The effect of Pheroid™ technology on the bioavailability of quinoline-based anti-malarial compounds in primates

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Had I the heavens embroidered cloths,
Enwrought with golden and silver light,
The blue and the dim and the dark cloths
Of night and light and the half-light,
I would spread the cloths under your feet:
But I, being poor, have only my dreams;
I have spread my dreams under your feet;
Tread softly because you tread on my dreams.

William Butler Yeats

Dedicated to my
family,
Frik, Joey and Eugene Gibhard

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~ Rom 5:3-5 ~

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ABSTRACT

Resistance against anti-malarial drugs remains one of the greatest obstacles to the effective control of malaria. The current first-line treatment regimen for uncomplicated *P.falciparum* malaria is based on artemisinin combination therapies (ACTs). However, reports of an increase in tolerance of the malaria parasite to artemisinins used in ACTs have alarmed the malaria community. The spread of artemisinin-resistant parasites would impact negatively on malaria control.

Chloroquine and amodiaquine are 4-aminoquinolines. Chloroquine and amodiaquine were evaluated in a primate model by comparing the bioavailability of these compounds in a reference formulation and also in a Pheroid® formulation. *In vivo* pharmacokinetic studies were conducted for chloroquine, and *in vitro* and *in vivo* drug metabolism and pharmacokinetic (DMPK) studies were conducted for amodiaquine. Pheroid® technology forms the basis of a colloidal drug delivery system, and it is the potential application of this technology in combination with the 4-aminoquinolines that was the focus of this thesis. Pheroid® is a registered trademark but for ease of reading will be referred to as pheroid(s) or pro-pheroid(s) throughout the rest of the thesis.

The non-human primate model used for evaluation of the pharmacokinetic parameters was the vervet monkey (*Chlorocebus aethiops*). Chloroquine was administered orally at 20 mg/kg. A sensitive and selective LC-MS/MS method was developed to analyze the concentration of chloroquine in both whole blood and plasma samples. The C_{max} obtained for whole blood was 1039 ± 251.04 ng/mL for the unformulated reference sample of chloroquine and 1753.6 ± 382.8 ng/mL for the pheroid formulation. The $AUC_{0\text{-}inf}$ was 37365 ± 6383 ng.h/mL (reference) and 52047 ± 11210 ng.h/mL (pheroid). The results indicate that the use of pheroid technology enhances the absorption of chloroquine. The effect of pheroid technology on the bioavailability of amodiaquine and *N*-desethylamodiaquine was determined in two groups of vervet monkeys, with the reference group receiving capsules containing the hydrochloride salt of amodiaquine and the test group receiving capsules containing a pro-pheroid formulation of amodiaquine. Amodiaquine was administered at 60 mg/kg. Blood concentrations of amodiaquine and *N*-desethylamodiaquine samples were monitored over 13 time points from 0.5 to 168 hours. Amodiaquine and pro-pheroid formulated amodiaquine were incubated *in vitro* with human and monkey liver (HLM and

MLM) and intestinal (HIM and MIM) microsomes and recombinant cytochrome P450 enzymes. The *in vitro* metabolism studies confirm the rapid metabolism of amodiaquine to the main metabolite *N*-desethylamodiaquine in monkeys. Although the pharmacokinetic parameters varied greatly, parameters for both the parent compound and main metabolite were lower in the test formulation compared to the reference formulation. For HLM, MLM and CYP2C8, the pro-pheroid test formulation showed significantly longer amodiaquine clearance and slower formation of *N*-desethylamodiaquine. However, the effect was reversed in MIM.

Pheroid technology impacts differently on the bioavailability of the various pharmaceutical classes of anti-malarials. Pheroid technology did not enhance the bioavailability of amodiaquine or *N*-desethylamodiaquine. This is contrary to the observed effects of pheroid technology on the pharmacokinetics of other drugs such as artemisone and chloroquine where it increases the area under the curve and prolongs the drug half-life.

Keywords: Malaria, Chloroquine, Amodiaquine, Pheroid® technology, *In vivo* pharmacokinetic analysis, *In vitro* metabolism, Non-human primates.

UITTREKSEL

Die ontwikkeling van weerstandbiedendheid teen anti-malaria geneesmiddels bly een van die grootste struikelblokke in die strewe na die effektiewe beheer van malaria. Artemisinien gebasseerde kombinasieterapie word tans aanbeveel as die eerste linie van behandeling van ongekompliseerde *P.falciparum* malaria. Onlangse verslae van 'n toename in die toleransie van malaria parasiete teen artemisinien gebasseerde kombinasieterapie wek kommer in die malaria gemeenskap. Die verspreiding van artemisinien-weerstandige parasiete sal die effektiewe beheer van malaria drasties bemoeilik.

Chlorokien en amodiakien word geklassifiseer as 4-aminokinoloonverbindings. Die biobeskikbaarheid van chlorokien en amodiakien was getoets in 'n primaat model deur 'n verwysings formulering met 'n Pheroid® formulering te vergelyk. *In vivo* farmakokinetiese studies was uitgevoer vir chlorokien, en *in vitro* en *in vivo* metaboliese en farmakokinetiese studies was uitgevoer vir amodiakien. Pheroid® tegnologie vorm die basis van 'n kolloïdale geneesmiddel aflewerings sisteem, en die moontlike toepassing van hierdie tegnologie in kombinasie met die 4-aminokinoloonverbindings was die fokus van hierdie tesis. Pheroid® tegnologie is 'n geregistreerde handelsmerk, maar vir gemak van lees sal verwys word na pheroid(s) of pro-pheroid(s) in die res van die tesis.

Die primaat model wat gebruik was vir die evaluering van die farmakokinetiese parameters was die Blouaap (*Chlorocebus aethiops*). Chlorokien was oraal toegedien teen 20 mg/kg. 'n Sensitiewe en selektiewe LC-MS/MS metode is ontwikkel om die konsentrasie van chlorokien in heelbloed en plasmamonsters te analiseer. Die heelbloed het 'n C_{maks} opgelewer van 1039 ± 251.04 ng/mL in die ongeformuleerde verwysing terwyl die pheroid formulering 'n C_{maks} van 1753.6 ± 382.8 ng/mL opgelewer het. Die $AUC_{0\text{-}inf}$ was 37365 ± 6383 ng.h/mL (verwysing) en 52047 ± 11210 ng.h/mL (pheroid). Die resultate dui daarop dat die gebruik van pheroid tegnologie die absorpsie van chlorokien vehoog. Die effek van pheroid tegnologie op die biobeskikbaarheid van amodiakien en *N*-desetielamodiakien is in twee groepe blouape bepaal, die verwysingsgroep het kapsules ontvang wat die hidrochloriedsout van amodiakien bevat het en die toets groep het kapsules ontvang wat 'n pro-pheroid formulering van amodiakien bevat het. Amodiakien is teen 60 mg/kg liggaamsmassa toegedien. Bloedkonsentrasies van amodiakien en *N*-desetielamodiakien is oor 13 tydsintervalle van 0,5 tot 168 uur gemonitor. Amodiakien en pro-pheroid geformuleerde amodiakien is *in vitro* met mens- en primaat lewer en intestinale mikrosome

en rekombinante sitochroom P450-ensieme geïnkubeer. Die *in vitro* metabloiese studies bevestig die vinnige metabolisme van amodiakien na *N*-desetielamodiakien in die primate. Alhoewel die farmakokinetiese parameters baie gevariëer het, het resultate aangetoon dat parameters vir beide amodiakien en *N*-desetielamodiakien laer was in die toets formulering in vergelyking met die verwysings formulering. Gedurende inkubasies met die mens- en primaatlewer mikrosome sowel as CYP2C8, het die pro-pheroid formulering 'n aansienlike langer amodiakien opruiming en stadiger vorming van *N*-desetielamodiakien getoon. Die bogenoemde effek was egter omgekeerd in die primaat intestinale mikrosoom inkubasies.

Pheroid tegnologie beïnvloed die biobeskikbaarheid van die verskillende farmaseutiese klasse anti-malaria geneesmiddels in 'n wisselende mate. Pheroid tegnologie het nie die absorpsie en biobeskikbaarheid van amodiakien en *N*-desetielamodiakien verbeter nie. Dit is in teenstelling met die waargenome gevolge van pheroid tegnologie op die farmakokinetika van ander geneesmiddels soos artemisoon en chlorokien, waar daar 'n verhoging in die area onder die kurwe en 'n verlenging in die geneesmiddel se halfleeftyd waargeneem is.

Sleutelwoorde: Malaria, Chlorokien, Amodiakien, Pheroid® tegnologie, *In vivo* farmakokinetiese analise, *In vitro* metabolisme, Primate

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LIST OF ABBREVIATIONS

All abbreviations are indicated and explained where they first appear in the text, where after only the abbreviation is used.

ACT	Artemisinin combination therapy
API	Active pharmaceutical ingredient
AQ	Amodiaquine
AUC_{0-inf}	Area under the concentration-time curve between time 0 and the time of the last sample collected
AUC_{0-last}	Area under the concentration-time curve between times 0 to infinity
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CL_H	Predicted hepatic clearance
CL_{int}	Intrinsic clearance
C_{max}	Peak drug concentration
CQ	Chloroquine
CQP	Chloroquine phosphate
CYP	Cytochrome P450
DEAQ	*N*-desethylamodiaquine
DV	Digestive vacuole
EROD	Ethoxyresorufin-O-deethylase
ESI	Electrospray ionization
FDA	Food and Drug Administration
G6PD	Glucose-6-phosphate dehydrogenase
HIM	Human intestinal microsomes
HIV	Human immunodeficiency virus
HLM	Human liver microsomes
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
Im	Intramuscular
LC–MS/MS	Liquid chromatography–mass spectrometry/mass spectrometry
LLOQ	Lower limit of Quantification
MDR	Multidrug-Resistant
MIM	Monkey intestinal microsomes

MLM	Monkey liver microsomes
MRM	Multiple reaction monitoring
N_2	Nitrogen
N_2O	Nitrous Oxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NH	Amino group
NHP	Non-human primate
NWU	North-West University
OH	Hydroxyl group
PCR	Polymerase-chain reaction
Phe	Pheroid
PK/PD	Pharmacokinetic/Pharmacodynamic
pLDH	*Plasmodium* lactate dehydrogenase
PO	Per os
QBS	Quantitative buffy coat method
QC	Quality Control
rCYP	Recombinanat human cytochrome P450
RDT	Rapid diagnostic test
Ref	Reference
RIA	Radioimmunoassay
ROS	Reactive oxygen species
STPHI	Swiss Tropical and Public Health Institute
$T_{1/2}$	Apparent elimination half-life
TB	Tuberculosis
T_{max}	Time to peak concentration
TRC	Toronto Research Chemicals
UCT	University of Cape Town
WHO	World Health Organization

CHAPTER 1

PROBLEM STATEMENT AND OBJECTIVES OF THE STUDY

Malaria, together with TB and HIV, remains one of the greatest burdens on humanity in the 21st century. Malaria is an infectious disease caused by parasites of the *Plasmodium* genus. The parasites are transmitted through the bites of infected female *Anopheles* mosquitoes. Humans are affected by five species of protozoan parasites of *Plasmodium*: *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* of which *P. falciparum* is the most prevalent and severe human pathogen (Gkrania-Klotsas & Lever, 2007; Wells *et al.*, 2009). These five species differ in their geographical distribution, morphology, immunology, relapse patterns as well as in their responses to different treatment regimens. It was estimated that in 2010, there were approximately 216 million cases of malaria worldwide with 655,000 deaths, mostly among children under the age of five and pregnant woman (WHO, 2011). However, the Institute for Health Metrics and Evaluation indicated that there were 1,238,000 deaths per year (95% confidence limits of 929,000 to 1 685,000) because of inclusion of deaths outside Africa and a large number of deaths among adult Africans (Murray *et al.*, 2012). Irrespective of the real figure, it is clear just how serious an infection malaria is, and there is an urgent need to institute an effective and long-lasting control (Greenwood & Owusus-Agyei, 2012).

The World Health Organization (WHO) currently recommends the use of artemisinin-based combination therapy (ACT) as the first-line treatment regimen for uncomplicated *P.falciparum* malaria in the face of wide spread resistance to chloroquine and fansidar (pyrimethamine and sulfadoxine). The pharmacokinetic-pharmacodynamic (PK/PD) rationale of ACT comprises a combination of the potent but short half-life artemisinin or one of its derivatives (artesunate, artemether, dihydroartemisinin) together with a longer half-life quinoline antimalarial drug, such as mefloquine, lumefantrine or amodiaquine (WHO, 2010). However, due to alarming reports of emerging tolerance of the malaria parasites to artemisinin, the core component of ACTs (Dondorp *et al.*, 2010; Cheeseman *et al.*, 2012), it is of utmost importance to develop novel drugs for the treatment of malaria, and to improve existing drug regimens so that these can be used more effectively and especially to have the ability to overcome the cause of resistance. An alternative approach to the development of novel drugs, which is extremely costly and time consuming, is the use of novel drug delivery technology, such as pheroid technology, which can be optimized to ensure effective delivery and enhanced bioavailability of existing drugs such as chloroquine and amodiaquine (Crowley & Martini, 2004). This thesis will describe studies concerned with the use of pheroid technology and its potential to enhance drug delivery to promote effective malaria treatment with chloroquine and amodiaquine.

Chloroquine and amodiaquine are 4-aminoquinolines. These drugs have been extensively used in the fight against malaria. Chloroquine (CQ), a well-tolerated and affordable anti-malarial drug was one of the most successful drugs used for chemoprophylaxis and treatment of malaria. However, since the emergence of chloroquine-resistant *P.falciparum* in 1957, the clinical use of CQ has declined (Ekland & Fidock, 2008). Amodiaquine first appeared in the 1950's. It is structurally similar to CQ, but incorporates an aromatic structure in the side chain that enhances the lipophilicity of the drug (Schlitzer *et al.*, 2007; O'Neill *et al.*, 1998). Upon oral administration, amodiaquine is rapidly converted to the main metabolite desethylamodiaquine (Li *et al.*, 2002; Li *et al.*, 2003). Furthermore, amodiaquine is currently used in ACT together with artesunate as a partner (WHO, 2010). The quinolines, especially chloroquine and amodiaquine, are discussed in section 2 of the literature review in Chapter 2.

