kurt-Jürgen Hoffmann and Michael Ashton.

   *LC-MS/MS quantitation of antimalarial drug piperaquine and metabolites in human plasma.*

   Accepted for publication in Journal of Chromatography B, 2017

II. **Mohd Yusmaidie Aziz**, Kurt-Jürgen Hoffmann and Michael Ashton.

   *Inhibition of CYP3A by Antimalarial Piperaquine and Its Metabolites in Human Liver Microsomes with IVIV extrapolation.*

   Submitted

III. **Mohd Yusmaidie Aziz**, Kurt-Jürgen Hoffmann and Michael Ashton.

   *Plasma protein binding of piperaquine and its metabolites: Binding to human serum albumin, α1-acid glycoprotein and plasma from healthy volunteers.*

   In manuscript

IV. **Mohd Yusmaidie Aziz**, Trinh Ngoc Hai, Emma Johansson, Le Minh Dao, Pham Thi Thinh and Michael Ashton

   *Dose- and time-independent pharmacokinetics of piperaquine and its metabolites in healthy male Vietnamese subjects after four escalating oral doses separated by one month.*

   In manuscript
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<th>Definition</th>
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<tbody>
<tr>
<td>ACTs</td>
<td>Artemisinin-based combination therapies</td>
</tr>
<tr>
<td>AGP</td>
<td>α1-acid glycoprotein</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the drug concentration-time curve</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CL/F</td>
<td>Apparent/oral clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum drug concentration</td>
</tr>
<tr>
<td>C&lt;sub&gt;u&lt;/sub&gt;</td>
<td>Unbound concentration</td>
</tr>
<tr>
<td>%CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug interaction</td>
</tr>
<tr>
<td>DHA</td>
<td>Dihydroartemisinin</td>
</tr>
<tr>
<td>E&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Maximal enzymes activity in the absence of inhibitor</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicine Agency</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>f&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Relative contribution of the enzyme</td>
</tr>
<tr>
<td>fu</td>
<td>Fraction unbound</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsome</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>I&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum Inhibition</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>IVIVE</td>
<td><em>In vitro-in vivo</em> extrapolation</td>
</tr>
<tr>
<td>K&lt;sub&gt;eff&lt;/sub&gt;</td>
<td>Binding affinity constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;inact&lt;/sub&gt;</td>
<td>Maximum rate of enzymes activation</td>
</tr>
<tr>
<td>K&lt;sub&gt;obs&lt;/sub&gt;</td>
<td>The rates of enzymes inactivation</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography/tandem mass spectrometry</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantitation</td>
</tr>
<tr>
<td>M1</td>
<td>Carboxylic acid cleavage product metabolite</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>M2</td>
<td>Mono-N-oxidated metabolite</td>
</tr>
<tr>
<td>M5</td>
<td>Double-N-oxidated metabolite</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NCA</td>
<td>Non compartmental analysis</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced form of NADP⁺</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>P450s</td>
<td>Cytochrome P450 enzymes</td>
</tr>
<tr>
<td>PQ</td>
<td>Piperaquine</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RAF</td>
<td>Relative activity factor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SPC</td>
<td>Summary Product Characteristic</td>
</tr>
<tr>
<td>TDI</td>
<td>Time-dependent inhibition</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>Elimination half-life</td>
</tr>
<tr>
<td>Tₘ₉₉</td>
<td>Time to reach Cₘ₉₉</td>
</tr>
<tr>
<td>Vᵢ</td>
<td>Rate of metabolism for each isoform</td>
</tr>
<tr>
<td>V/F</td>
<td>Apparent volume of distribution</td>
</tr>
<tr>
<td>WHO</td>
<td>The World Health Organization</td>
</tr>
</tbody>
</table>
### Definitions in Short

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioanalysis</td>
<td>The quantitative measurement of xenobiotics (drug, metabolites etc.) in biological matrices</td>
</tr>
<tr>
<td>Pharmacokinetics</td>
<td>The study of drug absorption, distribution, metabolism and excretion. ‘What the body does to the drug’</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Malaria Disease

Malaria is a life-threatening disease caused by parasites, transmitted to people through the bites of infected female Anopheles mosquitoes. The mosquitoes release Plasmodium parasites into human body. There are five parasite species that cause malaria in humans, and two of these species – P. falciparum and P. vivax – pose the greatest threat [1-3]

In 2015, 91 countries and areas had ongoing malaria transmission whereby nearly half of the world's population was at risk of malaria. Most malaria cases and deaths occur in sub Saharan Africa. According to the latest WHO estimates, released in December 2016, there were 212 million cases of malaria in 2015 and 429 000 deaths [4].

In areas with high transmission of malaria, children under five are particularly susceptible to infection, illness and death whereby more than two-thirds (70%) of all malaria deaths occur in this age group. Even though malaria death rates fall year by year, it remains a major killer of children under five years old, taking the life of a child every two minutes in Sub-Saharan Africa. Pregnant women in endemic regions are also of concern as being vulnerable to malaria transmission due to physiological changes during pregnancy [4-6].
1.1.1 Transmission of malaria

Malaria parasites spread by successively infecting two types of hosts: female *Anopheles* mosquitoes and humans (Fig. 1). When the mosquito feed on human blood to nourish her eggs, she releases the sporozoites from its salivary glands into the blood stream of the person. The sporozoites are rapidly transported to the liver and invade the hepatocytes. In all species of *Plasmodium*, these parasites develop to form schizonts, from which several thousand merozoites advance. In *P. vivax* and *P. ovale* only, a proportion of the liver-stage parasites known as hypnozoites remain dormant in the hepatocytes for months or several years [7, 8].

![Figure 1. Life cycle of the malaria parasite](image)

*Figure 1. Life cycle of the malaria parasite*  
*Reprinted from The Nature [9]*

When the liver cells rupture, the merozoites are released into the bloodstream where they rapidly invade the red blood cells. These
blood-stage parasites replicate asexually – rapidly attaining a high parasite burden and destroying the red blood cells, leading to the clinical symptoms of malaria. On the other hand, a small percentage of merozoites, differentiate into male and female gametocytes, which are taken up by the mosquito in her blood meal. These gametocytes cause the cycle of transmission to continue back to the mosquito. Male and female gametocytes fertilize within the mosquito forming diploid zygotes, which in turn become ookinetes. These ookinetes migrate to the midgut of the insect, pass through the gut wall and form the oocysts. Meiotic division of the oocysts occur and sporozoites are formed, which then migrate to the salivary glands of the female Anopheles mosquito ready to continue the cycle of transmission back to man [10-12].

1.2 Malaria Treatment

Malaria is an entirely preventable and treatable disease. The primary objective of treatment is to ensure the rapid and complete elimination of the *Plasmodium* parasite from the patient’s blood in order to prevent progression of uncomplicated malaria to severe disease and death. WHO recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria caused by the *P. falciparum* parasite. By combining two active ingredients with different mechanisms of action, ACTs are the most effective antimalarial medicines available today [13]. There are now five ACTs recommended for use against *P. falciparum* malaria and by fact, in 2015, ACTs had been adopted as first-line treatment in 81 countries. [14-18].

- Arthemether-Lumefantrine
- Artesunate-Amodiaquine
- Artesunate-Mefloquine
- Artesunate-Sulfadoxine-Pyremethamine
- Dihydroartemisinin-Piperaquine

*Five recommended ACTs by WHO*
Besides ACTs, antimalarial agents given as monotherapy are still in practice such as primaquine which is recommended in low transmission areas to reduce transmission of the infection [19]. Even though, primaquine is thought to induce hemolytic anemia in glucose-6-phosphate dehydrogenase, G6PD-deficient individuals, a single low dose of primaquine which effectively blocking transmission is unlikely to cause serious toxicity in individuals with any of the G6PD-deficiency variants [20]. Primaquine is also given to prevent relapses of *P. vivax* infections [21]. In case of severe malaria, injectable artesunate is given via intramuscular injections or intravenous infusion for at least 24 hours. Once the patient can tolerate oral medication, a complete 3-day course of an ACT will be added [22, 23]

### 1.2.1 Emergence of piperaquine

Piperaquine (PQ) is a synthetic bisquinoline antimalarial drug originally developed by Rhone-Poulenc (currently Aventis) in France in 1963 (Fig. 2). Its structure is related to chloroquine (CQ). PQ has a long-effective action against malaria and it were synthesized by imitation in China in 1965 [24, 25]. PQ was suggested to be active against the erythrocytic stage of malarial parasites with a long-term effect on the suppressive prophylaxis of malaria [26]. In mice, PQ was introduced and suggested to cause the interference to the structure of food vacuoles of *P. berghei* [27, 28].

![Figure 2. Molecular structure of piperaquine base (MW=535.52)](image-url)
Given its higher potency and tolerability compared to CQ, PQ superseded CQ as the antimalarial regimen recommended by the Chinese Malaria Control Programme in 1978. During that year, 200 tons of PQ phosphate, were dispensed in China, an equivalent of 140 million adult treatment doses [29]. Unfortunately, the campaigns of mass drug administration and the extensive use of PQ for both treatment and prophylaxis led to the emergence of *P. falciparum* PQ-resistant strains. The resistance rate of *P. falciparum* to the drug was high in the range of 73–96 %, with a wide distribution in the endemic area in southern China [30]. Therefore, the use of PQ as monotherapy and prophylactic agent for malaria control was abandoned in the late 1980s [31].

1.2.2 Combination of DHA-piperaquine

In the 1990s, Chinese scientists reconsidered piperaquine (PQ) as one of the potential partner components of the so-called artemisinin based combination therapies (ACTs). The first ACT containing PQ was used in the Vietnamese Malaria Control Programme in 2000 [32]. This ACT, named China-Vietnam 8 (CV8), combined PQ phosphate with dihydroartemisinin (DHA), trimethoprim and primaquine phosphate. The rationale of combining two or more drugs in ACT program is to counter the parasite resistance whereby the chance of parasites simultaneously developing resistance is much lower than the chance of parasites developing resistance to single drugs because of genetic mutations to two drugs with different modes of action [33]. However, concerns about the associated risk of red cell haemolysis owing to primaquine in G6PD-deficient populations and the questionable antimalarial potency of trimethoprim resulted in the removal of these two drugs in CV8. The remaining components of the regimen, DHA and PQ, provided a highly effective and relatively inexpensive combination, known as Artekín®, which is safe in curing malaria and providing prophylaxis for re-infection [34, 35]. DHA-PQ was then manufactured to meet GMP standard and marketed as Eurartesim®, and this combination was approved by the European Medical Agency (EMA) in 2011 [36].
In the last decade, several clinical trials evaluating the DHA-PQ combination for the treatment of uncomplicated *P. falciparum* and *P. vivax* malaria had been conducted in malaria endemic countries [29]. In Asia, 18 trials involving approximately two thousand patients treated with DHA-PQ took place between 2002 and 2009, showing an excellent safety profile and very high efficacy with the overall 28-day cure rates exceeded 95% in China, Vietnam, Cambodia, Thailand, Myanmar, Laos, Indonesia and Afghanistan [37-42]. In Africa, DHA-PQ was used in six large trials conducted between 2003-2009 for treating approximately one thousand patients with uncomplicated malaria in Rwanda, Burkina Faso, Uganda, Cameroon and Sudan [43-47]. The results were in close agreement with those obtained in the Asian studies but with a higher efficacy.

Concerning non-inferiority studies of DHA-PQ compared to other ACTs, DHA-PQ is as effective as artesunate-mefloquine at preventing further parasitaemia over 28 days follow-up in Asia. Both combinations contain partner drugs with very long half-lives and no consistent benefit in preventing new infections has been seen over 63 days follow-up [48, 49]. In Africa, DHA-PQ over 28 days follow-up is superior to artemether-lumefantrine at preventing further parasitaemia. DHA-PQ cures slightly more patients than artemether-lumefantrine, and it prevents further malaria infections for longer after treatment [50].

1.3 Properties of Piperaquine

1.3.1 Physico-chemical

Piperaquine (PQ) or chemically 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, is an antimalarial agent belonging to the 4-aminoquinolines. PQ is highly lipophilic (Log P = 6.2) at neutral and alkaline pH [51]. The molecular weight of PQ (base) is 535.52 g/mol.
Piperaquine is also available as PQ tetraphosphate tetrahydrate (MW=999.55 g/mol). Combination of PQ phosphate with DHA is quite a common practice in ACTs for malaria treatment [52]. PQ is a weak base with four pKa values of 8.6, 8.6, 6.5 and 6.5 [53]. The compound as the free base is poorly soluble in water, methanol and acetonitrile. At lower pH, PQ gets very hydrophilic due to formation of a salt like the phosphates and is easily soluble in polar solvents. In another study by Warhurst et al. (2007), PQ phosphate was reported with another four pKa i.e. 6.9, 6.2, 5.7 and 5.4 [54].

1.3.2 Mechanism of action

The exact mechanism of action of PQ is poorly understood, but it is likely to act in a similar way to chloroquine (CQ), by prevention of haem detoxification within the malaria parasite. CQ binds to heme, preventing the detoxification process of dimerization and crystallization, and producing complexes that are detrimental to both membranes and enzymes of the parasites. These are ultimately lethal to the parasite [55]. Despite structurally similar to CQ, in vitro experiments and clinical studies have shown PQ to be active against highly CQ-resistant *P. falciparum* [56, 57].

1.3.3 Metabolism

The metabolism of PQ could be of great importance in determining the pharmacological activity, clinical efficacy and toxicological profile [58, 59]. *In vitro* study by Lee TM et al. (2012) has suggested that the microsomal isoenzyme CYP3A4 is primarily responsible for the Phase I metabolism of PQ and to lesser extent, CYP2C8 and CYP2D6 [60]. Previously, a study by Tarning et al. (2008) has described five metabolites in human urine namely, carboxylic cleavage metabolite (M1), N-oxidated metabolite (M2), hydroxylated metabolite (M3 and M4) and double N-oxidated metabolite (M5) [58]. Additionally, a new
metabolic pathway via N-dealkylation was found later by Yang A et al (2016) while no phase II metabolites could be detected [61]. The biological effects or contribution of the metabolites has not been studied.

![Figure 3. Two major metabolites of PQ detected in human urine by Tarning et al. (2008). M1 is the metabolite from carboxylic acid cleavage and M2 is mono-N-oxidated metabolite.](image)

1.3.4 Drug interaction

Since CYP3A4 is suggested as the primary metabolizing isoenzyme for PQ, any co-administered drug having known designs of inhibition, induction or competition for CYP3A4 can potentially display pharmacokinetic interaction with PQ, knowing that PQ has long half-life approx. 1 month. The PK interaction may result in toxic effect or loss of antimalarial efficacy depending on plasma concentrations attained [62]. In general, studies regarding interaction of PQ-food intake or PQ-partner drug are well-documented [63-65] but such interaction with multiple medications need further investigations and establishments especially when DHA-PQ is planned for a massive scale deployment in Sub-Saharan African where malaria always coincides with HIV and tuberculosis. A recent study in pregnant Uganda women given the
combination and antiretroviral Efavirenz has led to significant decreases in exposure to both DHA and PQ [66].

1.3.5 Protein binding

Plasma protein binding of drugs might be important when the degree of the binding is higher (≥90%) which may influence PK parameters [67]. A high interindividual PQ PK variability has been reported after oral administration in patients as well as in healthy subjects [68, 69]. Currently, the only information available states that the binding degree for PQ is >99% [70]. Given the high protein binding of PQ, only the unbound fraction is pharmacologically active. The plasma binding of PQ metabolites has not been studied.

1.3.6 Pharmacokinetics

The first pharmacokinetic data of PQ in humans was published from studies in Cambodian children and adults with uncomplicated *P. falciparum* and *P. vivax* malaria treated with Artekin® tablets or granules used in younger children [71]. Using a population pharmacokinetic approach, a two-compartment open model with first-order absorption, with or without a lag time, was suggested to describe the pharmacokinetics. Absorption was slow, with mean absorption half-times (t½,abs) of 9.1 and 9.3 hours in adults and children, respectively. The mean terminal elimination half-life (t½,z) was long in both adults (543 hours) and children (324 hours), while the mean volume of distribution at steady state/bioavailability (Vss/F) was very large in adults (574 L/kg) and children (614 L/kg). Clearance/bioavailability (CL/F) was approximately twice as high in children (1.85 L/h/kg) compared with adults (0.9 L/h/kg).
Since that, numerous PK as well as population PK studies have been carried out to determine PK profile in healthy individuals, patients, children, and pregnant women [72-78]. All studies have an agreement of a long terminal half-life, a large volume of distribution and low clearance for PQ. Children with markedly higher clearance than that of adults have been proposed for some dose adjustment [79] and using high fat diet will also increase absorption of PQ [64, 80]. A recent population PK study in pooled of patients and healthy subjects has described PQ pharmacokinetics by a three-compartment disposition model with flexible absorption whereby the body weight influenced clearance and volume parameters significantly [69].