Pheroid technology is based on a colloidal drug delivery system. The use of this technology in a number of applications is described in 8 patents filed over a period ranging from 1998 to 2006. Pheroid technology has been shown to enhance the absorption of orally administered anti-infective drugs (Steyn *et al.*, 2010) and topical applications (Saunders *et al.*, 1999). Applications of this technology have been patented in various countries (Saunders *et al.*, 1999, Meyer, 2002, Grobler & Kotzé, 2006; Grobler, 2007; Grobler *et al.*, 2009). There are currently four pheroid related topical products and one bio-agricultural product on the market. The primary components of pheroid technology are ethyl esters of essential fatty acids, pegylated ricinoleic acid, α-tocopherol, and nitrous oxide-saturated water (Saunders *et al.*, 1999, Meyer 2002, Grobler, 2008). The details of pheroid technology as well as the applications thereof, as applied to chloroquine and amodiaquine, will be discussed in section 3 of the literature review in Chapter 2.

Chloroquine and amodiaquine act as rapid blood schizonticides. Treatment of the intra-erythrocytic stages of the malaria parasite relies on transport of the drug through the erythrocyte membrane, and then through the parasite outer membrane, followed by transport to the intra-parasitic site of action. In addition, the use of formulations to overcome resistance of the malaria parasites is of crucial importance to each of chloroquine and amodiaquine.

Pharmacokinetics is the science of kinetics of drug absorption, distribution, metabolism, elimination and toxicity (Shargel & Yu, 1999). Preclinical pharmacokinetic studies provide critical information on the biological activity of a drug, including how well a drug

is absorbed into the circulation, and how the drug is metabolized and eliminated from the body (Weerts & Fantegrossi, 2007). For an drug to be effective, the active pharmaceutical ingredient (API) in any formulation of a drug has to be available at the site of action (Shargel & Yu, 1999). *In vivo* bioavailability of any API can be defined as the rate and extent by which a therapeutically active drug reaches the systemic circulation and is available at the site of action. *In vivo* bioavailability is greatly influenced by the dosage form, since the formulation plays an important role in the stability of the API as well as the route of administration and the site of absorption and absorption rate. Furthermore, the systemic absorption of the drug from the gastrointestinal tract or from any other extravascular site is dependent on the physiochemical properties of the drug, the dosage form used, as well as the anatomy and physiology of the absorption site (Shargel & Yu, 1999). While systemic bioavailability may be increased by a delivery system, this does not necessarily translate into increased therapeutic efficacy. The therapeutic efficacy still requires that the API is delivered to the site of action, has a good bioavailability and a desirable duration of action (Lin & Lu, 1997).

Preclinical pharmacokinetic studies, especially *in vivo* pharmacokinetic studies, are used to estimate the potential success of subsequent clinical trials by contributing to producing safe, cost-effective and clinically sustainable drugs (Korfmacher, 2009). Traditionally, rodents such as rats and mice have been used as animal models during *in vivo* preclinical studies, because of their accessibility through rapid breeding, and their relatively short lifespan (Martignoni *et al.*, 2009). However, due to dissimilarities in various physiological functions of these species compared to humans, the pharmaceutical industry is required to include one or more non-rodent species in preclinical studies, depending upon the type of drug. Non-rodent species include mini-pigs, dogs and non-human primates (Sharer *et al.*, 1995). Non-human primates are usually particularly useful in predicting human pharmacokinetics due to their evolutionary proximity with humans (Chiou & Buehler, 2002). Among non-human primates, cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*) are widely used. For the study described in this thesis, the African green or vervet monkey (*Chlorocebus aethiops*) was used as a preclinical pharmacokinetic model. The relevance of this model is discussed in section 4 of the literature review in Chapter 2.

A number of previous *in vitro* efficacy studies and *in vivo* bioavailability and efficacy rodent studies have been conducted at both the North-West University (NWU) and

Swiss Tropical and Public Health Institute (STPHI). During these studies chloroquine and amodiaquine were administered as pheroid formulations. Some of these results are discussed in section 3 of Chapter 2.

The *in vitro* efficacy studies conducted at the NWU and STPHI illustrated that when CQ was entrapped in vesicles contained in a pheroid formulation, an enhanced efficacy was obtained against both chloroquine-sensitive and especially, -resistant strains of *P.falciparum*. While there was no observable improvement in bioavailability *in vivo* in rodents in studies conducted at the NWU, an enhancement in the *in vivo* efficacy was observed with the pheroid formulations used in the 'Peter's 4-day suppressive test' conducted at the University of Cape Town (UCT). Therefore, the first objective of the study is to investigate the effect of entrapment of anti-malarial quinolines in the vesicles contained in pheroid formulations on the bioavailability and intra-erythrocytic levels of chloroquine in the non-human primate model; and more specifically the African green or vervet monkey (*Chlorocebus aethiops*). The impact of entrapment of chloroquine in pheroid vesicles on the bioavailability and its modulation of efficacy need to be elucidated.

The *in vitro* studies conducted at NWU and STPHI also illustrated an enhanced efficacy for both chloroquine-resistant and -sensitive *P.falciparum* strains when amodiaquine was formulated using pheroid technology. The use of pheroid formulations rendered a two-fold improvement of bioavailability in rodents, as established in studies conducted at the NWU (Langley, 2010). Therefore, the second objective of the study is to establish the bioavailability of amodiaquine in the African green or vervet monkeys (*Chlorocebus aethiops*). This will establish if the results obtained from the rodent model are comparable to those of the non-human primate model. As previously mentioned, there are dissimilarities in physiological function between rodents and humans, and hence the inclusion of non-rodent species, such as the vervet monkey is required by the pharmaceutical industry.

It has been established that amodiaquine undergoes rapid metabolism to *N*-desethylamodiaquine. A third objective of the study is to investigate the *in vitro* metabolism of amodiaquine formulated as pheroids in comparison with the non-formulated amodiaquine. Here, human and monkey liver and intestinal microsomes, and recombinant human CYPs will be used. These studies are required in order to evaluate inter-species differences in the metabolism of amodiaquine.

For the *in vivo* bioavailability studies the following pharmacokinetic parameters will be calculated using non-compartmental models:

- The peak drug concentration (C_{max}) ng/mL
- Time to peak concentration (T_{max}) hours;
- Apparent elimination half-life ($T_{1/2}$) hours;
- Area under the concentration-time curve between time 0 and the time of the last sample collected (AUC_{0-last}) ng.h/mL and
- Area under the concentration-time curve between times 0 to infinity (AUC_{0-inf}) ng.h/mL

For the *in vitro* metabolism studies the following parameters will be calculated:

- Intrinsic clearance (CL_{int}) µL/min/mg protein;
- Predicted hepatic clearance (CL_H) L/kg/h.

The experimental data will be presented in article format in Chapters 3 – 5.

This thesis consist of three manuscripts:

- Manuscript 1 (Chapter 3): Submitted to *Journal of Pharmaceutical and Biomedical Analysis*

- Manuscript 2 (Chapter 4): Submitted to *Antimicrobial Agent and Chemotherapy*

- Manuscript 3 (Chapter 5): Submitted to *Drug Metabolism and Disposition*

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CHAPTER 2

LITERATURE REVIEW

1. Malaria

Malaria is a mosquito-borne infectious disease caused by a protozoan parasite of the genus *Plasmodium.* It was Charles Louis Alphonso Laveran who discovered malaria parasites in 1880, after which the Englishman Ronald Ross together with the Italian Batista Grassi discovered that malaria parasites are transmitted by the female Anopheles mosquito. In 1911, Ronald Ross wrote "*malaria can be completely extirpated in a locality by the complete adoption of any of the three great preventative measures, namely personal protection, mosquito reduction, and treatment*". However, he soon realized that "*it will never be possible for any general community to adopt or enforce any one of these measures completely*" (Ross, 1911). Somehow all of these truths still apply today. In humans, malaria infection is caused by one of five species of *plasmodium*: *P.falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi* of which *P. falciparum* is the most prevalent and virulent (Gkrania-Klotsas & Lever, 2007; Wells *et al.*, 2009). Furthermore, the disease burden is heaviest in African children under the age of 5 years and in pregnant woman. Children have frequents attacks and poor immunological protection, and malaria in pregnancy contributes to a substantial number of maternal deaths. Infant deaths resulting from a low birth weight are also prevalent (Greenwood *et al.*, 2008).

1.1 Current malaria epidemiology

Over recent decades, the global morbidity and mortality caused by malaria have fluctuated but it is estimated that approximately 40 % of the world population remains at the risk of contracting this infectious disease (Figure 1.1). Overall in the past decade, the incidence of malaria has slightly decreased.

During 2010 there were an estimated 216 million cases and 655 000 deaths due to malaria worldwide. The vast majority of cases (81%) and deaths (91%) were in the African region. The top five countries accounting for most deaths due to malaria in the African region are Nigeria, Democratic Republic of the Congo, Burkina Faso, Mozambique, Cote d'Ivoire and Mali (WHO, 2011; GMAP, 2008). The African region is followed by South-East Asia with 13% cases and 6% deaths, and eastern Mediterranean regions with 5% cases and 3% deaths. Furthermore, it is estimated that *P. falciparum* were responsible for 91% of malaria cases observed during 2010 and that 86% of deaths occurred in children under the age of five (WHO, 2011). Despite some geographic overlap, each *Plasmodium* species has a defined area of endemicity, and 5-7% of patients with malaria are infected with more than a single *Plasmodium* species (Marchand *et al.*, 2011)
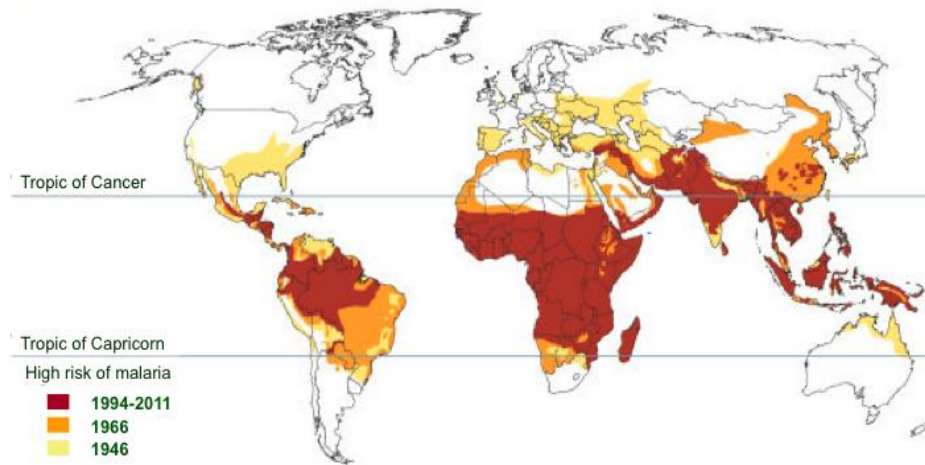
Figure1.1: Global distribution of malaria (Adapted from Sachs & Malaney, 2002:681 with permission from Nature Publishing Group).

The persistence of malaria worldwide is due to various factors. Firstly, attempts to control mosquito vectors by the widespread distribution and usage of insecticide treated mosquito nets and indoor residual spraying, plays a crucial role in preventing malaria (WHO, 2010). Programmes to drain breeding grounds for mosquitoes were successful in many areas, but have been hampered by the development of insecticide resistant mosquitoes. Secondly, *Plasmodium* species have consistently demonstrated their ability to develop resistance to available anti-malarial drugs. No effective vaccine is currently available for the chemoprophylaxis and treatment of malaria. The physiology of malaria parasites is complex, with a layer of complexity added by the parasite-host interaction. The development of an effective vaccine is, therefore, a great challenge. However, despite these challenges, there are currently promising vaccines in clinical trials. For instance, in a phase 3 study of 15,460 children with the candidate malaria vaccine RTS,S/AS01 in seven African countries, the RTS,S/AS01 vaccine provided on average 45% protection against both clinical and severe malaria in African children (The RTS,S Clinical Trails Partnership, 2011).

1.2 Clinical symptoms of malaria

Uncomplicated malaria usually presents with characteristic fever patterns. *P. falciparum, P. vivax* and *P. ovale* present tertian fever spikes (Figure 1.2), while *P. malariae* presents a quartan fever pattern (WHO, 2010; Ashley *et al.*, 2006) and non-specific symptoms, which resemble flu-like symptoms. The symptoms include chills, headache, fatigue, food

aversion, arthralgia, malaise and gastro-intestinal symptoms such as vomiting and/or diarrhoea (Shapiro & Goldberg, 2006; Greenwood *et al.*, 2008).

Plasmodia infections cause hypoglycemia and lactic acidosis, lysis of infected and uninfected erythrocytes, suppression of hematopoiesis, and increased clearance of erythrocytes by the spleen, which in turn results in anemia and sometimes in splenomegaly. End-organ disease, specifically in the central nervous system, lungs, and kidneys, may develop in patients with *P falciparum* infection, probably as a result of cytokines profiles and a high burden of parasites. Long-term malaria infection may also cause thrombocytopenia (Taylor *et al.*, 2012; Gkrania-Klotsas & Lever, 2007; Greenwood *et al.*, 2008).

1.3 Diagnosis

Rapid and accurate diagnosis of malaria plays a critical role in the effective management of the disease, especially in vulnerable population groups such as children under the age of five in which the disease could have fatal consequences. The diagnosis of malaria is based on clinical suspicion and the presence of malaria parasites in the blood. Furthermore, it is important that the diagnosis of malaria is of high quality in order to prevent unnecessary treatment with anti-malarials, which in turn will minimize the emergence of resistance (Bloland, 2001). Initial diagnosis of malaria is based on clinical presentation followed by confirmatory parasitological diagnosis (WHO, 2010).

The symptoms of uncomplicated malaria are non-specific and clinical suspicion of the disease is most often based on the presence or history of a fever. However, the disease should always be confirmed with parasitological diagnosis (WHO, 2010). The World Health Organization (WHO) recommends that in settings where the risk of contracting malaria is low the diagnosis of uncomplicated malaria should be based on a history of fever in the previous three days with the absence of manifestation of any other severe diseases as well as the degree of possible exposure to malaria. In high-risk areas a history of a fever in the previous 24 hours should form the basis of malaria diagnosis. Other signs that should also be included are anemia and in young children the colour of the palms (WHO, 2006; WHO, 2010).