1.4 Bioanalytical Method

A summary of PQ bioanalysis is tabulated in Table 1 [51, 53, 73, 81-90]. The reliability of data generated by an analytical method is of great importance as measuring the concentration of PQ correctly will facilitate the interpretation of pharmacokinetic findings. The process by which a specific bioanalytical method is developed, validated and used in routine sample analysis can be divided into [91]

i. reference standard preparation
ii. bioanalytical method development and establishment of assay procedure
iii. application of validated bioanalytical method within acceptance criteria for the analytical run

FDA (2001) and EMA are the most widely referred guidelines for the validation of bioanalytical method even though there are a plethora of guidelines available from the different pharmaceutical organizations or countries [92].
Table 1 shows the literatures where bioanalysis of PQ is successfully accomplished in human matrices. Recent method development by H Liu et al. (2017) has included the determination of N-oxide metabolite in rat plasma as well.

<table>
<thead>
<tr>
<th>Author</th>
<th>Sample volume</th>
<th>Extraction procedure</th>
<th>Mobile phase</th>
<th>Detection</th>
<th>LLOQ (ng/mL)</th>
<th>ULOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. Lindergardh et al. (2003)</td>
<td>500 µL whole blood</td>
<td>SPE</td>
<td>Acetonitrile 0.1M ; Phosphate buffer pH 2.5</td>
<td>UV</td>
<td>53.55</td>
<td>1606.5</td>
</tr>
<tr>
<td>T.Y. Hung et al. (2003)</td>
<td>1 mL plasma</td>
<td>LLE</td>
<td>Acetonitrile; Water with 0.025% TFA, 0.1% sodium chloride and 0.008% TEA</td>
<td>UV</td>
<td>5</td>
<td>1000</td>
</tr>
<tr>
<td>N. Lindergardh et al. (2005)</td>
<td>250/1000 µL plasma</td>
<td>SPE</td>
<td>Acetonitrile 0.1M ; Phosphate buffer pH 2.5</td>
<td>UV</td>
<td>10</td>
<td>5000</td>
</tr>
<tr>
<td>N. Lindergardh et al. (2005)</td>
<td>500 µL plasma</td>
<td>SPE</td>
<td>Acetonitrile 0.1M ; Phosphate buffer pH 2.5</td>
<td>UV</td>
<td>13</td>
<td>2678</td>
</tr>
<tr>
<td>C. Liu et al. (2007)</td>
<td>500 µL plasma</td>
<td>LLE</td>
<td>Acetonitrile; 0.1% TCA; Phosphoric acid</td>
<td>UV</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>Singhal P et al. (2007)</td>
<td>50 µL plasma</td>
<td>SPE</td>
<td>Acetonitrile; 2.5 mM Ammonium bicarbonate pH 10</td>
<td>MS/MS</td>
<td>1.5</td>
<td>500</td>
</tr>
<tr>
<td>N. Lindergardh et al. (2008)</td>
<td>50 µL plasma</td>
<td>PPT</td>
<td>Methanol 10 mM; Ammonium acetate, formic acid, ammonia solution</td>
<td>MS/MS</td>
<td>1</td>
<td>250,20</td>
</tr>
<tr>
<td>Authors</td>
<td>Sample Volume</td>
<td>Extraction Method</td>
<td>Solvent/Buffer Details</td>
<td>Detection Method</td>
<td>Sensitivity</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------</td>
<td>-------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>-----------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Malm et al. (2004)</td>
<td>100 µL blood spot</td>
<td>SPE</td>
<td>Acetonitrile 0.1M; Phosphate buffer pH 2.5</td>
<td>UV</td>
<td>1338.7</td>
<td></td>
</tr>
<tr>
<td>Tarning J et al. (2006)</td>
<td>1 mL urine</td>
<td>SPE</td>
<td>Acetonitrile 0.1M; Phosphate buffer pH 2.5</td>
<td>UV</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>Satish G. Pingale (2011)</td>
<td>500 µL plasma</td>
<td>LLE</td>
<td>Methanol with 0.1% acetic acid; 0.1% ammonia in water</td>
<td>MS/MS</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>E.M. Hodel et al. (2009)</td>
<td>200 µL plasma</td>
<td>PPT</td>
<td>20 mM ammonium formate; acetonitrile; 0.5% formic acid</td>
<td>MS/MS</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>Kjellin LL et al. (2014)</td>
<td>25 µl plasma</td>
<td>PPT</td>
<td>20 mM ammonium formate with 0.14% TFA, pH 2.96; 0.1% TFA in MeCN</td>
<td>MS/MS</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>Wahajuddin M et al. (2016)</td>
<td>100 µL plasma</td>
<td>PPT</td>
<td>Acetonitrile; methanol; ammonium formate buffer (10 mM, pH 4.5)</td>
<td>MS/MS</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>H Liu et al. (2017)</td>
<td>40 µL plasma</td>
<td>PPT</td>
<td>Acetonitrile; 2 mm ammonium acetate, 0.15% FA and 0.05% TCA</td>
<td>MS/MS</td>
<td>400</td>
<td></td>
</tr>
</tbody>
</table>
2. Aims

This thesis will outline several objectives

- The simultaneous quantitation of PQ and its metabolites M1 and M2 for the first time in human plasma by developing a new bio-analytical method of LC-MS/MS analysis. (Paper I)

- *In vitro* metabolism study focusing on the potential of PQ and its metabolites to inhibit the main metabolizing enzymes, CYP3A4/5 in human liver microsomes followed by *IVIV* extrapolation using Simcyp simulation application. (Paper II)

- *In vitro* identification of CYPs enzymes involved in PQ metabolism using recombinant enzymes. (incorporated in Paper IV)

- Protein binding of PQ and its metabolites in pooled- and individual- healthy plasma, serum albumin (HSA) and α1-acid glycoprotein by using ultrafiltration method. (Paper III)

- Pharmacokinetics of PQ and its metabolites in Vietnamese healthy subjects after four escalating oral doses separated by one month, using non compartment analysis (Paper IV)
3. Methods

3.1 Bioanalysis (Paper I)

3.1.1 Instrumentation

Schematic diagram of LC-MS/MS system operation
(Illustrated by Daniel Norena-Caro, 2017)

3.1.2 Mass spectrometry detection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declustering potential (DP)</td>
<td>130 V</td>
</tr>
<tr>
<td>Collision energy (CE)</td>
<td>55 V</td>
</tr>
<tr>
<td>Entrance potential (EP)</td>
<td>10 V</td>
</tr>
<tr>
<td>Collision cell exit potential (CXP)</td>
<td>20 V</td>
</tr>
<tr>
<td>Collision gas (CAD)</td>
<td>4 psi</td>
</tr>
<tr>
<td>Curtain gas (CUR)</td>
<td>20 psi</td>
</tr>
<tr>
<td>Ion source gas 1 (GS1)</td>
<td>40 psi</td>
</tr>
<tr>
<td>Ion source gas 2 (GSI)</td>
<td>45 psi</td>
</tr>
<tr>
<td>Ionspray voltage (IS)</td>
<td>5500 V</td>
</tr>
<tr>
<td>Temperature (TEM)</td>
<td>600°C</td>
</tr>
</tbody>
</table>

Optimization of mass spectrometric settings
3.1.3 Chromatographic condition

<table>
<thead>
<tr>
<th>Column</th>
<th>Ascentis® Express C18 column (3 cm x 2.1 mm, 5 µm) with SecurityGuard C18 column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>Mobile phase A = 0.1% formic acid in water</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Mobile phase B = 0.1% formic acid in acetonitrile</td>
</tr>
<tr>
<td>Elution</td>
<td>Gradient ; 0.1% mobile phase B increasing linearly to 100%</td>
</tr>
<tr>
<td>Flow rate</td>
<td>400 µl/min</td>
</tr>
<tr>
<td>Running time</td>
<td>7 minutes</td>
</tr>
<tr>
<td>Injection volume</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

3.1.4 Preparation of standards and QC samples

- Stock solutions – 1 mM PQ tetraphosphate tetrahydrate and metabolite were prepared in water solution (1% formic acid).
- Stock solution – 1 mM M2 was prepared in acetonitrile (1% formic acid).
- Stock solutions were mixed and diluted with water containing 1% formic acid to form two working stock solutions (100 µM & 10 µM).
- Plasma calibration standards – 3.9 to 2508 nM.
- Quality control (QC) samples – 15.6, 750 and 1880 nM.
- Internal standard – Deuterated PQ-d₆ (1 µM).
- Stock solutions, working stocks solutions and plasma standards – stored at -80°C until use.

3.1.5 Sample preparation

- Samples volume – 100 µL from thawed plasma.
- Acidification with 300 µL 1% formic acid-water (internal standard included).
- Centrifuged at 17000g for 15 minutes.
- Supernatants (250 µL) were transferred to polypropylene autosampler vials.
- Thirty microlitres injected onto the LC-MS/MS system.

### 3.1.6 Method validation

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>The lower limit of quantitation (LLOQ) - S/N-ratio was &gt; 5 and the intra and inter-day coefficient of variation below 20%.</td>
<td></td>
</tr>
<tr>
<td>Carry-over effects - blank plasma samples were analysed after injecting the highest calibration standards.</td>
<td></td>
</tr>
<tr>
<td>Intraday accuracy and precision - five replicates of each of the three different quality control (QC) concentrations, as well as five calibration standards at both the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were analyzed on the same day together with one set of calibration standards.</td>
<td></td>
</tr>
<tr>
<td>Interday accuracy and precision – The set of intraday was repeated on two additional days.</td>
<td></td>
</tr>
<tr>
<td>Relative recovery - comparing the peak area of extracted plasma sample to peak areas of extracted blank plasma samples spiked with the analytes.</td>
<td></td>
</tr>
<tr>
<td>Matrix effects (qualitative) - post-column infusion using a ‘T’ connector where the standard solution of analytes in mobile phase was directly infused into the mass spectrometer and blank plasma simultaneously injected onto the LC column.</td>
<td></td>
</tr>
<tr>
<td>Matrix effect (quantitative) - comparing the analytical response for extracted blank plasma sample, postspiked with analytes with the analytical response of standard aqueous solution.</td>
<td></td>
</tr>
</tbody>
</table>
### 3. METHODS

- Freeze and thaw stability - three cycles of freeze and thaw.
- Short-term (24 hour) stability at ambient room temperature.
- Long-term up to 3 months when stored at -80°C.
- Autosampler stability was tested up to 24 hours.
- Stock solutions stability after 6 hours at room temperature and after a week stored at 4°C and -80°C respectively.
3.2 CYP3A inhibition studies (Paper II)

3.2.1 Type and mechanism of inhibition

**Reversible inhibition**

PQ, M1 & M2 at final concentrations of 0.01, 0.1, 0.25, 1, 2.5, 10 and 20 µM were incubated in incubation mixture with substrate midazolam (1.56 µM) for IC$_{50}$ determination.

The incubation mixtures consist of HLM (0.5 mg protein/mL) and Tris–HCl buffer (50 mM Tris, 150 mM KCl and 10 mM MgCl$_2$, pH 7.4).

The incubation mixture as prescribed above was repeated with difference midazolam's concentration at 0.5, 5, 10 and 20 µM for $K_i$ determination.

**Time dependent inhibition**

The incubation mixtures were pre-incubated with 0.01, 0.1, 0.25, 1, 2.5, 10 or 20 µM PQ or M2 for 30 minutes in the presence of NADPH after which midazolam was added. As control, no NADPH was incubated in the pre-incubation step. Time dependent inhibition (TDI) was indicated by IC$_{50}$ shift.

For $K_i$ and $K_{inact}$ determination, the incubation mixtures in HLM containing PQ or M2 and NADPH were pre-incubated at 0, 10 and 30 minutes. Following pre-incubation, 10% of the aliquot was then added to mixture solution composed of midazolam and NADPH and incubated for 10 minutes at 37°C.
3.2.2 Metabolite quantitation.

- Measurement of 1’-hydroxymidazolam (1’-OH-MDZ) metabolite by LC-MS/MS and chlorpropamide was used as internal standard.
- *m/z* transitions used for 1’-OH-MDZ and the internal standard were 342→324 and 277→175, respectively.
- Lower and upper limits of quantification for 1’-OH-MDZ were 1 nM and 500 nM, respectively.
- Retention time of 1’-OH-MDZ and internal standard were 1.6 and 2.3 minutes, respectively.

3.2.3 Data Analyses.

The remaining CYP3A activity was quantified by the formation rate of 1’-hydroxymidazolam relative to the control CYP3A activity. *IC*$_{50}$ values for test compounds were determined by fitting an inhibitory effect *I*$_{max}$ model (Eq. 1) to the percentage of CYP3A remaining activity at different test compound concentrations using WinNonlin.

\[
I(\%) = I_0 - \frac{I_{Max} \cdot C}{IC_{50} + C} \quad \text{(Eq. 1)}
\]

where $I_0$ represents the maximal enzyme conversion rate in the absence of inhibitor, C is the concentration of test compound and $I_{max}$ denotes the maximum inhibition. Data were analyzed by naïve pooled regression analysis of triplicate data.

The reversible inhibition constant, $K_i$, was estimated by fitting different inhibition models (competitive, non-competitive, uncompetitive and mixed inhibition model) to the data using GraphPad Prism. Model selection was based on residual plots, the Akaike information criterion (AIC) and parameter precisions (%CV). TDI was assumed when *IC*$_{50}$ shifts were larger than 1.5-fold during the pre-incubation period, in which
case inactivation parameters $K_1$ and $k_{\text{inact}}$ were estimated as follows. The observed rates of CYP3A inactivation ($k_{\text{obs}}$) at different inhibitor concentrations were determined from the negative slopes of linear regression analysis of the natural logarithm of the remaining activity at 0, 10 and 30 minutes of pre-incubation. The inhibitor concentration at which half of the maximal rate of inactivation occurs ($K_I$) and the maximum rate of enzyme inactivation ($k_{\text{inact}}$) values were calculated by nonlinear regression (WinNonlin) of the relationship

$$k_{\text{obs}} = \frac{k_{\text{inact}} \cdot I}{K_I + I} \quad \text{(Eq. 2)}$$

where I is the initial concentration of the inhibitor.

3.2.4 IVIV extrapolation

The degree of PQ-CYP3A inhibition with midazolam was studied. In vitro-to-in vivo extrapolation (IVIVE) was performed using the Simcyp population-based ADME simulator where

- *In vitro* $K_i$, $K_I$ and $k_{\text{inact}}$ values were incorporated into the model.
- Midazolam model parameters were default Simcyp values.
- PQ pharmacokinetic parameters were set as to mimic the clinically observed multi-phasic plasma concentration-time profile.
- Ten trials were simulated, each with a virtual population of 10 healthy individuals.
- The oral dosage regimens were 960 mg daily intake of PQ phosphate corresponding to 960 mg base for three consecutive days followed by 5 mg oral intake of midazolam on the days 1-14 after treatment initiation.
- A drug-drug interaction (DDI) between midazolam and PQ was indicated when midazolam AUC increased by $\geq 1.25$ fold (FDA, 2012) [93]
3.3 CYP identification

3.3.1 Incubations with recombinant enzymes

- CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP219, CYP2D6, CYP3A4 or CYP3A5 were incubated with PQ.
- Mixtures containing 10 µM PQ and a NADPH regenerating system (2.6 nM NADP+, 6.6 mM glucose-6-phosphate, 6.6 mM MgCl and 4 U/mL glucose-6-phosphate dehydrogenase in 100 mM pH 7.4 phosphate buffer) were pre-incubated at 37°C for 5 minutes in a gently agitating water bath.
- Reactions were initiated by the addition of enzyme to a final concentration of 100 nM in the 500 µL reaction mixture.
- Incubations proceeded for 2 hours and 100 µL aliquots sampled at ≈0, 30, 60 and 120 minutes.
- Reactions were quenched by the addition of 300 µL ice-cold acetonitrile containing 0.1% formic acid and 80 nM PQ-d₆ (IS).
- After centrifugation at 10000g for two minutes, supernatants were transferred to new tubes and stored at -80°C until LC-MS/MS analysis for the presence of M1 and M2.
- Metabolite formation rates were calculated for each time interval. Negative controls consisted of the basal non-transfected cell line at a protein concentration corresponding to that in the active incubations.