Various techniques exist or are under development to confirm the diagnoses of malaria.

i. Microscopy: Light microscopy examination of Giemsa-stained thin and thick blood smears by skilled microscopists and the identification of asexual stages within erythrocytes provide a high degree sensitivity and specificity. It is the most widely

practiced method and golden standard for the diagnosis of malaria against which sensitivity and specificity of other methods must be assessed. This method allows the identification of the infecting species as well as quantification of malaria parasites. Additionally, when a leucocyte count is included, it can also assist in diagnosis of bacterial or viral infection and determine whether fever in a patient is attributable to the manifestation of malaria or some other infection, thereby ensuring that the patient receives the correct treatment (Bloland, 2001; Ashley *et al.*, 2006; Rieckmann, 2006; WHO, 2010).

ii. The so-called quantitative buffy coat method (QBC) is a modification of light microscopy in which acridine orange is utilized to stain malaria parasites. This technique is, however, non-specific as it stains nucleic acids from all cell types (Bloland, 2001; Ashley *et al.*, 2006).

iii. Rapid diagnostic test: Rapid diagnostic test (RDT) involves the rapid detection of parasite antigens or enzymes with the aid of immunochromatographic techniques in a finger-prick blood sample. A number of the RDT kits have certain ability to differentiate between species of human malaria parasites (*P. vivax, P. ovale, P. malariae and P. knowlesi*). Commercially available rapid diagnostic immunochromatographic tests have been evaluated in returning travelers with the majority of them generating a sensitivity and specificity values over 85-90 % for *P. falciparum* species which is in compliance with the recommendations of the WHO, which also maintains a list of RDT manufacturers and ISO 13485:2003 certification as evidence of quality of manufacture. Current assays are based on the detection of various antigens including histidine-rich protein II (HRP-II) which is specific for *P.falciparum*; species-specific or pan-specific lactate dehydrogenase (pLDH) from the parasites glycolytic pathway found in all species of *plasmodium* that are known to infect humans, and pan-specific adolase (Ashley *et al.*, 2006; WHO, 2010). False negative results at low parasite densities as well as poor detection of non-*falciparum* species still occur (WHO, 2010). It is, therefore, important that the diagnosis obtained from RDT kits should be confirmed with microscopy when possible to ensure correct treatment (Ashley *et al.*, 2006).

iv. Molecular test: Detection of parasite genetic material through polymerase-chain reaction (PCR) techniques is becoming a more frequently used tool in the diagnosis of malaria, as primers have been developed for four of the malaria-causing species. This technique is highly sensitive and very useful for detecting mixed infections at low parasite densities as well as the surveillance of drug resistance (Bloland, 2001; WHO, 2010).

1.4 Biology of malaria infections

Among the five *Plasmodium* species responsible for the manifestation of malaria in humans, *Plasmodium falciparum* is the most prevalent and responsible for the vast majority of deaths. *Plasmodium vivax* is less deadly and is responsible for 25 – 40% of the malaria burden worldwide, particularly in Central and South America, and in South and South-East Asia (Price *et al.*, 2007). *P. vivax* is not that prevalent in Africa due to the absence of the Duffy blood group antigen, which is a necessary receptor for *P. vivax* invasion. The ability of parasites from *P. vivax* and *P. ovale* to remain dormant in the form of hypnozoites in the liver for periods ranging from weeks to several years makes it difficult to eliminate these parasites. Re-emergence from the liver results in relapses of infection. *P. malariae* does not form hypnozoites but it can persist as an asymptomatic blood stage infection for decades. A fifth species, *P. knowlesi,* originally described as a primate malaria of long-tailed macaque monkeys, has shown to infect humans in Malaysia, and could possibly be life-threatening if not treated rapidly (Gkrania-Klotsas & Lever, 2007; Rosenthal & Miller, 2001; Greenwood *et al.*, 2008).

The life cycle of *Plasmodium* species is illustrated in Figure 1.2. Infection of the human host with the parasite is initiated during a blood meal of an infected female *Anophele*s mosquito during which saliva containing sporozoites is inoculated into the host. The sporozoites are rapidly taken up into the liver where they pass through the Kupffer cells and infect hepatocytes. They undergo asexual multiplication, eventually generating schizonts which rupture and release merozoites into the circulation. The merozoites enter erythrocytes and initiate the intra-erythrocytic cycle during which the intra-erythrocytic parasites undergo asexual development proceeding through young ring form stages to trophozoites to mature schizonts. The latter rupture and release merozoites and cell debris, a process, which causes the classical episodes of fever in the host. Within minutes, the merozoites invade new red blood cells and the cycle continues. After several cycles, some of the intra-erythrocytic parasites develop into sexual stage gametocytes, which are ingested when a female mosquito takes a blood meal from an infected host. The male gametocytes are activated and form gametes, which fuse with the female gametes resulting in zygotes which develop into oocsts that in turn release sporozoites that migrate to the salivary glands of the mosquito to be passed on to another individual (Shapiro & Goldberg, 2006; Wells *et al.*, 2009).
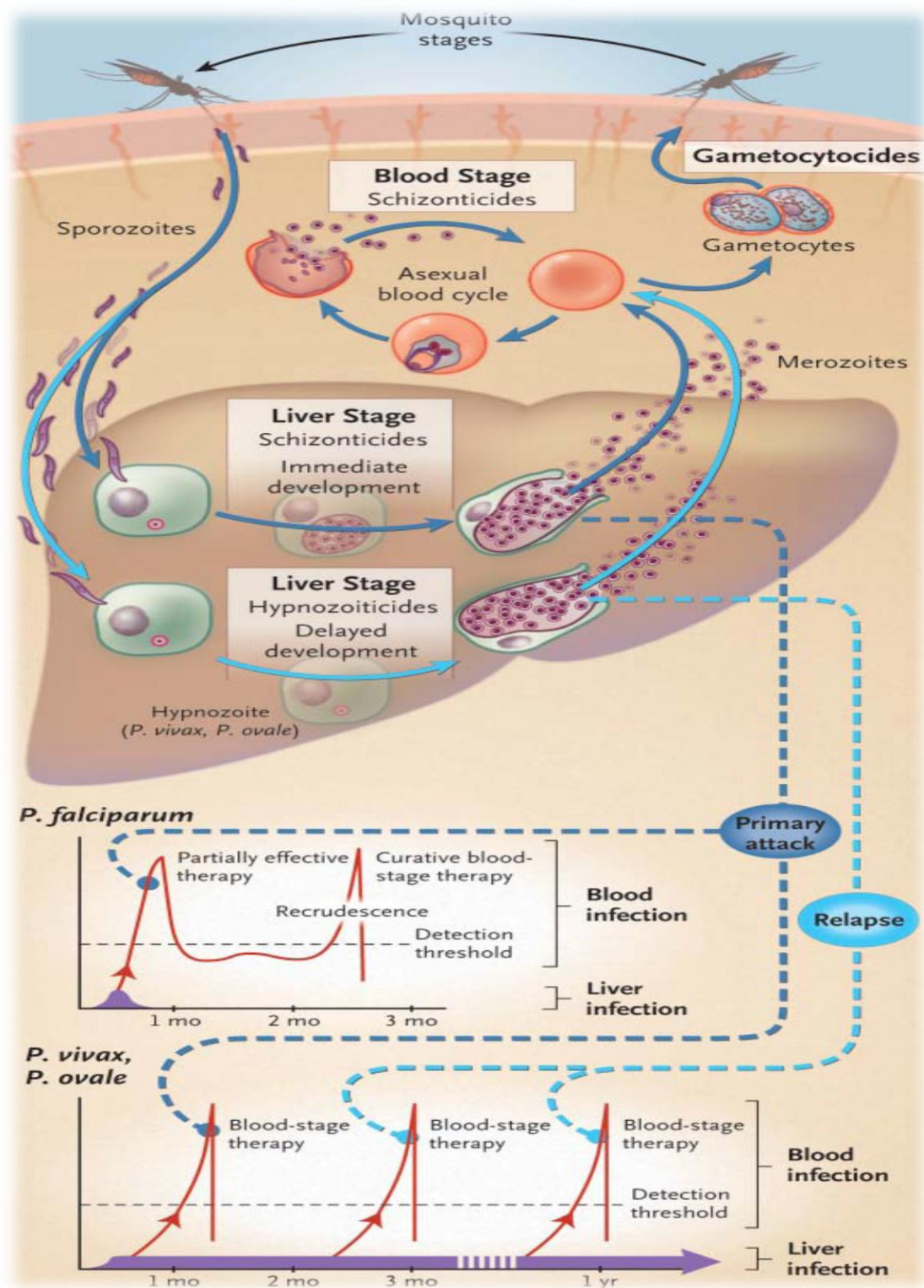
Figure1.2: Life cycle of malaria (Reprinted from Wellems & Miller, 2003:1497 with permission from the Massachusetts Medical Society).

1.5 Chemoprophylaxis against malaria

Paraphrasing a missionary child in 1950, Barbara Kingsolver (1998) wrote: "*Malaria is our enemy number one. Every Sunday we swallow quinine tablets so bitter your tongue wants to turn itself inside out like a salted slug. But Mrs. Underwood warned us that pill or no pills, too many mosquito bites could still overtake the quinine in our blood and spell doom*". These truths still apply today as personal protective measures as well as environmental modifications in combination with chemoprophylaxis can only reduce the risk of contracting malaria. The existing prophylactic regimens if taken correctly only provide 75 - 95% protection; therefore, none of the existing prophylactic regimens provide total protection.

In deciding on a prophylaxis regimen the risk of contracting malaria should be weighed against the risk of experiencing adverse reactions due to the administered anti-malarials. When assessing the risk the following factors should be taken in consideration:

- The geographical location to be visited;
- The duration of the visit;
- Degree of exposure and
- Resistance patterns (Gkrania-Klotsas & Lever, 2007, Chen *et al.*, 2006).

The chemoprophylactic medication should be taken prior to departure and for the duration of the visit in the malaria risk area and should only be discontinued four weeks after returning from the destination. A brief summary of the most current chemoprophylactic regimens for adults and special groups is given in Table 1.1

Table 1.1: Malaria chemoprophylaxis in adults and special groups (adapted from Gkrania-Klotsas & Lever, 2007)

Drug	Usage	Adult dose	Pregnant & breastfeeding dose	Pediatric dose
Chloroquine phosphate	Chloroquine-sensitive *P. falciparum*	300 mg base: once a week or two tablets weekly (one tablet = 150 mg base)	Safe (See adult dose)	5 mg base per kg, po once/week up to max adult dose. Chloroquine syrup: ≤ 4.5 kg: 2.5 mL 4.5 - 7.9 kg: 5.0 mL 8 - 10.9 kg: 7.5 mL 11 - 14.9 kg: 10 mL 15.0 - 16.5 kg: 12.5 mL
Hydroxychloroquine sulphate	Chloroquine-sensitive *P. falciparum*	310 mg base, po, once a week	Safe (See adult dose)	5 mg base per kg, po once/week up to max adult dose.
Atovaquone/ proguanil	Chloroquine-resistant or mefloquine-resistant *P. falciparum*	250 mg Atovaquone and 100 mg proguanil hydrochloride: 1tablet daily	Not recommended	Pediatric tablets contain 62.5 mg atovaquone and 25 mg proguanil hydrochloride: 11 - 20 kg: 1 tablet 21 - 30 kg: 2 tablets 31 - 40 kg: 3 tablets ≥ 40 kg: 1 adult tablet daily
Doxycycline	Chloroquine-resistant or mefloquine-resistant *P. falciparum*	100 mg, po daily	Not recommended (Contraindicated)	≥ 8 years of age: 2 mg/kg up to max adult dose

Table 1.1: Malaria chemoprophylaxis in adults and special groups continued (adapted from Gkrania-Klotsas & Lever, 2007)

Drug	Usage	Adult dose	Pregnant & breastfeeding dose	Pediatric dose
Chloroquine/ Proguanil	Chloroquine-resistant *P. falciparum*	2 tablets weekly (150 mg base/tablet) plus 2 tablets daily (100 mg/tablet)	Safe (See adult dose)	Chloroquine phosphate: 5 mg base per kg, po, once/week, up to max adult dose PLUS proguanil: ≤6 kg: ¼ tablet 6 - 9.9 kg: ½ tablet 10 - 15.9 kg: ¾ tablet 16 - 24.9 kg: 1 tablet 25 - 44.9 kg: 1½ tablets ≥ 45 kg: adult dose
Mefloquine	Chloroquine-resistant *P. falciparum*	228 mg base, po, once a week	Not recommended in first trimester, but safe for second and third trimester (See adult dose)	Mefloquine 5 mg/kg body weight once weekly: 5 - 10 kg: ⅛ tablet 10 - 20 kg: ¼ tablet 21 - 30 kg: ½ tablet 31 - 45 kg: ¾ tablet ≥ 45 kg: 1 tablet
Primaquine	Primary prophylaxis	30 mg base, po, taken daily	Not recommended	0.6 mg/kg base up to adult dose, po - primary prophylaxis
Primaquine	*P. vivax and P. ovale*	30 mg base, po, taken once a day for 14 days	Not recommended	0.6 mg/kg base up to adult dose, po once/day for 14 days after departure from malarious area

1.6 Treatment of uncomplicated *P. falciparum* malaria

Uncomplicated malaria is defined as symptomatic malaria without signs of severity or evidence of vital organ dysfunction (WHO, 2010). Treatment of malaria depends on many factors including the species of malaria parasite responsible for the manifestation of the infection as well as the geographical location where the infection was acquired and disease severity. The former two characteristics help determine the probability of resistance to certain anti-malarial drugs. Additional factors such as age, weight, and pregnancy status may limit the available options for malaria treatment.

In 1955, the WHO launched a programme to eradicate malaria, based on a dual therapeutic and vector control approach. In this programme, chloroquine was used for the chemoprophylaxis and treatment of malaria infections and DDT (dichlorodiphenyltrichloroethane) was used for vector control. This effort included some important successes. However, with the emergence of chloroquine-resistant *P.falciparum* parasites and DDT-resistant *Anopheles* mosquitoes, the programme was ended in 1972, which resulted in an increase in the burden of malaria worldwide. Following the emergence of resistance to chloroquine, the WHO recommended the use of sulfadoxine-pyrimethamine as first-line therapy. Resistance to this anti-malarial developed more rapidly than that of chloroquine and resulted in a search for more effective treatments. During 2001 the WHO recommended the use of artemisinin combination therapy (ACT) as first-line treatment therapy for uncomplicated *falciparum* malaria. The success of artemisinin-based combination therapies has raised the hope for global eradication of malaria. However, because of emerging tolerance of the malaria parasite to artemisinin in the ACT, it is of utmost priority to both develop new anti-malarial drugs or to improve existing drug regimens (Dondorp *et al.*, 2010; Cheeseman *et al.*, 2012). The availability of genome sequences for humans, *Anopheles* mosquitoes as well as *Plasmodium* parasites may play a critical role in identifying drug targets to eradicate malaria (Greenwood *et al.*, 2008; Bosman & Mendis, 2007).