3.3.2 Isoform contribution by the relative activity factor (RAF) approach

The RAF was determined for all CYP isoforms that were capable of forming the metabolites, using substrate specific data obtained from the vendor where RAF is the ratio of metabolite formation rate in HLM relative to formation rate in recombinant enzymes. The contribution of each isoform (fᵢ, %) was calculated as
Where $v_i$ is the rate of metabolism for a specific isozyme during the first 30 minutes of incubation.

3.4 Protein binding (Paper III)

3.4.1 Non-specific binding (NSB)

- Mixed stock solution of PQ, M1 and M2 was prepared in aqueous solution.
- Final concentration was 25 nM for each compound.
- Samples in triplicates were incubated for 30 minutes in a gently agitating water bath at 37°C.
- Samples were transferred into filtration device (Centrifree®), centrifuged at 1500 g for 5 minutes.
- Ultrafiltrates were taken out, transferred into vials containing PQ-d₆ as internal standard.
- LC-MS/MS analysis

The percentage (%) of non-specific binding (NSB) was calculated using the equation, adapted from Dow et al [94]. The recoveries of compounds in plasma after ultrafiltration was also determined adapted from Wanga and Williams method [95]. Those equations or methods were described in Paper III.
3.4.2 Unbound fractions in pooled blank human plasma

- Pooled plasma solution containing PQ (10 µM), M1 (1µM) or M2 (1µM) were prepared as triplicates (≤ 5% dilution of compounds).
- Incubation for 30 minutes at 37°C.
- Samples were transferred into filtration device (Centrifree®).
- Centrifugation at 1500 g at 5, 10 and 15 minutes.
- Ultrafiltrates were collected, added with internal standard PQ-d₆.
- Quantitation of compounds by LC-MS/MS analysis.
- Total drug concentrations were determined from the plasma samples after incubation.
- Fraction unbound in plasma was calculated from the unbound concentration relative to total drug concentration [96].
- The ultrafiltrates post analyses were added with trichloroacetic acid (TCA) for determination of protein leakage.

3.4.3 Unbound fractions in individual human plasma

- Blank plasma from individuals were added with ≤ 5% of plasma stock solution to yield the final concentration of PQ (10µM), M1 (1µM) or M2 (1µM).
- Samples in triplicates were incubated and transferred into filtration device (Centrifree®).
- Centrifugation at 500 g for 5, 10 and 15 minutes.
- The ultrafiltrates were collected for the analysis of protein bound as described in 3.4.2.

3.4.4 Apparent binding affinity (K_{aff}) to HSA and AGP

- Human serum albumin (HSA) and α1-acid glycoprotein (AGP) solutions were prepared at concentrations of 602 µM and 23 µM, respectively.
• PQ, M1 and M2 from aqueous standard solutions were spiked into either HSA or AGP solutions to yield final concentration of each compound at 0.25, 0.51, 0.82 or 1.01 µM.
• Samples were incubated in water bath to reach the temperature of 37°C and transferred into filtration device (Centrifree®).
• Centrifugation at 1500 g for 5 minutes.
• Ultrafiltrates were collected for the analysis.
• The affinity constant, \( K_{aff} \) of PQ, M1 and M2 in HSA and AGP were modeled, assuming for a non-saturatable binding (WinNonlin).

\[
C_{tot} = C_u \cdot K_{aff} \cdot [P] + C_u \quad \text{(Eq. 4)}
\]

where \( C_{tot} \) is the determined concentration in the protein solution, and \( C_u \) the concentration in the filtrate, whereas \([P]\) is the nominal protein concentration in the incubated solution.

Given an estimate of \( K_{aff} \) values, the effects of varying AGP concentrations on the unbound fraction of PQ and metabolites were simulated as equation 5. HSA and AGP from individual samples were sent to the Department of Clinical Chemistry at Sahlgrenska University Hospital, Gothenburg for measurement.

\[
f_u = \frac{1}{1 + [\text{HSA}] \cdot K_{aff,\text{HSA}} + [\text{AGP}] \cdot K_{aff,\text{AGP}}} \quad \text{(Eq. 5)}
\]
3.5 Pharmacokinetics (Paper IV)

3.5.1 Study Design

The trial was a single-center, randomized and single dose-escalation Phase I study in healthy male Vietnamese subjects and was conducted in accordance with Good Clinical Practice procedures and the principles of the Helsinki Declaration. The study was performed at National Institute of Malariology, Parasitology and Entomology (NIMPE) Hanoi under the approval of Ministry of Health, Vietnam. Thirteen healthy male subjects received a single tablet of 320 mg PQ phosphate + 40 mg DHA (Artekin®) in the morning. Additional doses of 2, 3 and 4 tablets were administered in escalating sequence one month apart. Samples were obtained at time zero (pre-dosing) 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 hours, days 3, 5, 7, 14, 21 and 28. Prolonged dose samplings after the last dose were at days 49, 63, 91 and 150. The quantitation of PQ and metabolite M1 or M2 in the clinical samples were prepared as described in 3.1.5. (pg.15-16)

3.5.2 Protein binding analysis

- In anticipation of a very high binding degree making quantitation of unbound, ultrafiltrate concentrations unfeasible at clinical concentrations, PQ and metabolites were externally added.
- The stock solution of PQ and M2 (1 mM in aqueous) was diluted (≤ 1%) in each individual plasma (500 µL).
- Samples were transferred into filtration device (Centrifree®) and centrifuged at 1500 g for 5 minutes.
- The ultrafiltrates were collected and transferred into HPLC vials containing internal standard PQ-d6.
- Quantitative analysis was performed using the LC-MS/MS.
3.5.3 PK and statistical analyses

Non-compartmental analysis (NCA) analysis was carried out to estimate the PK properties of PQ and metabolites using WinNonlin® v6.1. The maximum observed drug concentration ($C_{\text{max}}$) and the corresponding time to reach the maximum concentration ($t_{\text{max}}$) were obtained directly from the concentration-time data. The apparent terminal phase elimination rate constant ($\lambda_z$) was determined by least squares regression analysis using at least three of the last concentration data points, with the terminal half-life ($t_{1/2,z}$) calculated as $\ln(2)/\lambda_z$. AUCs were calculated by the log-linear trapezoidal method. Area extrapolation beyond the last measurable sample time-point was by $C_{\text{pred, last}}/\lambda_z$.

Results are presented as geometric means with 95% confidence intervals (CIs) calculated based on the natural logarithmic distribution of variables and t-distributions. Dose- and time-dependent pharmacokinetics were investigated by repeated measures ANOVA of dose-normalized, natural logarithm–transformed exposure parameters ($\text{AUC}_{0-t}$ and $\text{AUC}_{0-\infty}$) (MS Excel 2010, SPSS version 19). $p$-values <0.05 were deemed to indicate statistical significance.
4. Results

4.1 Bioanalysis- LC-MS/MS (Paper I)

4.1.1 Optimization

PQ, M1 and M2 peak separations were successfully accomplished using an Ascentis® Express C18 column with the following precursor-product ion pairs; m/z 535/288 (PQ), 320/205 (M1), 551/258 (M2) and 541/294 (PQ-d6). The chromatogram in Fig. 4 shows the separation of the analytes within a run time of 7 min.

![Chromatogram](image)

*Fig. 4. Chromatographic peaks of piperaquine (PQ), metabolites M1, M2 at 400 nM, respectively and deuterated internal standard (PQ-d6).*
4.1.2 Sample preparation

Acidification with 1% formic acid in water was selected as a work up method for plasma samples. Even though this resulted in diluting the samples, the method allowed measurement of clinical concentrations of PQ with a LLOQ comparable with previous assays [72, 97, 98].

4.1.3 Validation

Validation based on FDA guidelines as described in 3.16 (pg.16) was completed successfully. The LLOQ was set to 3.91 nM for PQ, M1 and M2 in plasma, providing adequate accuracy and precision with signal-to-noise ratio >5 (Table 2). All compounds were stable under validation experiments. Recoveries were ≈80% and matrix effects were insignificant.
Table 2. Intra- and inter-day accuracy and precision for the analytes in human plasma (mean ± SD)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Analyte nominal concentration (nM)</th>
<th>Intra-day (n=5)</th>
<th>Inter-day (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed concentration (nM)</td>
<td>Accuracy</td>
<td>% CV</td>
</tr>
<tr>
<td>PQ</td>
<td>3.91</td>
<td>4.72 ± 0.32</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>17.9 ± 1.6</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>715 ± 17</td>
<td>95.3</td>
</tr>
<tr>
<td></td>
<td>1880</td>
<td>1892 ± 15</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>2508</td>
<td>2372 ± 20</td>
<td>94.6</td>
</tr>
<tr>
<td>M1</td>
<td>3.91</td>
<td>3.88 ± 0.36</td>
<td>99.2</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>17.8 ± 1.4</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>784 ± 15</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1880</td>
<td>1800 ± 24</td>
<td>95.7</td>
</tr>
<tr>
<td></td>
<td>2508</td>
<td>2468 ± 25</td>
<td>98.4</td>
</tr>
<tr>
<td>M2</td>
<td>3.91</td>
<td>4.32 ± 0.32</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>14.8 ± 0.8</td>
<td>94.9</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>705 ± 16</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>1880</td>
<td>1880 ± 28</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2508</td>
<td>2464 ± 40</td>
<td>98.2</td>
</tr>
</tbody>
</table>

4.2 CYP3A inhibition – IVIVE (paper II)

4.2.1 Reversible inhibition

Estimated IC₅₀ values for PQ and its M2 metabolite were 0.76 and 0.043 μM, respectively. Metabolites M1 and M5 showed no appreciable inhibitory effect. Of the different inhibition models tested, goodness-of-fit indicated a competitive model as appropriate for PQ and mixed inhibition model for M2 with estimated Ki values of 0.68 μM and
0.057 μM respectively. The inhibited formation of 1´-hydroxymidazolam by PQ and M2 is shown in Figure 5.

![Graph](image1.png)

**Fig. 5.** Inhibition of 1’hydroxymidazolam formation by PQ and M2 in pooled human liver microsomes. Lines represent the fit of an inhibitory $I_{\text{max}}$ model to triplicate data.

### 4.2.2 Time-dependent inhibition

The $IC_{50}$ of PQ was about 50% lower when pre-incubated 30 minutes with NADPH, compared with in the absence of NADPH, with values estimated to be 0.32 and 0.76 μM, respectively. The shift was 2.4-fold indicating PQ to be a time dependent inhibitor. No $IC_{50}$ shift was observed for M2 with $IC_{50}$ values almost identical at 0.043 and 0.041 μM. PQ $k_{\text{inact}}$ and $K_1$ values were estimated to be 0.024 min$^{-1}$ and 1.63 μM, respectively.
4.2.3 IVIV extrapolation

The simulated average *in vivo* increase in midazolam AUCs was approx. 2-fold (average) from the first day until the third and last day of PQ treatment (Figure 6). The predicted average increases in midazolam AUCs became less than 1.25 fold on day 4 whereas the upper, 95% percentile, prediction limit decreased below this value on day 5.

Fig. 6. Simulation of AUC ratio of midazolam in healthy subjects given 3 days course of antimalarial PQ treatment. The shaded area represents 95th and 5th quartile. Inset; simulated profile of PQ.

Simulations were carried out assuming either reversible inhibition or TDI, or a simultaneous combination of both. Inclusion of only a TDI mechanism resulted in slightly but not significant changes to midazolam AUCs.
4.3 CYPs identification

4.3.1 CYPs activity in recombinant enzymes

Metabolite M2 was formed in the incubations containing recombinant CYP1A2, CYP2C8, CYP2D6, CYP3A4 and CYP3A5 (Table 3). CYP3A4 showed the highest rate of M2 formation, followed by CYP3A5, CYP2D6 and CYP2C8 in descending order. The concentrations of M2 in other isoenzymes were close to zero. Metabolite M1 could not be detected in any of the samples.

Table 3. Formation rates of M2 after incubation of PQ with recombinant enzymes.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Time (min)</th>
<th>Formation rate (pmol M2/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>30</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>-3.57</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>-0.537</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>30</td>
<td>6.34</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.77</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1.62</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>30</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>15.1</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>30</td>
<td>747</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>365</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>287</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>30</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>54.3</td>
</tr>
</tbody>
</table>
4.3.2 Relative contribution of CYPs – RAF

Using relative activity factor (RAF), CYP3A4/5 was estimated to be responsible for 98.9% of the total M2 formation (Table 4).

Table 4. Calculated values of the relative activity factor and relative contribution for the isozymes involved in M2 metabolism.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>RAF (mg/pmol CYP)</th>
<th>Relative contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C8</td>
<td>21.7</td>
<td>0.7</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>1.44</td>
<td>0.4</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>21.5</td>
<td>98.9</td>
</tr>
</tbody>
</table>

4.4 Protein binding (Paper III)

The non-specific binding (NSB) of PQ, M1 and M2 were found low where calculated NSB for all the analytes were ≤ 6%. Recoveries of compounds in plasma were high, ranged 98.2-102 %. Precipitation of protein was not observed when TCA was added to ultrafiltrates post analysis. Filtered volumes of pooled and individual plasma from different filtration duration were ranged 8.9-31%.

4.4.1 Unbound fractions in human plasma

Results from in vitro assay for unbound fractions (fu) of PQ, M1 and M2 in pooled plasma and individual plasma are given in Table 5.
4.4.2 Binding affinities to HSA and AGP

PQ, M1 and M2 exhibited high binding to HSA and AGP in protein solutions. PQ had a high apparent affinity ($K_{\text{aff}}$) for AGP compared to HSA (8-folds) yet due to the relative abundance of albumin in human plasma. From incubations with pure HSA or AGP, affinity constants for PQ of 0.221 µM$^{-1}$ and 1.67 µM$^{-1}$, respectively, were estimated. M1 and M2 also showed less affinity towards HSA (0.00124 µM$^{-1}$; 0.0271 µM$^{-1}$) than AGP (0.0312 µM$^{-1}$; 0.127 µM$^{-1}$).

4.4.3 Predictive effects of AGP on protein binding

The unbound fractions PQ, M1 and M2 decreased when AGP concentrations were elevated to 2-4 folds whereby HSA concentrations were kept constant at 600 µM.

Clinical HSA concentrations were measured 690 and 630 µM for individual 1 and individual 2, while AGP concentrations were 26.4 and 24 µM, respectively.
4.5 Pharmacokinetics (Paper IV)

4.5.1 Non-compartmental analysis (NCA)

The pharmacokinetic parameters of PQ showed multi-phasic kinetics with a very long terminal half-life, a large volume of distribution (V/F) and intermediate oral clearance (CL/F) (Table 6). In terms of total systemic exposure, M2 total plasma AUCs was about 50% of the parent compound. Many M1 concentrations were found below LLOQ.
Table 6. Pharmacokinetic parameter values from non-compartmental analysis of piperaquine (PQ), carboxylic acid cleavage product metabolite (M1) and mono-N-oxide metabolite (M2) and in twelve Vietnamese healthy male volunteers after receiving a single oral administration of Artekin® in an ascending dose manner one month apart.