1.6.1 Anti-malarial combination therapy

Anti-malarial combination therapy refers to the simultaneous use of two or more blood schizontocidal medicines with independent modes of action, and thus, unrelated biochemical targets in the parasite. The use of anti-malarial combination therapy contributes greatly to improving therapeutic efficacy and also aids in delaying the development of resistance to the individual drugs in the combination (Davis *et al.*, 2005;

WHO, 2010). The artemisinin-combination therapies (ACTs) are the most important of these therapies (WHO, 2010).

1.6.2 Artemisinin-based combination therapy (ACT)
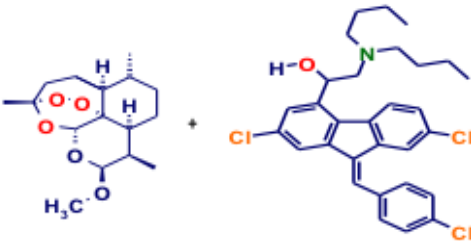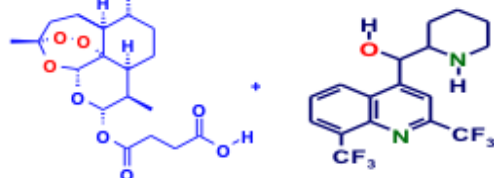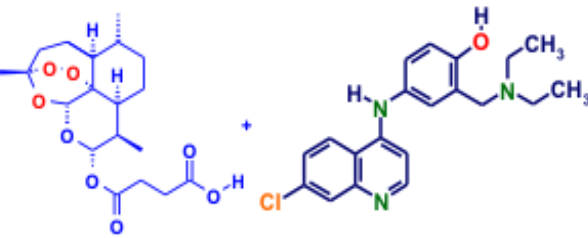
Currently 84 countries worldwide have employed ACTs as first line treatment for uncomplicated *P. falciparum* malaria (WHO, 2011). ACTs are combinations of an artemisinin or its derivatives (artesunate, artemether, dihydroartemisinin) and another structurally unrelated and more slowly eliminated anti-malarial drug. ACTs are responsible for rapid clearance of parasites resulting in rapid abrogation of symptoms and high cure rates; furthermore, they are generally well tolerated and possess additional gametocytocidal activity. The additional benefit of gametocytocidal activity aids in reducing parasite transmissions (Greenwood *et al.*, 2008). The current recommended 3-day treatment course of ACTs exposes 2 asexual cycles, which result in an one hundred million-fold decrease in the number of parasites (WHO, 2010; Bosman & Mendis, 2007). ACTs currently recommended for the treatment of uncomplicated *falciparum* malaria by the WHO as well as the dosing schedules of the various ACTs are shown in Table 1.2 below. Dihydroartemisin-piperaquine, developed in China has recently been added to the recommended list of ACTs (Ashley *et al.*, 2004; WHO, 2010).

1.6.3 Monotherapy and non-artemisinin-based combination therapy
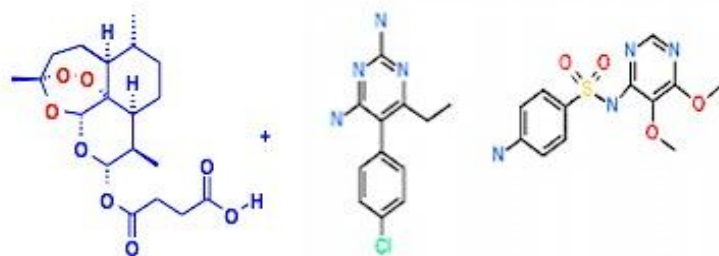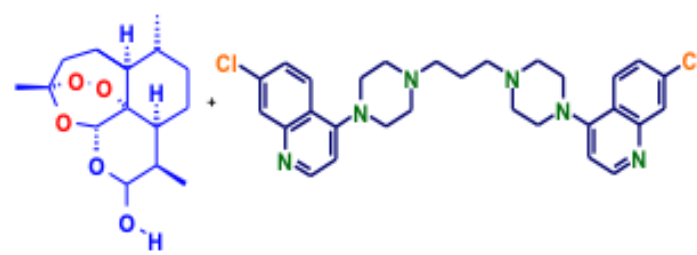
Monotherapy refers to therapy in which a single anti-malarial drug is administered. In contrast, non-artemisinin-based combination therapy refers to the simultaneous administration of more than one anti-malarial drug, with the exclusion of any artemisinin-based partner drugs. Several non-artemisinin-based combination treatments existed prior to the development of ACTs and include quinine-tetracycline, sulfadoxine pyrimethamine-chloroquine, sulfadoxine pyrimethamine-amodiaquine and atovaquone-proguanil (Davis *et al.*, 2005; Sayang *et al.*, 2009; WHO, 2010). The recommended dosing schedule of the various monotherapies and non-artemisinin-based combination therapies are given in Table1.3.

Table 1.2: Artemisinin-based combination therapies (WHO, 2006; WHO, 2010; Sigma-tau, 2010)

Artemether + Lumefantrine							
Age	**Number of tablets/time of dosing**						
	0h	**8h**	**24h**	**36h**	**48h**	**60h**	
< 3 years	1	1	1	1	1	1	
≥ 3 – 8 years	2	2	2	2	2	2	
≥ 9 – 14 years	3	3	3	3	3	3	
> 14 years	4	4	4	4	4	4	
Artesunate + Mefloquine							
Age	**Artesunate (50 mg)**			**Mefloquine (250 mg)**			
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**	
5 – 11 months	25 (½)	25	25	–	125 (½)	–	
≥ 1 – 6 years	50 (1)	50	50	–	250 (1)	–	
≥ 7 – 13 years	100 (2)	100	100	–	500 (2)	250 (1)	
> 13 years	200 (4)	200	200	1 000 (4)	–	500 (2)	
Artesunate + Amodiaquine							
Age	**Artesunate (50 mg)**			**Amodiaquine (153 mg)**			
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**	
5 – 11 months	25 (½)	25	25	76 (½)	76	76	
≥ 1 – 6 years	50 (1)	50	50	153 (1)	153	153	
≥ 7 – 13 years	100 (2)	100	100	306 (2)	306	306	
> 13 years	200 (4)	200	200	612 (4)	612	612	

Table 1.2: Artemisinin-based combination therapies continued (WHO, 2006; WHO, 2010; Sigma-tau, 2010)

Artesunate + Sulfadoxine-pyrimethamine							
Age	Artesunate (50 mg)			Sulfadoxine-Pyrimethamine (number of 500/25 mg tablets)			
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
< 1 year	25 (½)	25	25	125/6.25(¼)	–	–	
≥ 1 – 4 years	50 (1)	50	50	500/25 (1)	–	–	
≥ 5 – 8 years	100 (2)	100	100	750/37.5(1½)	–	–	
≥ 9 – 14 years	150 (3)	150	150	1000/50 (2)	–	–	
≥ 15 years	200 (4)	200	200	1500/75 (2)	–	–	
Dihydroartemisinin + Piperaquine							
Body weight (kg)	Dihydroartemisinin			Piperaquine			
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
5 - < 7	10(¼)	10	10	80 (¼)	80	80	
7 - <13	20 (½)	20	20	160(½)	160	160	
13 - <24	40 (1)	40	40	320 (1)	320	320	
24 - < 36	80 (2)	80	80	640 (2)	640	640	
36 - < 75	120 (3)	120	120	960 (3)	960	960	
75 – 100	160(4)	160	160	1280 (4)	1280	1280	

Dosing for the current recommended ACTs are given in milligrams (mg) and the corresponding number of tablets in brackets

Table 1.3: Monotherapies and non-artemisinin-based combination therapies (Adapted from CDC, 2011, WHO 2010)

Clinical Diagnosis/ *Plasmodium* species	Drug	Adult dose	Pediatric dose
Uncomplicated malaria / *P. falciparum* acquired in areas of chloroquine-resistance	Atovaquone/proguanil	2 adult tablets, po taken twice daily **or** 4 adult tablets, po once daily for 3 days	≤ 5 kg: not indicated 5 - 8 kg: 2 pediatric tablets once daily for 3 days 9 - 10 kg: 3 pediatric tablets once daily for 3 days 11 - 20 kg: 1adult tablet once daily for 3 days 21 - 30 kg: 2 adult tablets once daily for 3 days 31 - 40 kg: 3 adult tablets once daily for 3 days ≥ 40 kg: 4 adult tablets once daily for 3 days
	Quinine sulfate **plus**	542 mg base, po taken three times a day for 3-7 days	8.3 mg base/kg taken 3 times a day for 3 - 7 days
	doxycyline **or**	100 mg taken twice daily for 7 days	2.2 mg/kg, po taken every 12 hours for 7 days
	tetracycline **or**	250 mg taken four times a day for 7 days	6.25 mg/kg taken 4 times a day for 7 days
	clindamycin	6.67 mg/kg taken 3 times as day for 7 days	6.67 mg/kg taken 3 times as day for 7 days
	Mefloquine	750 mg followed 12 hrs later by 500 mg	15 mg/kg followed 12 hours later by 10 mg/kg
Uncomplicated malaria / *P.falciparum* acquired in areas of chloroquine-sensitive *P.falciparum* and Uncomplicated malaria *P.malariae* or *P.knowlesi*	Chloroquine phosphate	600 mg base po immediately followed by 300 mg base po at 6, 24 and 48 hours	10 mg base/kg po immediately, followed by 5 mg base/kg po at 6, 24 and 48 hours
	Hydroxychloroquine	620 mg base po immediately followed by 310 mg base po at 6, 24 and 48 hours	10 mg base/kg po immediately, followed by 5 mg base/kg po at 6, 24 and 48 hours

Table 1.3: Monotherapies and non-artemisinin-based combination therapies continued (Adapted from CDC, 2011, WHO, 2010)

Clinical Diagnosis/ *Plasmodium* species	Drug	Adult dose	Pediatric dose
Uncomplicated malaria/ *P. vivax or P. ovale*	Chloroquine phosphate **plus** primaquine phosphate	Chloroquine phosphate: Treatment as above Primaquine phosphate: 30 mg base, po, taken once daily for 14 days	Chloroquine phosphate: Treatment as above Primaquine phosphate: 0.5 mg base/kg, po, taken once daily for 14 days
	Hydroxychloroquine **plus** primaquine phosphate	Hydroxychloroquine: Treatment as above Primaquine phosphate: Treatment as above	Hydroxychloroquine: Treatment as above Primaquine phosphate: 0.5 mg base/kg, po, taken once daily for 14 days
Uncomplicated malaria/ *P. vivax* acquired in areas of chloroquine resistant *P. vivax*	Quinine sulfate **plus**	542 mg base, po taken three times a day for 3-7 days	8.3 mg base/kg taken 3 times a day for 3 - 7 days
	doxycyline **plus** primaquine phosphate **or**	Doxycycline: 100 mg taken twice daily for 7 days Primaquine phosphate: Treatment as above	Doxycycline: Treatment as above Primaquine phosphate: Treatment as above
	tetracycline **plus** primaquine phosphate **or**	Tetracycline: 250 mg taken four times a day for 7 days Primaquine phosphate: Treatment as above	Tetracycline: Treatment as above Primaquine phosphate: Treatment as above
	Mefloquine **plus** primaquine phosphate	Mefloquine: 750 mg followed 12 hrs later by 500 mg Primaquine phosphate: Treatment as above	Mefloquine: Treatment as above Primaquine phosphate: Treatment as above

Table 1.3: Monotherapies and non-artemisinin-based combination therapies for the treatment of uncomplicated malaria in pregnant woman (Adapted from CDC, 2011, WHO 2010)

Clinical Diagnosis/ *Plasmodium species*	Drug	Adult dose
Uncomplicated malaria alternatives for pregnant woman / in areas of chloroquine-sensitive *P. falciparum or P. vivax* or *P. ovale*	Chloroquine phosphate	600 mg base po immediately followed by 300 mg base po at 6, 24 and 48 hours
	Hydroxychloroquine	620 mg base po immediately followed by 310 mg base po at 6, 24 and 48 hours
	Chloroquine phosphate **plus** primaquine phosphate	Chloroquine phosphate: Treatment as above Primaquine phosphate: 30 mg base, po, taken once daily for 14 days
Uncomplicated malaria alternatives for pregnant woman / in areas of chloroquine-resistant *P. falciparum and P. vivax*	Quinine sulfate **plus** clindamycin	Quinine sulfate: 542 mg base, po taken three times a day for 3-7 days Clindamycin: 6.67 mg/kg taken 3 times as day for 7 days
	Mefloquine	750 mg followed 12 hrs later by 500 mg

1.6.4 Treatment of severe *P.falciparum* malaria

Severe malaria is almost always the result of poor treatment of uncomplicated malaria or of the presence of drug resistant parasites of *P.falciparum*. High-risk groups include children under the age of 5 years and adults from low transmission areas. Severe malaria is classified by the WHO as the presence of one or more of the clinical or laboratory features (Table 1.4), with the exclusion of any other severe febrile disease. Severe malaria accounts for approximately 5% of global malaria cases reported each year (WHO, 2010; Trampux *et al.*, 2003).

Table 1.4: Clinical and laboratory features of severe malaria (WHO, 2010)

One or more of the following clinical or laboratory features
Clinical features
Impaired consciousness
Prostration
Failure to feed
Multiple convulsions
Respiratory distress or acidotic breathing
Circulatory collapse or shock
Jaundice
Organ dysfunction
Haemoglobinuria
Abnormal bleeding
Pulmonary oedema
Laboratory features
Hypoglycaemia (blood glucose < 2.2 mmol/L)
Metabolic acidosis (plasma bicarbonate < 15 mmol/L)
Renal impairment (serum creatinine > 265 µmol/L)
Severe normocytic anemia (Hb < 5 g/dL, packed cell volume < 15%)
Haemoglobinuria
Hyperparasitaemia (> 2%/100 000/µL in low transmission areas and > 5% in high transmission areas)

Prompt and accurate administration of anti-malarial treatment plays a crucial role in ensuring a positive outcome and curing severe malaria. Two classes of anti-malarial drugs are available for the treatment of severe malaria (Table 1.5) and include quinine, which has been the gold standard for treatment of severe malaria, and the artemisinin derivatives; artesunate, and artemether (Dondorp *et al.*, 2005).