<table>
<thead>
<tr>
<th>Parameters / Dose (PQ phosphate)</th>
<th>320 mg</th>
<th>640 mg</th>
<th>960 mg</th>
<th>1280 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PQ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(_{0-24}) (h•µM)</td>
<td>1.49 (1.20 ; 1.88)</td>
<td>4.35 (2.88 ; 6.59)</td>
<td>7.19 (5.14 ; 10.0)</td>
<td>10.3 (8.64 ; 12.4)</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (h•µM)</td>
<td>14.4 (8.65 ; 23.9)</td>
<td>19.5 (11.6 ; 32.6)</td>
<td>33.0 (20.5 ; 53.1)</td>
<td>52.3 (24.2 ; 64.6)</td>
</tr>
<tr>
<td>% extrapolation to ∞</td>
<td>30.6 (29.1 ; 32.1)</td>
<td>11.6 (10.4 ; 12.7)</td>
<td>15.6 (14.3 ; 16.8)</td>
<td>12.7 (11.6 ; 13.8)</td>
</tr>
<tr>
<td>C(_{\text{max}}) (nM)</td>
<td>141 (98.9 ; 183)</td>
<td>512 (354 ; 670)</td>
<td>960 (686 ; 1233)</td>
<td>1025 (771 ; 1280)</td>
</tr>
<tr>
<td>T(_{1/2,z}) (hr)</td>
<td>22.2 (19.7 ; 24.8)</td>
<td>32.9 (28.1 ; 37.7)</td>
<td>33.9 (30.6 ; 37.1)</td>
<td>24.5 (23.6 ; 25.3)</td>
</tr>
<tr>
<td>T(_{\text{max}}) (hr)(^a)</td>
<td>2.0 (2 - 4)</td>
<td>2.5 (1 - 5)</td>
<td>3.0 (2 - 6)</td>
<td>3.0 (1 - 8)</td>
</tr>
<tr>
<td>V(_{z}/F) (L)</td>
<td>6396 (3053 ; 9739)</td>
<td>5129 (439 ; 5867)</td>
<td>4655 (4399 ; 4911)</td>
<td>5860 (4717 ; 7003)</td>
</tr>
<tr>
<td>V(_{\text{ss}}/F) (L)</td>
<td>28208 (26187 ; 30230)</td>
<td>18161 (16592 ; 19731)</td>
<td>20629 (19125 ; 22134)</td>
<td>15396 (14943 ; 15849)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>M1</strong></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_{0-24}) (h•µM)(^b)</td>
<td>0.382 (243 - 561)</td>
<td>0.638 (128 - 1312)</td>
<td>0.494 (94.6 - 1473)</td>
<td>0.632 (153 - 3444)</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (h•µM)(^b)</td>
<td>0.441 (255 - 609)</td>
<td>1.25 (162 - 3027)</td>
<td>0.954 (175 - 2826)</td>
<td>1.09 (220 - 5028)</td>
</tr>
<tr>
<td>% extrapolation to ∞(^b)</td>
<td>23.8 (4.61 - 89.84)</td>
<td>38.8 (19.7 - 56.7)</td>
<td>45.5 (33.9 - 67.8)</td>
<td>34.2 (24.2 - 72.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>M2</strong></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_{0-24}) (h•µM)(^d)</td>
<td>0.833 (0.493 ; 1.41)</td>
<td>0.978 (0.650 ; 1.48)</td>
<td>1.69 (1.01 ; 2.83)</td>
<td>1.55 (1.09 ; 2.19)</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (h•µM)(^d)</td>
<td>4.28 (1.18 ; 15.5)</td>
<td>5.08 (1.94 ; 13.3)</td>
<td>11.4 (3.81 ; 34.0)</td>
<td>10.3 (5.49 ; 19.5)</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (h•µM)(^d)</td>
<td>7.12 (2.16 ; 23.5)</td>
<td>8.69 (3.29 ; 22.9)</td>
<td>22.7 (7.23 ; 71.5)</td>
<td>18.4 (8.75 ; 38.5)</td>
</tr>
<tr>
<td>% extrapolation to ∞(^d)</td>
<td>35.5 (32.9 ; 38.0)</td>
<td>34.3 (32.0 ; 36.8)</td>
<td>33.4 (27.4 ; 39.4)</td>
<td>31.5 (28.8 ; 34.2)</td>
</tr>
<tr>
<td>C(_{\text{max}}) (nM)</td>
<td>74.2 (25.7 ; 123)</td>
<td>70.0 (32.1 ; 108)</td>
<td>124 (63.4 ; 184)</td>
<td>124 (81.3 ; 167)</td>
</tr>
<tr>
<td>T(_{1/2,z}) (hr)</td>
<td>324 (147 ; 367)</td>
<td>335 (174 ; 670)</td>
<td>809 (224 ; 1056)</td>
<td>493 (277 ; 832)</td>
</tr>
<tr>
<td>T(_{\text{max}}) (hr)(^e)</td>
<td>3.0 (3 - 7)</td>
<td>4.0 (3 - 7)</td>
<td>5.0 (3 - 10)</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Data are geometric mean (95% confident interval from ln-transformed distribution) for pharmacokinetic data unless otherwise stated. \(^a\) Median (minimum - maximum). \(^b\) Geometric mean (range). \(^c\) 3≤N<9, \(^d\) 5≤N<11 Abbreviations: AUC\(_{0-24}\), area under the plasma concentration-time curve from time zero to 24 hours; AUC\(_{0-\infty}\), area under the plasma concentration-time curve from time zero to last measurable point; C\(_{\text{max}}\), maximum observed plasma concentration; CL/F, oral total plasma clearance; T\(_{1/2,z}\), terminal half-life; T\(_{\text{max}}\), time to C\(_{\text{max}}\); V\(_{z}/F\), oral volume of distribution V\(_{\text{ss}}/F\), oral volume of distribution at steady-state.
Both PQ and M2 were highly protein bound with unbound fractions (geometric means) of 0.362% for PQ and 2.49% for M2. The percentage of filtered volumes by ultrafiltration was ranged 9.3-11.8%. Average ratios of $\text{AUC}_{u,\text{M2}}/\text{AUC}_{u,\text{PQ}}$ were 3-fold and was not increased- or decreased with dose.

Based on total plasma concentrations and unbound concentrations of either PQ or M2, there was no evidence of either a dose- or time dependency with respect to piperaquine. Dose-adjusted AUCs were not statistically different between doses (Fig. 7).

![Figure 7: Box plots summarising comparisons between dose-normalized, ln-transformed total and unbound AUC$_{0-\infty}$ of PQ and M2. Circles represent outliers.](image)
5. General Discussion

A major setback for antimalarial treatment nowadays is the presence of parasite resistance particularly *P. falciparum* which has increased the treatment failure among patients. Pertaining to the issue, even the combination of DHA-PQ has been found ineffective in some studies in Southeast Asia regions such as Cambodia and Thailand. Despite the emergence of locally antimalarial resistance for DHA-PQ, the combination is still highly effective with high cure rates in Sub-Saharan Africa where there is a growing interest in DHA-PQ for potential use in mass-drug-administration (MDA) campaigns supporting national elimination of malaria. In parallel, the research community shares the same idea to consider DHA-PQ as a possible alternative for intermittent preventive treatment in pregnancy. Some countries are also working with clinical trials to collect evidence that will be critical for informing future policy making DHA-PQ as recommended first-line treatments (e.g. India). Because of that, the DHA-PQ combination is still highly relevant as the first-line treatment option for malaria.

PQ by its own is viewed as an ineffective drug for monotherapy but is thought to be useful when combining with other antimalarial (e.g. DHA). However, the disposition and kinetics of PQ are not yet fully established, leading to some queries of inter-individuality kinetics, drug interactions and possible contribution of the formed metabolites. Therefore, the works of this thesis are focused to bring new knowledge or findings that might be essential to contribute, establish or verify the efficacy and safety issues of PQ. Given the PQ properties with long half-life approximately 1 month, it has become such an interest to study the remaining PQ and its metabolites as they are expected to remain longer in the body even after the treatment ends.

Two major PQ metabolites, namely M1 and M2 that were previously found in urine and human plasma were investigated meticulously and
quantitatively about their presence in human plasma and the effects of CYPs inhibitory potential. The insight of these studies is enlightened in Paper I and Paper II. For the first time, the chromatographic separation via LC-MS/MS was developed to quantitate the metabolites M1 and M2 simultaneously with its parent compound, PQ in human plasma (Paper I). The method was successfully validated based on FDA guidelines to confirm its reliability and reproducibility. The concern of this bioanalytical method was the sample preparation. In our bioanalysis, we were using precipitation of plasma proteins with acidified water. During this workup procedure, the analytes were diluted affecting the sensitivity of the LC-MS/MS method. For that prospect, modification of this sample preparation may improve the sensitivity to enable measuring the compounds at very low concentration than our current LLOQ (3.9 nM). The carry-over and matrix effects were another obstacles and should be carefully paid attention during the analysis. Even though, the carry over- and matrix effects were solved in the present method, one should be cautious to understand that the settings of instrument, bench work experiment and choice of solvents will probably have some effects by taking the fact that, PQ and metabolites are unstable when in contact with plastic or metal surface. After all, the LC-MS/MS method developed in this study was suitably adequate to quantitate PQ and metabolite M1 and M2 in human plasma samples from the clinical trial.

The role of CYP3A is undeniably essential and very significant in most of the drug metabolism including antimalarial PQ. From our observation, the presence of metabolite M2 was confirmed in vitro by CYPs identification experiment with the major involvement of CYP3A4/5. Relatively, almost 99% of M2 formation was associated with CYP3A4/5 activity and the remaining formation of M2 was contributed by CY2C8 and 2D6. Metabolite M1 however was not found in incubation with CYPs isoenzyme, both in recombinant enzymes and in human liver microsomes. By considering the major contribution of CYP3A towards PQ metabolism, further investigation was carried out to determine the behaviour of PQ and metabolites for CYPs activity (Paper II). Apparently, PQ and M2 were found to reversibly inhibit CYP3A activity in human liver microsomes with potent values of $IC_{50}$ and $K_i$. PQ also
exhibited time-dependent inhibition (TDI) but it could not be established whether this was due to mechanism-based inhibition or the formation the potent inhibitor M2 during microsomal pre-incubation. *In silico* approach of *IVIV* extrapolation has predicted the interaction with midazolam during 3 days PQ treatment of malaria in healthy subjects given clinical dose. When the probability of DDI exists, this should be a focus area when treating malarial patient with multiple medication. However, our findings are limited in that context of *in vitro* to *in silico* approaches. The clinical trials are warranted to confirm *in vivo* DDI in individuals.

Protein binding of PQ has been reported to be >99% by EMA SPC. Unfortunately, the degree of PQ plasma protein binding is not fully studied albeit several decades of clinical usage. Since PQ is assumed to be highly bound to proteins, any changes to the binding may cause some effects on disposition and kinetics of PQ. Therefore, the degree of PQ protein binding and its metabolites by using ultrafiltration method was investigated (Paper III). Fraction unbounds were determined from filtered volumes of filtration. PQ, M1 and M2 were found highly bound to plasma protein in pooled blank plasma and fresh plasma in two healthy volunteers (87-99% bound). This finding is in agreement with EMA SPC for PQ and for the first time the determination of metabolite’s protein binding. PQ and metabolites have demonstrated high apparent affinity towards two main constituents of plasma protein, HSA and AGP where the binding to AGP is more prominent in term of $K_{aff}$ value. Thus, considering that the AGP concentration will be dramatically increased during malaria infection, the effect of protein binding when antimalarial PQ given for the treatment, should be monitored carefully which may or may not affecting PK parameters. It was predicted as well as the other protein such as lipoprotein might involve with PQ and metabolite’s binding. Within this experiment, compounds were spiked into plasma, make these *in vitro* results could be real or exaggerated when translating into *in vivo* interpretation.

The pharmacokinetics (PK) of PQ and metabolites are another aspect of this thesis where samples from clinical trials conducted in healthy Vietnamese subjects were analysed (Paper IV). The PK parameters
derived from non-compartmental analysis (NCA) demonstrated linear pharmacokinetic of PQ with respect to dose escalation in ascending order (320-1280 mg). Nevertheless, PQ was found to be dose- and time-independent kinetics whereby the distribution and elimination of PQ were not affected by increasing the dose. PQ exhibited a long half-life, a large volume of distribution and intermediate oral clearance as expected from the previous PK studies. Metabolite M1 that appears to be principal product in urine, was hardly detected in clinical plasma samples. On the other hand, total plasma concentration of M2 was found 50% of parent PQ, suggesting M2 to be the circulating metabolite in human beings with similar half-life to its parent compound. As unbound fractions of clinical samples were also determined, ratio of unbound AUC of M2 relative to unbound AUC PQ was approx. 3 fold. This indicates significance whereby clinical exposure to M2 could exert significant potency considering its ability to inhibit CYP3A4.
6. Conclusion

**PQ** - Overall, the thesis presents the new knowledge of antimalarial PQ regarding the reversible- or time dependent inhibitory potential and predictive effect towards CYP3A, one of the important metabolizing enzymes. By using *IVIVE* approach, PQ has shown positive DDI potential when midazolam was used simultaneously during PQ treatment. It was also demonstrated that PQ was highly bound to protein, with the unbound fractions ≤1% where the binding to AGP was greater than albumin which may or may not associated with reported PK PQ variability. Pharmacokinetic parameters of PQ are in agreement with previous studies in healthy subject and PQ appears to be dose- and time independent kinetics.

**M2** - Bioanalytical assay for PQ and two of its metabolites was successfully developed enabling simultaneous quantitation of these compounds from clinical samples. This has opened up for the possibility to embark on quantitative studies of the metabolites for the first time. Two major metabolites were highlighted as our interest in this thesis, but only one metabolite (mono-N-oxide PQ @ M2), had an interesting outlook. Metabolite M2 was characterized as a reversible potent CYP3A inhibitor *in vitro*, greater than PQ. Given the similar plasma half-life of M2 to PQ, it exhibited formation-rate limited kinetics. Exposure of unbound M2 also was found greater than the parent compound, indicating its significance. **M1** - Metabolite (M1) however, had shown no CYP3A inhibitory potential and hardly found in plasma clinical samples. Most often, M1 was below LLOQ. **M5** - Metabolite M5 or double-N-oxidated PQ, suggested as the secondary metabolite was only studied for CYP3A inhibition and showed no potential of inhibition.
7. Future Perspective

Despite the emergence of *plasmodium* resistance, combination of DHA-PQ is still relevant and highly recommended for malaria treatment especially in Africa regions where high efficacy of DHA-PQ is recorded. Argumentatively, the studies on PQ should not be understated in terms of its efficacy or toxicity issues.

In this thesis, PQ has been described to show a predictive positive DDI with midazolam during the treatment. This needs to be confirmed with clinical studies. Besides, the interaction with other medications should also be carefully investigated such as antiretroviral or tuberculosis agents. Nevertheless, we have not demonstrated the inhibitory effects of metabolite M2 which is more potent than PQ *in vitro*. In order to simulate the inhibitory effect of M2, more information regarding physicochemical properties and PK of M2 are warranted. From another perspective, PQ is also associated with QT prolongation (leading to arrhythmias) but the effect of metabolites on QT prolongation is unknown. Therefore, the developed bioanalytical method is hopefully could be feasible enough to study the contribution of PQ metabolites in this area.

PQ or metabolites have never been reported as unbound concentrations previously. As unbound concentration become subject of interest in the future, the thesis has provided an effective and workable *ex vivo* experimental procedure to measure unbound concentration of these compounds. A modeling approach of population PK is possibly done and recommended to obtain and predict more kinetics information in Vietnamese population in order to improve malaria treatment with DHA-PQ combination.
Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor, Prof. Michael Ashton for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I have the same feeling for Dr. Kurt Jürgen Hoffman, who has been with me on this journey, through thick and thin and without his support, I cannot imagine myself to reach at this final lap.

My sincere thanks also goes to Dr. Angela Arbelo, Dr. Sofea Bigersson, and my fellow labmate, Jesper Sundell, who continuously gave me a lot of support during my lab days. Without their precious support, it would not be possible to conduct my research efficiently. Not to be forgotten, my previous colleagues who had graduated before me, Dr.- Carl, Emile, Richard and Therese, thank you for welcoming me with great smile from the first time I stepped my foot at this lab few years ago. Many thanks also to Dr. Elin Karlson, who had significant contribution for my admission to university and continuous support. Not to be forgotten, Master students in the lab who have significant contribution toward this thesis, Emma and Marija as well as the practical students, Winnie and Rocco.

I have my big appreciation and special thanks to Ministry of Higher Education Malaysia and Universiti Sains Malaysia for the financial support throughout my studies here in Sweden.

Last but not the least, I would like to thank my family: my parents and to my brothers and sister and my friends (in Malaysia and Sweden) for supporting me spiritually and sharing many ideas throughout my whole journey. I am unable to jot down all the names here, but you know who you are. Wink ! #SayangKetatSendatPadatPadu. Thank you very much for this unconditionally love and continuous encouragement. Without all these people, this book, this thesis means absolutely nothing.

After all, this is just a beginning …. 
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