Table 1.5: Treatment of severe malaria in adults and special groups (adapted from CDC, 2011, Lalloo *et al.*, 2007)

Drug	Adult dose	Pediatric dose
Quinine gluconate plus one of the following:	Loading dose IV of 6.25 mg base/kg over 1 - 2 hours, followed by 0.0125 mg base/kg/min continues infusion for 24 hours. Alternative regimen: 15 mg base/kg loading dose IV infused over 4 hours every 8 hours, starting 8 hours after the loading dose. At a parasitemia of ≤ 1 % the patient can take the following oral medication: Quinidine/quinine course for 3 - 7 days	Loading dose IV of 6.25 mg base/kg over 1 - 2 hours, followed by 0.0125 mg base/kg/min continues infusion for 24 hours. Alternative regimen: 15 mg base/kg loading dose IV infused over 4 hours every 8 hours, starting 8 hours after the loading dose. At a parasitemia of ≤ 1 % the patient can take the following oral medication: Quinidine/quinine course for 3 - 7 days
Doxycycline	100 mg IV every 12 hours and then switch to oral doxycycline. 100 mg taken twice daily for 7 days	100 mg IV every 12 hours and then switch to oral doxycycline. 2.2 mg/kg, po taken every 12 hours for 7 days
Tetracycline	250 mg taken four times a day for 7 days po	6.25 mg/kg taken 4 times a day for 7 days po
Clindamycin	6.67 mg/kg taken 3 times as day for 7 days po. If the patient is not ab take oral medication: 10 mg base/kg loading dose IV followed by 5 mg base/kg IV every 8 hours.	6.67 mg/kg taken 3 times as day for 7 days po
Artesunate in conjunction with doxycycline	IV at a dose of 2.4 mg/kg administered at 0, 12 and 24 hours followed by one daily thereafter. Doxycycline treatment as described above	n/a

2. Quinolines and related compounds

Quinolines are heterocyclic aromatic organic compounds and have been the mainstay for anti-malarial chemotherapy starting with quinine nearly 3 centuries ago. Quinolines mostly exert their activity during the intra-erythrocytic stages of the parasites life cycle, with the exception of primaquine, which has the ability to eradicate hepatic stages as well. It is, therefore often used for the treatment of *P. vivax* (Baird & Rieckman, 2003; Kaur *et al.*, 2010). During the last century many related compounds have been synthesized and tested for anti-malarial activity. A number of drugs that are useful for the chemoprophylaxis and treatment of malaria have resulted from these programmes (Shapiro & Goldberg, 2006). For the purpose of this thesis only the literature relevant to the 4-aminoquinoline, chloroquine, and the Mannich base 4-aminoquinoline, amodiaquine, will be discussed in detail.

2.1 History and the development of 4-aminoquinoline drugs from quinine

Powdered bark from the cinchona tree containing the quinoline alkaloids, quinine (1) and quinidine (2) was the first medicine to be used against malaria. The bark was introduced from South America into Europe by the Jesuits during the 17th century. To this day, quinine is still an indispensable malaria medication (Schlitzer, 2007; O'Neill *et al.*, 1998; Foley & Tilley, 1998; Jensen & Mehlhorn, 2009; Lee & Solomon, 2009).

In 1820 quinine was isolated by Pelletier and Caventou (Foley & Tilley, 1998; Lee & Solomon, 2009). The structure of quinine was elucidated in 1907 (Rabe *et al.*, 1907). The total synthesis of quinine was achieved by Woodward and Doering in 1944 (Woodward and Doering, 1944; Woodward and Doering, 1945). The work of Woodward and Doering was based on the relay synthesis of quinine performed by Rabe and Kindler (Rabe, 1911; Rabe & Kindler, 1918). Paul Ehrlich noticed that methylene blue (3) was particularly effective in staining malaria parasites. He also realized that this dye may also be selectively toxic to the parasite and in 1891 Ehrlich and Guttman cured two malaria patients with methylene blue, which became the first synthetic drug ever to be used in therapy. It subsequently played a crucial role in the development of synthetic anti-malarial drugs.

Cinchona *ledgeriana*

1: Quinine

2: Quinidine

3: Methylene Blue

4: Pamaquine

5: Primaquine

6: Mepaquine

7: Chloroquine

8: Amodiaquine

9: Mefloquine

11: Piperaquine

10: Lumefantrine
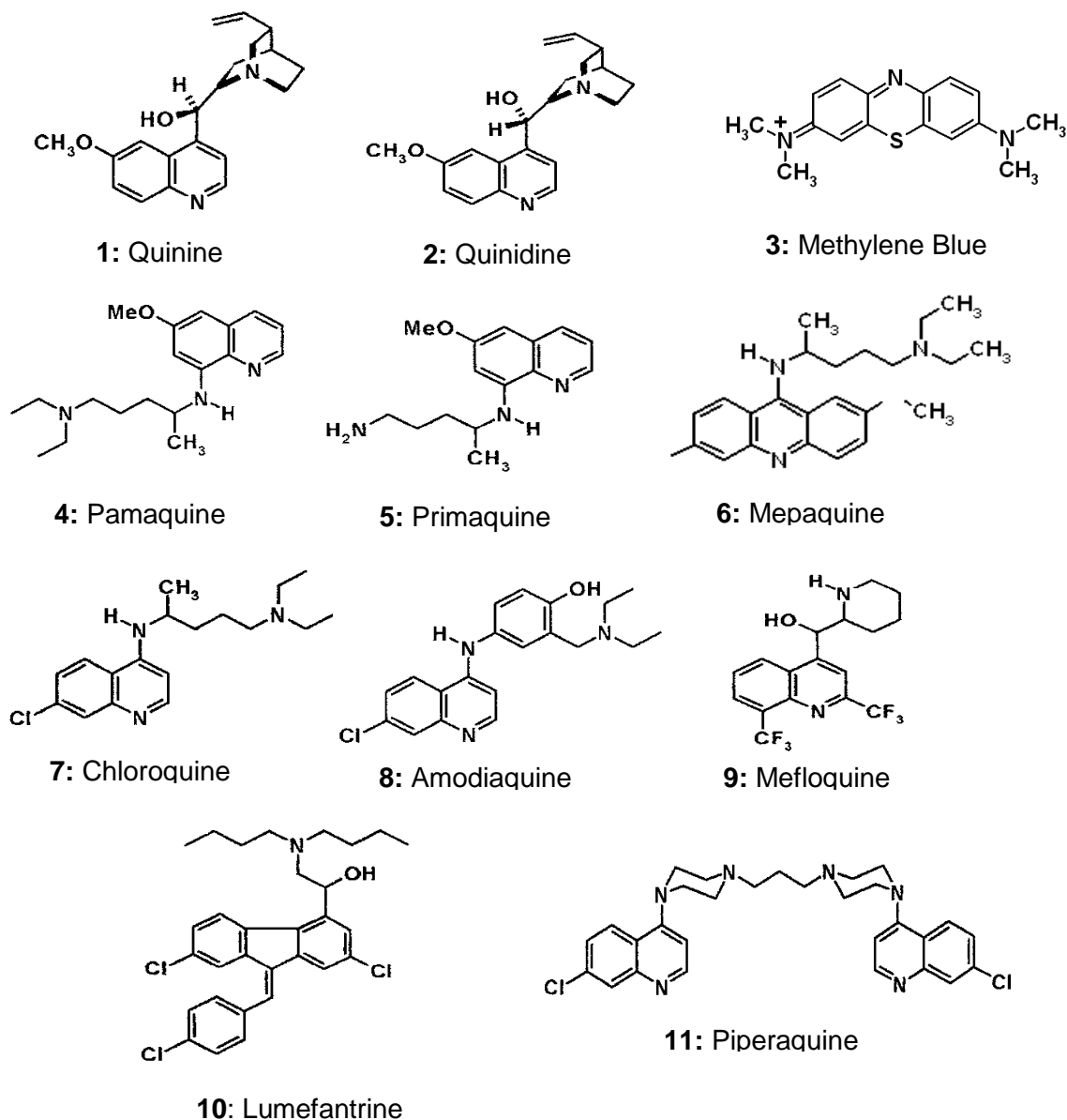
Figure 2.1: Structures of quinolines and related compounds.

During the 1920s an anologue of methylene blue was synthesized by replacing one-methyl group with the basic dialkylaminoalkyl side chain. Chemists at Bayer attempted to make methylene blue more lipophilic by replacing the cationic scaffold. They blended the quinoline ring of quinine with the longer side chain analogues of methylene blue.

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Subsequently this side chain was connected with different heterocyclic systems such as the quinoline system, which resulted in pamaquine (4), an 8-aminoquinoline, which was demonstrated to be more effective than quinine in eradicating the liver stages of the malaria parasites in humans (Lee & Solomon, 2009; Rieckmann, 2006; O'Neill *et al.*, 1998). Unfortunately pamaquine displayed a multitude of side effects when taken at the effective dose levels, and it appeared to lose its effectiveness when better-tolerated lower doses were administered. The latter problems led to the introduction of primaquine (5) in 1952, which became the most important of the 8-aminoquinolines. Primaquine is still the only drug available today that can prevent relapses of *P. vivax* malaria. Primaquine has marked activity against the mature gametocytes of *P.falciparum* (Rieckmann, 2006). In 1932 mepacrine (6) was introduced as Atebrine (Schlitzer, 2007). Atebrine had substantial activity against the intra-erythrocytic stages of the malaria parasites. Furthermore, it was eliminated much more slowly than quinine, and hence a shorter treatment period could be used (Rieckmann, 2006).

A major success was achieved by Hans Andersag and colleagues at Bayer in 1934 with the introduction of resochin. However, after *in vivo* testing on the avian model it was concluded that the efficacy of resochin was similar to that of atebrine but considered to be somewhat more toxic. The first human trial was conducted by Franz Sioli with results apparently mirroring those observed in the avian model. It was, therefore, concluded to be too toxic for use in humans (Coatney, 1963). As a result resochin was not subjected to any further clinical studies and was ignored for a decade. In 1936 the structurally closely related sontoquine was prepared in the Bayer laboratories, and used by German troops serving in North Africa and other malarious areas. Sontoquine and resochin were licensed to Winthrop Chemical Company in the USA, under a pre-war disclosure agreement between Bayer and Winthrop. In 1943 the US government set up the Board for the Coordination of Malaria studies with the goal of developing new anti-malarial medications. Then, the promising effects of resochin were rediscovered, though this occurred via an unintentionally circuitous route of a parallel development of the substance SN-7619. The test substance SN-7619 then received the name chloroquine (7), but it was identical to the previous established resochin. From 1946 onward, chloroquine became available for treatment of malaria. Numerous clinical studies were initiated with chloroquine manufactured by Winthrop and it soon emerged that the drug was clinically effective and well tolerated (Coatney, 1963; Jensen & Mehlhorn, 2009). In 1950, 5 years after the end of the war, Bayer AG in

Leverkusen (Germany) began to manufacture and distribute chloroquine again and chloroquine became the cornerstone of malaria therapy.

Attempts to enhance the lipophilicity of the side chain of chloroquine through incorporation of an aromatic structure in the side chain resulted in the emergence of another 4-aminoquinoline, amodiaquine (Olliaro & Taylor, 2003; Schlitzer, 2007). Amodiaquine (8) was introduced as an alternative to chloroquine (Foley & Tilley, 1998). Other drugs that were developed in various parts of the world include mefloquine (9), lumefantrine (10) which was developed in China, and piperaquine (11), which was first made in France and then used in China. Currently compounds 8 to 11 (i.e. amodiaquine, mefloquine, lumefantrine and piperaquine) play an important role as they are combined with artemisinin derivatives in combination therapies (section 1.6.2).

2.2 Chloroquine

Chloroquine (CQ) is classified as a 4-aminoquinoline and has been the most successful single drug for the chemoprophylaxis and treatment of malaria (Schlitzer, 2007; Kaur *et al.*, 2010). It was the drug of choice in the WHO Global Eradication Programme. Chloroquine is a relatively well-tolerated drug as long as it is used in the recommended therapeutic regimens. The therapeutic index is relatively narrow, between 10 and 20 mg/kg body weight (Schlitzer, 2007). However, widespread resistance to the drug has now rendered it virtually useless against chloroquine-resistant *P.falciparum* infections in most parts of the world, although it still maintains considerable efficacy for treatment of chloroquine-sensitive *P.falciparum* infections and *P. vivax* infections (Shapiro & Goldberg, 2006).

2.2.1 Pharmacokinetics of chloroquine

The pharmacokinetic properties of chloroquine have been well characterized in humans. Peak plasma concentrations can vary considerably. In humans, chloroquine has a bioavailability of approximately 80% and is extensively distributed into body tissues, including the placenta and breast milk. It has an enormous total apparent volume of distribution of 132-261 L/kg. Fifty to sixty percent of chloroquine is bound to plasma proteins, and the drug is eliminated slowly from the body via the kidneys, with an estimated terminal half-life of 1 – 2 months (Krishna & White, 1996; Shapiro & Goldberg, 2006). Publications of pharmacokinetic properties of chloroquine for non-human primates are

limited (McChesney *et al.*, 1967; McChesney *et al.*, 1966; Prasad *et al.*, 1985). The pharmacokinetic properties of chloroquine in various non-rodent species available in the literature are summarized in Table 2.1.

2.2.1.1 Metabolism of chloroquine

Metabolism aids in preventing accumulation of drugs in an organism. The total metabolism of any drug is dependent on all the metabolic pathways involved in the metabolism of the drug. In 1947, Williams proposed that drug metabolism occurred through phase I and phase II metabolic reactions (Williams, 1947). Phase I reactions include the introduction of a new polar functional group into the parent drug (oxidation), or modification of an existing functional group to be more polar (reduction), or unmasking of existing polar functional groups (hydrolysis). The most common polar groups that are exposed during phase I reactions include hydroxyl (-OH), amino (-NH), and carboxylic acid (-COOH) groups. Phase II reactions include glucuronidation, sulfonation, glycine/glutamine conjugation, acetylation, methylation and glutathione conjugation. These are substitution reactions, which link a new group to either the parent drug or to the metabolite (Martignoni *et al.*, 2009; King, 2009).

Cytochrome P450 (CYP) represents a superfamily of iso-enzymes responsible for biotransformation of drugs, steroids, fatty acids and environmental pollutants via oxidation. In humans the CYP superfamily consists of a large number of drug-metabolizing enzyme genes: 57 functional genes, and 58 pseudogenes (Nelson *et al.*, 2004). These genes have been divided into CYP gene families (e.g. CYP1, CYP2, CYP3, etc.) where the members have more than 40% amino acid sequence identity. Each family is divided into subfamilies (labeled with letters A, B, C etc.) where the amino acid sequence of the members is more than 55% identical, and finally by Arabic numbers which represents the allele or variant categories (King, 2009).

Table 2.1: Pharmacokinetic properties of chloroquine in various species

Species	Matrix	N	Drug	Route	Dose	C_{max}	T_{max}	$T_{1/2}$	AUC (0-last)	AUC (0-inf)	Reference
					(mg/kg)	(ng/ml)	(h)	(h)	(ng.h/ml)	(ng.h/ml)	
Rabbit	Whole blood	4	CQS	po	40	-[a]	-[a]	159	-[a]	101172.5	Aderounmu *et al.*, 1987
Dog	Whole blood	7	CQP	po	150	-[a]	-[a]	348	-[a]	32482	Aderounmu & Fleckenstein, 1983
Rhesus monkey	Plasma	6	CQP	po	20	320.8	2	18.76	-[a]	-[a]	Prasad *et al.*, 1985
Rhesus monkey	Plasma	4	CQP	im	4	183.3	3	-[a]	-[a]	-[a]	McChesney *et al.*, 1966
Rhesus monkey	Whole Blood	4	CQP	im	4	560	3	76	-[a]	-[a]	McChesney *et al.*, 1966
Rhesus monkey	Plasma	2	CQ	im	3.7	262	3	81.6	-[a]	-[a]	McChesney *et al.*, 1967
Human	Plasma	11	CQP	po	12	73	3.3	207	4990	-[a]	Gustafsson *et al.*, 1983
Human	Whole Blood	7	CQP	po	25	838	-[a]	150	122000	-[a]	Na-Bangchang *et al.*, 1994
Human	Plasma	14	CQ	po	10	374	5	192	18609	-[a]	Walker *et al.*, 1987

a no data available

Abbreviations: **AUC (0-last)** = area under the plasma-concentration curve from zero to the last sample measured; **AUC (0-inf)** = area under die plasma-concentration curve from 0 to infinity; **CQ** = chloroquine; **CQP** = chloroquine diphosphate; **CQS** = chloroquine sulfate; **im** = intramuscular administration; **po** = oral administration

The enzymes are heme-containing membrane proteins, that are located in the endoplasmic reticulum of several tissues. Although the majority of iso-enzymes are located in the liver, extra-hepatic metabolism also occurs in the kidneys, skin, lungs and gastro-intestinal tract. The liver and intestinal epithelia represent the predominant sites for CYP-mediated drug metabolism (Martignoni *et al.*, 2009). CYP enzymes bind two atoms of oxygen, resulting in the formation of a water molecule and the metabolite, which is more polar than the parent drug (Martignoni *et al.*, 2009). In humans CYP1-3 families are responsible for the metabolism of the vast majority of drugs (Bjornsson *et al.*, 2003; Nelson *et al.*, 2004; Iwasaki & Uno, 2009; Martignoni *et al.*, 2009). Chloroquine is mainly metabolized in the liver by de-ethylation of the side chain, resulting in the major metabolite *N*-desethylchloroquine and minor metabolite bisdesethylchloroquine.
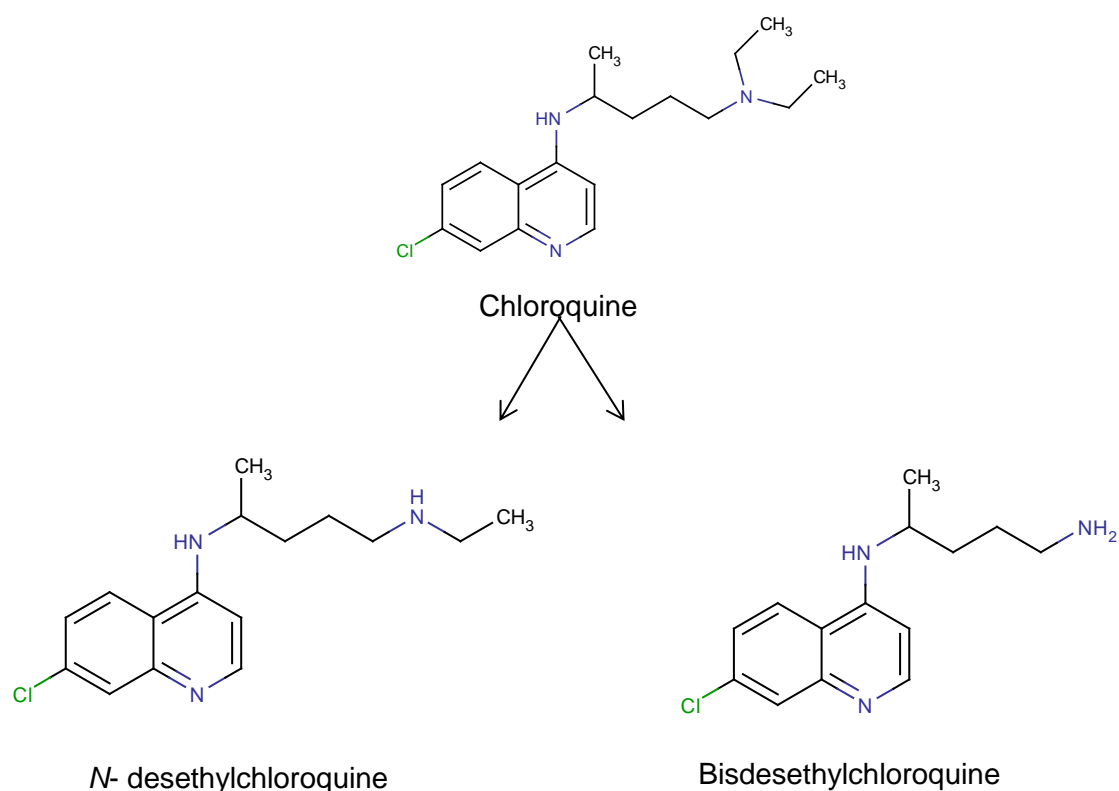
Figure 2.2: Metabolism of chloroquine

The main cytochrome P450 isoforms affected by or involved in the biotransformation of chloroquine to *N*-desethylchloroquine are CYP3A and CYP2D6. The pharmacokinetic profile of *N*-desethylchloroquine as well as its anti-malarial properties are similar to that of chloroquine (Ducharme & Farinotti, 1996).

2.2.1.2 Chloroquine toxicity

Chloroquine is a safe drug when taken at therapeutic doses of 10 – 20 mg/kg. Side effects include headache, various skin eruptions and gastrointestinal disturbances, such as nausea, vomiting and diarrhoea. Urticarial pruritus may occur. However, the therapeutic window of chloroquine is narrow and a single dose of 30 mg/kg may result in cardiovascular effects including hypotension, vasodilatation, suppressed myocardial function, cardiac arrhythmias, and eventual cardiac arrest. Confusion, convulsion and coma indicate CNS dysfunction. Chronic use may lead to eye disorders, including keratopathy and retinopathy. Other uncommon effects include myopathy, reduced hearing, photosensitivity and loss of hair. Blood disorders, such as aplastic anemia have been reported, but are extremely uncommon (Shapiro & Goldberg, 2006; Jaeger & Flesch, 1994).

2.2.2 Drug interactions and contraindications

Chloroquine should be used with caution in patients with hepatic, gastrointestinal, neurological, or blood disorders. In patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, administration of chloroquine may result in hemolysis. Chloroquine is an inhibitor of CYP2D6 (Shapiro & Goldberg, 2006). Chloroquine is not recommended for treating individuals with epilepsy, myasthenia gravis, psoriasis and porphyria cutanea tarda (Taylor & White, 2004). Other general drug interactions when chloroquine is co–administered with the following drugs include:

- Halofantrine: increase risk of ventricular arrhythmias;
- Mefloquine: increased risk of convulsions;
- Anti-acids: reduced absorption of chloroquine;
- Cimetidine: reduced metabolism and clearance of chloroquine;
- Metronidazole: increased risk of dystonic reactions;
- Ampicillin and praziquantel: reduced bioavailability of ampicillin and praziquantel;
- Phenylbutazone: tendency to cause dermatitis;
- Carbamazepine and sodium valproate: reduced therapeutic effects of carbamazepine and sodium valproate, and
- Cyclosporine: increased plasma concentrations of cyclosporine (Jaeger & Flesch, 1994; Shapiro & Goldberg, 2006; WHO, 2010).

2.2.3 Mechanism of action of chloroquine

The mechanism of action of CQ has been the subject of ongoing debate for many years. Various mechanisms have been proposed for the action of CQ, either intra or extra vacuolar. Early studies suggested that CQ interacts with DNA, proteases, phospholipases or metabolic enzymes. However, these proposed mechanisms would require higher concentrations of CQ than those that can be achieved *in vivo* (Olliaro, 2001). CQ is classified as a rapid acting blood schizontocide and therefore exerts it action on the intra-erythrocytic stages of the malaria parasite life cycle (Figure 2.2).
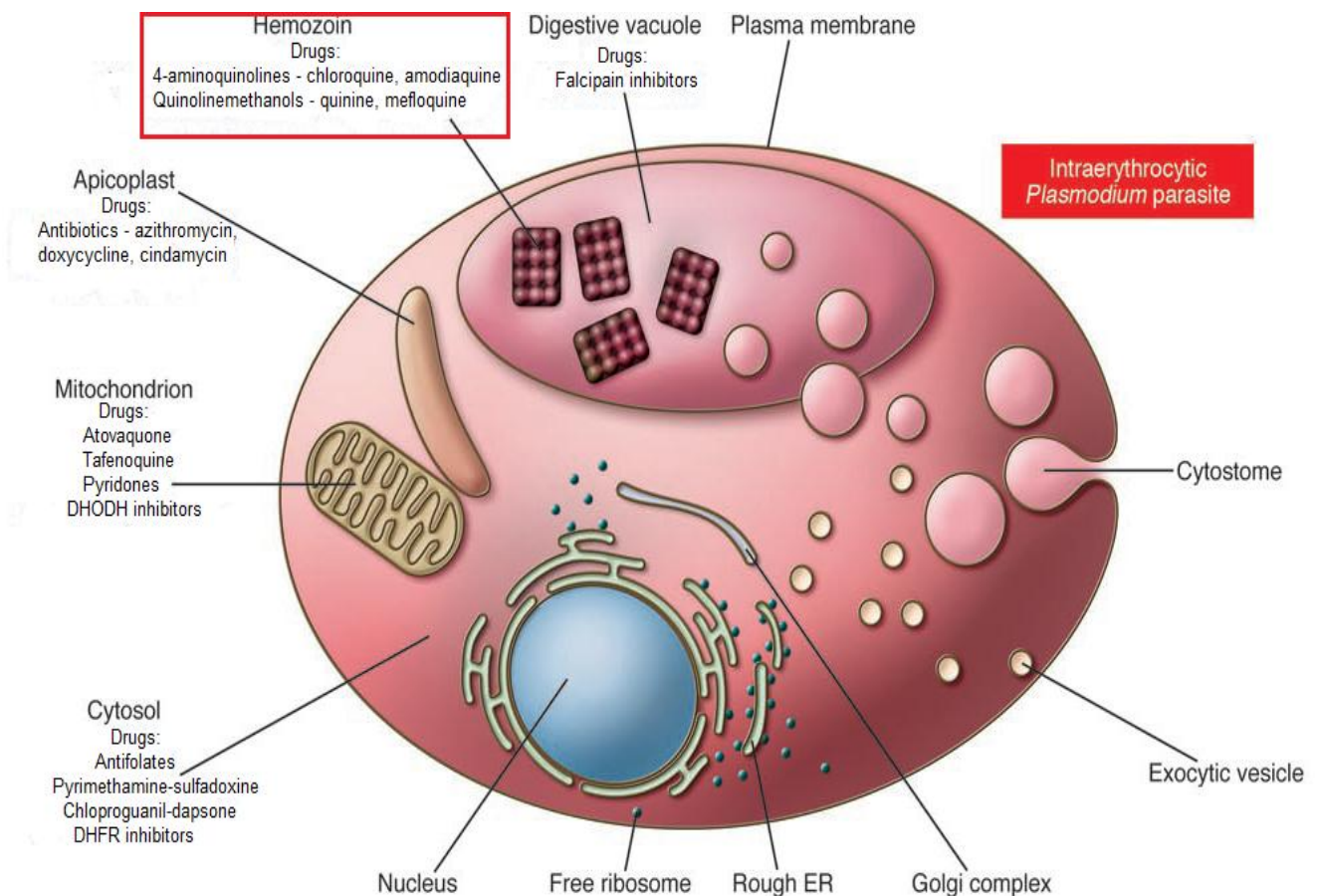
Figure 2.3: Anti-malarial drugs mediate their effects by disrupting processes or metabolic pathways in different subcellular organelles (Reprinted from Greenwood *et al.*, 2008:1270 with permission from the American Society for Clinical Investigation).

During the mid-ring (Bakar *et al.*, 2010) and early trophozoite stages, the malaria parasite actively degrades hemoglobin in the digestive vacuole (DV) of the malaria parasite (Sullivan, 2002; Kumar *et al.*, 2007; Dubar *et al.*, 2010). Ferriprotoporphyrin IX (heme-Fe^{3+}) is released during the degradation process and is cytotoxic (Monti *et al.*, 1999) and responsible for generating reactive oxygen species (ROS) that induces peroxidation of lipid membranes and may induce cell lysis (Kumar *et al.*, 2007; Dubar *et al.*, 2010; Schmitt *et al.*, 1993).

The malaria parasite possesses an efficient heme-Fe^{3+} detoxification mechanism to protect itself from oxidative stress. The most important mechanism is that operating in the DV wherein heme-Fe^{3+} is normally detoxified by a biomineralization process to malaria pigment hemozoin or β-haematin (Kumar *et al.*, 2007; Klonis *et al.*, 2007).

CQ exerts its mode of action through interference with the sequestration of heme-Fe^{3+}. CQ binds to heme-Fe(III) to prevent the formation of hemozoin (Müller & Hyde, 2010; Fitch, 2004; Egan *et al.*, 2006; Stiebler *et al.*, 2010). Solid-state spectroscopic studies have elucidated the structure of the CQ-heme-Fe^{3+} complex (Asghari-Khiavi *et al.*, 2011) and X-ray crystallography has confirmed the structures of the quinine-heme-Fe^{3+} complex (De Villiers *et al.*, 2012). Further, studies relating to the physiochemical properties of quinolines and their abilities to accumulate in the DV or inhibit hemozoin formation have been meticulously carried out (Kaschula *et al.*, 2002**;** Dubar *et al.*, 2010; Otelo *et al.*, 2011). A positive correlation has also been established between the extent of free heme present in the parasite and the amount of CQ applied (Combrinck *et al.*, 2012). The inhibition of hemozoin formation results in accumulation of heme in the DV, which in turn enhances the diffusion the CQ-heme-Fe^{3+} complex across a steeper concentration gradient into the cytosol (Haynes *et al.*, 2012). It is apparent that the CQ-heme-Fe^{3+} complex and other complexes must diffuse out of the relatively acidic DV (pH ~5.2) (Bakar *et al.*, 2010) into the cytosol where at a higher pH (pH ~ 7.4) they dissociate into the deprotonated heme and chloroquine. The latter may re-enter the DV to cycle more heme into the cytosol. Therefore, CQ causes redistribution of monomeric heme within the parasite such as to intensify cytotoxicity (Haynes *et al.*, 2012).

2.3 Amodiaquine

Amodiaquine, discovered in 1950, is a Mannich base 4-aminoquinoline that is closely related to chloroquine (Olliaro & Taylor, 2003). The structural difference is that amodiaquine carries a 4-hydroxyanilino aromatic side chain (O'Neill *et al.*, 1998). This side chain is vital for anti-malarial activity of the drug (Schlitzer, 2007). Amodiaquine was first introduced as an alternative to chloroquine due to its effectiveness against chloroquine resistant *P. falciparum*

parasites (Krishna & White, 1996). However, during the 1970s it was withdrawn from clinical use because of its toxicity, including agranulocytosis and hepatitis, which were observed during chemoprophylactic use (Neftel *et al.*, 1986). Despite these concerns amodiaquine is currently recommended by the WHO as a partner drug in the artesunate-amodiaquine combination therapy for the treatment of uncomplicated malaria (Schlitzer, 2007; German & Aweeka, 2008; WHO, 2010).

2.3.1. Pharmacokinetics of amodiaquine

The literature on the pharmacokinetic properties of amodiaquine (AQ) is limited, and is believed to be similar to that of chloroquine due to the structural similarities. Differences that exist between the two drugs include a smaller volume of distribution (17 – 34 L/kg) and a shorter terminal elimination half-life (1 to 3 weeks) for amodiaquine and *N*-desethylamodiaquine (DEAQ). Up to 90% of AQ and DEAQ are bound to plasma proteins (Krishna & White, 1996). Table 2.2 summarizes the relevant pharmacokinetic properties of amodiaquine and *N*-desethylamodiaquine available in the literature.

2.3.1.1 Metabolism of amodiaquine

2.3.1.1.1 Microsomal metabolism

Following oral administration, amodiaquine (AQ) (Fig 2.4;1) is rapidly absorbed from the gastro-intestinal tract and extensively metabolized to the main metabolite *N*-desethylamodiaquine (Fig 2.4;2), resulting in low exposure levels of the parent compound in plasma (Figure 2.4).

Table 2.2: Pharmacokinetic properties of orally administered amodiaquine in different animal models

Species	Matrix	N	Drug	Dose	C_{max}	T_{max}	$T_{1/2}$	AUC (0-last)	AUC (0-inf)	Reference
				(mg/kg)	(ng/ml)	(h)	(h)	(ng.h/ml)	(ng.h/ml)	
Cynomolgus monkey	Whole Blood	2	AQ-13	20	356,26	7,95	36,3	12438,71	-[a]	Ramanathan-Girish
Cynomolgus monkey	Whole Blood	2	AQ-13	100	5694,4	1,9	38,2	124422,6	-[a]	Ramanathan-Girish
Humans	Plasma	12	AQ	10	29,2	2,32	5,3	162,4	-[a]	Orrell *et al.*, 2008
Humans	Plasma	7	AQ	10	32	0.5	5.2	99	154	Winstanley *et al.*, 1987
Humans	Whole Blood	7	AQ	10	60	0,5	-[a]	148	-[a]	Winstanley *et al.*, 1987
Cynomolgus monkey	Whole Blood	2	AQ-13	20	1441	12	21,5	43935,86	-[a]	Ramanathan-Girish *et al.*, 2004
Cynomolgus monkey	Whole Blood	2	AQ-13	100	5516	48	57,75	697670,8	-[a]	Ramanathan-Girish *et al.*, 2004
Humans	Plasma	12	AQ	10	268.7	3.68	240.8	12041	-[a]	Orrell *et al.*, 2008
Humans	Plasma	7	AQ	10	181	3.4	-[a]	2304	8037	Winstanley *et al.*, 1987
Humans	Whole Blood	7	AQ	10	561	2,2	-[a]	6811	20074	Winstanley *et al.*, 1987
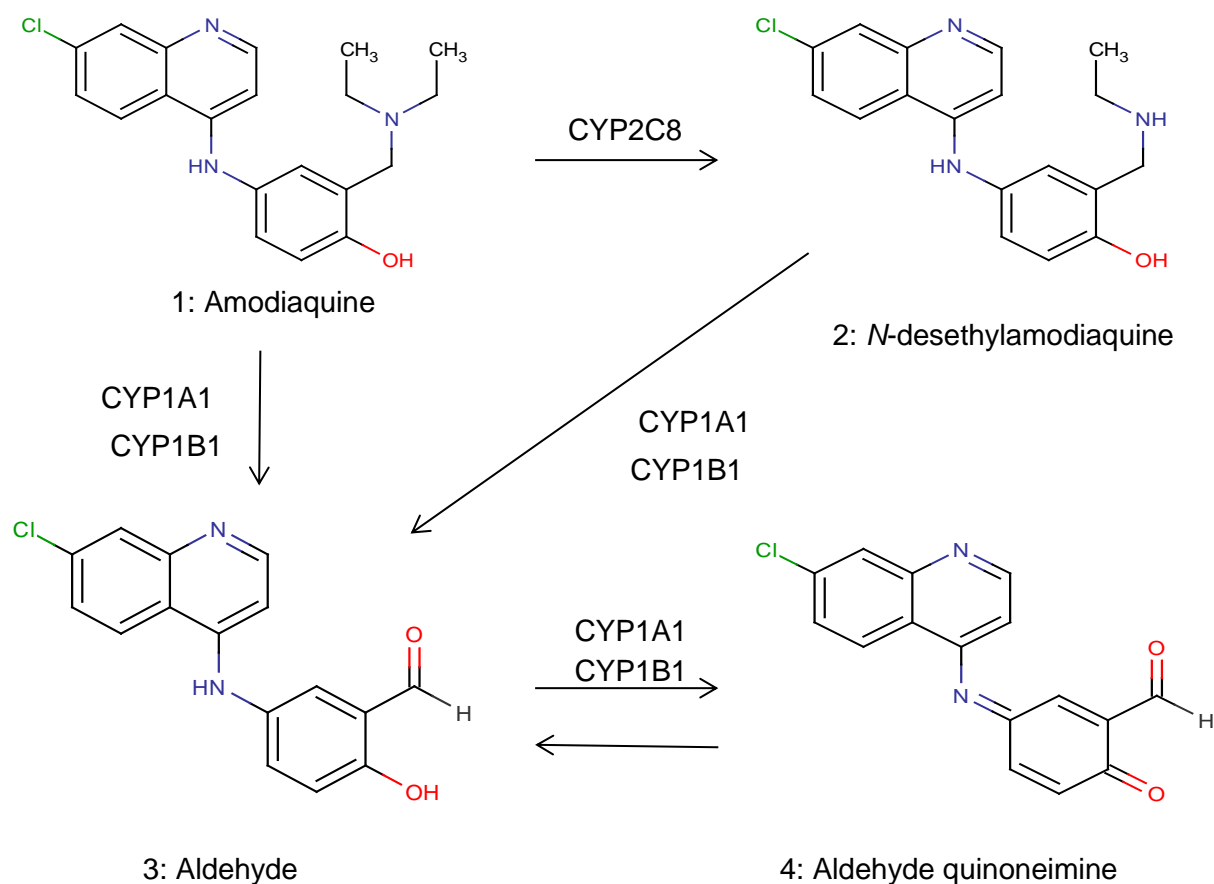
Figure 2.4: Metabolism of amodiaquine (Adapted from Johansson *et al*., 2009:576 with permission from the American Society of Pharmacology and Experimental Therapeutics).

Other minor metabolites include bisdesethylamodiaquine and 2-hydroxydesethylamodiaquine (Churchill *et al*., 1985; Laurent *et al*., 1993). Both the parent compound and main metabolite *N*-desethylamodiaquine have anti-malarial activity (Churchill *et al*., 1985). Amodiaquine is three times more active than *N*-desethylamodiaquine. An *in vitro* study using human liver microsomes and two sets of recombinant human CYP isoforms showed that CYP2C8 is mainly responsible for the oxidation of amodiaquine to *N*-desethylamodiaquine in the liver. Additionally, during the screening of 13 recombinant expressed CYPs the formation of an unknown metabolite mediated by CYP1A1 and CYP1B1 was observed. The unknown metabolite was not present in incubations with human liver microsomes (Li *et al*., 2002; Li *et al*., 2003). This novel metabolic pathway of amodiaquine was confirmed in a study performed by Johansson *et al*. (2009) in which the formation of additional metabolites by CYP1A1 and CYP1B1 that were not present in the human liver microsome incubations was observed. The metabolites include an aldehyde (Fig 2.4;3) and an aldehyde quinoneimine (Fig 2.4;4).

This may be explained by the fact that CYP1A1 and CYP1B1 are mainly expressed in extra-hepatic tissues *e.g.*, in leucocytes, which may be of interest in relation to the known ability of amodiaquine to induce agranulocytosis (Johansson *et al.*, 2009).

The therapeutic value of amodiaquine is significantly decreased by the biotransformation of its 4-hydroxyanilino aromatic moiety into a quinoneimine (Fig 2.4;4). The quinoneimine is highly susceptible to nucleophilic attack, mainly by thiols. This is the apparent cause for the severe hepatotoxicity with an incidence of 1 in 15,500 as well as the agranulocytosis with an incidence of 1 in 2100. These adverse effects have been observed after chemoprophylactic use of amodiaquine (Schlitzer, 2007).

2.3.1.1.2 Species differences between monkey and human cytochrome P450-mediated drug metabolism

In this thesis, the pharmacokinetic studies were performed with a non-human primate model. It is, therefore, important to discuss the difference between monkey and human cytochrome P450 mediated metabolism of amodiaquine with respect to the CYP isoforms involved.

Animal models are commonly utilized to predict the metabolic behaviour of various drugs in humans during the preclinical development programme. It is important to realize that humans differ from animals with regard to isoform composition, expression and catalytic activities of drug metabolizing enzymes (Nelson *et al.*, 2004; Martignoni *et al.*, 2009; Uno *et al.*, 2011).

Non-human primates have often been used in biomedical research, including drug metabolism studies because of their evolutionary proximity to humans (Uno *et al.*, 2011). Among non-human primates, cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*) are widely used for these studies (Iwasaki & Uno, 2009). However, the oral bioavailability of some drugs is significantly lower in non-human primates when compared to humans. The latter could be explained by extensive first-pass metabolism in the intestine of non-human primates (Takahashi *et al.*, 2009; Akabane *et al.*, 2010). Species differences in intestinal metabolic activities of the CYP3A4 family have been well characterized; however, species differences in the intestinal metabolic activities of other CYP isoforms between non-human primates and humans are not well documented (Nishimuta *et al.*, 2011). CYP studies have been reported for cynomolgus monkeys, rhesus monkeys and vervet monkeys (Komori *et al.*, 1992; Uno *et al.*, 2011), with the largest

number of CYP studies reported for the cynomolgus monkey. A total number of 23 CYP cDNAs from cynomolgus monkeys in the CYP1-4 families are deposited in GenBank. These sequences all show high homology (≤ 93%) to human CYP cDNAs. Seventeen rhesus CYPs have been deposited in GenBank, all of which are >99% identical to cynomolgus CYP cDNA. Only five CYP cDNAs have been identified in the vervet monkey. It should be noted that in each subfamily the CYPs of these primate species are much closer to humans than CYPs of the dog, rat or mouse (Uno *et al.*, 2011). Among monkey CYP cDNAs identified, some CYPs have been subjected to functional characterization, including conformation of metabolic activity measured using human CYP substrates. Most monkey CYPs metabolize typical substrates of human CYPs in the same subfamilies.

2.3.1.1.3 CYP1A

This subfamily consists of two members, namely CYP1A1 and CYP1A2 in the monkey and human. CYP1A1 is expressed at very low levels in human liver and it is essentially an extra-hepatic enzyme that is predominantly present in the intestine, lung, placenta and kidneys. Furthermore, there are no reports on CYP1A1 activity in monkey intestine (Martignoni *et al.*, 2009). CYP1A isoforms illustrate a strong conservation among species (Sharer *et al.*, 1995). Cynomolgus CYP1A1 and CYP1A2 cDNAs are 95% homologous to human CYP1A1 and CYP1A2 cDNA respectively (Komori *et al.*, 1992; Sakuma *et al.,* 1998; Uno *et al.*, 2011).

Cynomolgus CYP1A1 is involved in *7-ethoxy-resorufin O-deethylation* (EROD) (Komori *et al.*, 1992). Similarly, human CYP1As also catalyze EROD and mediate activation of heterocyclic amines (Kim & Guengerich, 2005). However, varying results have been described in the literature regarding the catalytic activities of the CYP1A isoforms of monkeys and humans. Edwards *et al.* (1994) found that the livers of cynomolgus monkeys lacked detectable expression of CYP1A1 by immunoblot analysis. In contrast, Iwasaki and Uno (2009) found that the catalytic activity of CYP1A1 was very similar to that of humans and Sharer *et al.* (1995) illustrated that the catalytic activities of cynomolgus monkey CYP1A1 are significantly higher when compared to those of humans (Sharer *et al.*, 1995). Interestingly there have been reports that CYP1A1 mRNA is expressed much more abundantly than CYP1A2 mRNA in the liver of cynomolgus monkeys (Sakuma *et al.*, 1998). In contrast, CYP1A2 is expressed much more abundantly than CYP1A1 in human liver (Shimada *et al.*, 1994). These results suggest that cynomolgus monkey CYP1A generally have enzyme characteristics similar to human CYP1As, but functional differences have been observed between species despite the high sequence identities (Sharer *et al.*, 1995).

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These differences could be explained by differences in the expression and affinity of the CYP1A family between humans and monkeys or the possible involvement of additional cynomolgus monkey cytochrome P450 isoforms in the catalytic activity of EROD (Edwards *et al.*, 1994; Sharer *et al.*, 1995). Whether this difference is caused by genetic factors or environmental factors is unknown (Sharer *et al.*, 1995).

2.3.1.1.4 CYP2C

The CYP2C subfamily is the most complex subfamily of the CYPs found in both human and animal species, with several different isoforms of these specific enzymes (Martignoni *et al.*, 2009). CYP2C is present in rodent and non-human primate species, and its expression in extra-hepatic tissues is isoform specific. In humans the CYP2C family is involved in the metabolism of about 16% of drugs currently on the market (Kings, 2009).

In the cynomolgus monkey, CYP2C8 cDNA is 95% homologous to human CYP2C8 cDNA (Ohi *et al.*, 1989; Komori *et al.*, 1992; Uno *et al.*, 2006). Furthermore, the CYP2C8 cDNA of the African green monkey is highly similar to cynomolgus CYP2C8 cDNA. The CYP2C8 of the African green monkey still needs to be characterized (Uno *et al.*, 2011). Cynomolgus CYP2C8 mRNA is predominantly expressed in the liver (Uno *et al.*, 2011), but its mRNA has also been detected in the duodenum (Uno *et al.*, 2007; Martignoni *et al.*, 2009; Klose *et al.*, 1999). The latter were also supported by the results of a study performed by the group of Nishimuta *et al.* (2011) in which they observed that the intestinal CL_{int} values of the CYP2C8 substrate (amodiaquine) were significantly higher in the cynomolgus monkey compared to the intestinal CL_{int} in humans. Amodiaquine was metabolized in the cynomolgus monkey intestine and the metabolic activities were inhibited by all anti-human CYP2C antibodies but not by anti-human CYP3A antibodies. CYP2C8 may, therefore, be involved in greater intestinal first-pass metabolism in the cynomolgus monkey. In addition, there is a possibility that similar CYP2C isoforms may be involved in the metabolic activities of the intestine and liver, because the monkey intestinal CL_{int} values correlated well with those of the liver (Nishimuta *et al.*, 2011).

2.3.1.2 Non-microsomal metabolism

Amodiaquine can be oxidized by non-microsomal metabolism. In a study by Maggs *et al.* (1988) the *in vitro* metabolism of amodiaquine was investigated with specific reference to the formation of chemically reactive quinoneimines. Results from the study showed that amodiaquine and *N*-desethylamodiaquine is susceptible to autoxidation to reactive

quinoneimines, in a solution at physiological pH under air. It is presumed that the presence of the aromatic *N*-substituent is responsible for the autoxidation of the 4-hydroxyanilino aromatic moiety in amodiaquine (Maggs *et al.*, 1988; Winstanley *et al.*, 1990; Jurva *et al.*, 2008).

2.3.1.3 Amodiaquine toxicity

The adverse effects of amodiaquine do have a parallel with those of chloroquine, although adverse effects recorded for amodiaquine are more severe, and in general, amodiaquine is regarded as a more toxic drug. After a single oral administration of amodiaquine the main adverse effects include nausea, vomiting, abdominal discomfort, diarrhoea headache, skin irritations, dizziness, blurring of vision and fatigue. Amodiaquine is associated with a lesser degree of pruritus and is generally more palatable compared to chloroquine. It is associated with agranulocytosis and hepatotoxicity when used for chemoprophylaxis. The mechanism involved in agranulocytosis and hepatotoxicity has not yet been fully elucidated, but it has been proposed that the reactive quinoneimine could be responsible by exerting its toxicity through covalent bindings to various cell components, leading to direct disruption of its functions and/or triggering an immunological response. An overdose is associated with cardiotoxicity, syncope spasticity, convulsions and involuntary movements (Temple, 2007; WHO, 2010).

2.3.2 Drug interactions and contraindications

Limited literature is available regarding the drug interactions and contraindications of amodiaquine. Amodiaquine should be used with caution in patients with hepatic or blood disorders (Temple, 2007). Chemoprophylactic use of amodiaquine is associated with agranulocytosis and hepatotoxicity. Amodiaquine significantly decreases the activity of CYP2D6 and CYP2C9 (Wennerholm *et al.*, 2006). Amodiaquine co-administered with antiretroviral drugs (efavirenz, saquinavir, lopinavir and tipranavir) inhibits CYP2C8, the main CYP P450 isoform responsible for the biotransformation of amodiaquine (Parikh *et al.*, 2007).

2.3.3 Mechanism of action of amodiaquine

Amodiaquine and chloroquine are not only structurally related; amodiaquine also competitively inhibits CQ accumulation and *vice versa*. It is, therefore, suggested that these compounds share a similar mode of action. Like CQ, amodiaquine is a diprotic weak

base with lower pK_a values (pK_{a1} = 7.1 and pK_{a2} = 8.1) compared to those of CQ (pK_{a1} = 10.8 and pK_{a2} = 8.3). This raises the expectation that amodiaquine will accumulate less efficiently within the parasite DV compared to CQ. However, the accumulation of amodiaquine in the DV of the parasite is in fact more efficient than that of CQ, which indicates that the uptake of amodiaquine is enhanced by additional mechanisms (Foley & Tilley, 1998). Furthermore, amodiaquine interferes with ferriprotoporphyrin IX detoxification by binding to heme-Fe^{3+} and inhibiting the polymerization thereof (Mungthin *et al.*, 1998; Egan & Marques, 2001; Olliaro, 2001; Kumar *et al.*, 2007). This inhibition results in heme-Fe^{3+} induced oxidative stress (Kumar *et al.*, 2007). A recent alternative hypothesis (discussed in section 2.2.3) involving drug-assisted transport of heme-Fe(III) out of the DV into the cytosol may also play a role in the mode of action of AQ (Haynes *et al.*, 2012).

2.4 Chloroquine and amodiaquine drug resistance

Drug resistance in malaria is an important public health concern. Drug resistance has been reported in *P. falciparum, P. vivax* and *P. malariae*. Anti-malarial drug resistance in malaria is defined by the WHO as "… the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those normally recommended but within the tolerance of the subject" and its posterior addendum "the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action" (WHO, 2010). Several factors contribute to the development and spread of anti-malarial drug resistance including interactions of drug patterns, characteristics of the drug itself, and human host factors.

2.4.1 Development of chloroquine and amodiaquine resistance

The development of resistance against CQ and AQ, especially CQ, has been one of the biggest obstacles to emerge in the fight against malaria for the last few decades (WHO 2010). Figure 2.4 depicts the emergence of CQ and AQ resistance.

AQ resistance has been reported in South-America, Asia and East Africa (Muntabingwe *et al.*, 2005; Beshir *et al.*, 2010). During the 1990's AQ was removed from the approved anti-malarial list, due to concerns regarding the serious side effects, but was reinstated in 1996 (dashed border) (Ekland & Fidock, 2008).
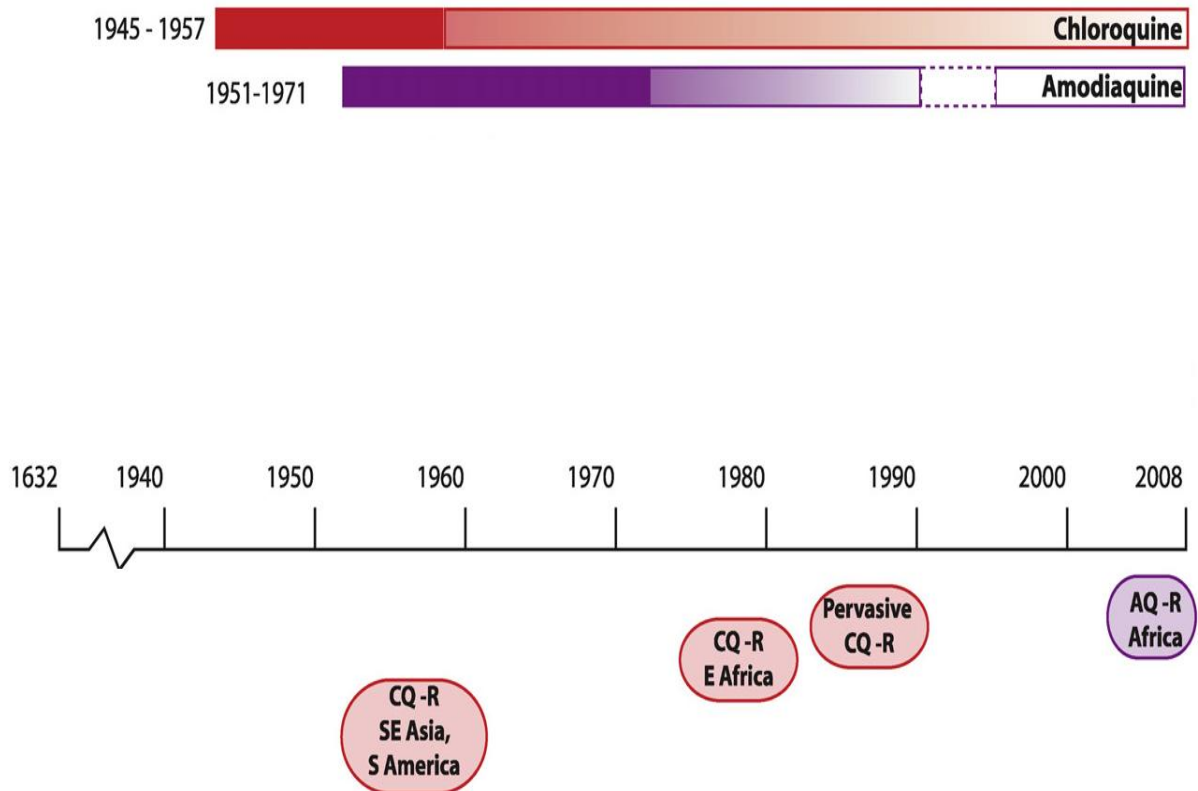
Figure 2.5: Emergence of CQ and AQ resistance (Adapted from Ekland & Fidock, 2008 with permission from Elsevier). The red bar represents CQ, the purple bar AQ. The years to the left of each bar represent the date the drug was introduced as treatment and the date to the right represents the first documented cases of resistance. The ovals below the time line indicate the approximate time frame when drug resistance spread through different geographical regions worldwide.

Resistance of *Plasmodium falciparum* to CQ was first observed in 1957 on the Thai-Cambodian border and in Colombia and spread across Africa during the 1980s. Today CQ resistance is virtually omnipresent and occurs almost everywhere where *P. falciparum* infections occur. However, in recent reports from Malawi, the first country to abandon CQ treatment in 1993 because of high rates of treatment failure of uncomplicated malaria, a gradual decrease in the prevalence of the P*F*CRT molecular marker of CQ resistance has been observed from 1992 to 2002, with complete disappearance in 2001 (Kublin *et al.*, 2003). Furthermore, a more recent study by Laufer *et al.*, (2006) supported the former findings, indicating a possibility of re-introduction of this safe, inexpensive and long-acting drug. Such a re-introduction will have to occur in combination with other drugs to prevent the re-emergence of resistance.

It will be important to consider cross-resistance between AQ and CQ, as several clinical and *in vitro* reports have shown cross-resistance between CQ and AQ (Bloland & Ruebush, 1996; White, 1996). AQ is a partner drug for artemisinin-based combination therapies in many African countries. Laufer and colleagues have suggested that one way to determine cross-resistance would be to compare the prevalence of CQ resistance (P*f*CRT) in areas where CQ treatment was withdrawn and replaced with ACT, and in areas where CQ was replaced with anti-malarial drugs unrelated to CQ (Kobbe *et al.*, 2007).

Many studies have been devoted to elucidating the mechanism responsible for CQ resistance. CQ-resistant parasites accumulate significantly less CQ than CQ-sensitive parasites (Fitch, 1970; Krogstad *et al.*, 1987). The latter suggests that drug-resistance results, at least in part, from exclusion of the CQ from the site of action. Point mutations in two genes of the *Plasmodium falciparum* genome have been associated with CQ resistance. The two genes include the *Plasmodium falciparum* CQ resistant transporter (*pfcrt*) gene and to a lesser extent the *Plasmodium falciparum* multi drug resistance 1 (*pfmdr1*) gene (Wongsrichanalai *et al.*, 2002; Ochong *et al.*, 2003).

The decreased accumulation of CQ in CQ-resistant parasites can be attributed to mutations in the *pfcrt* gene, which is located on chromosome 7, and codes for *P. falciparum* CQ-resistant transport protein (P*f*CRT) (Wongsrichanalai *et al.*, 2002). P*f*CRT is located on the membrane of the digestive vacuole of malaria parasites, and it has been proposed that the mutant form of the protein transports CQ out of the digestive vacuole (Sanchez *et al.*, 2005). Among the amino acid changes observed in this protein, the lysine to threonine change at codon 76 is the most strongly associated with CQ resistance. A recent study by Martin *et al.* (2009) illustrated that a mutation in P*f*CRT contributes to CQ resistance. They showed that in the parasite the presence of a mutant P*f*CRT on the digestive vacuole allows the transport of the protonated drug down its electrochemical gradient, hence out of the digestive vacuole and away from its site of action (Martin *et al.*, 2009). It has been illustrated that in CQ-resistant parasites the addition of CQ results in an enhanced leak of H^+ ions from the digestive vacuole, which is consistent with the drug in the protonated form effluxing from the CQ-resistant parasites (Lehane *et al.*, 2008). In a more recent study by Lehane and Kirk (2010), the authors illustrated that the same is true for a range of other anti-malarial drugs including AQ, as well as various chloroquine resistant reversers (Lehane & Kirk, 2010). The group of Ochong *et al.* (2003) demonstrated that P*f*CRT is a marker for CQ and AQ resistance *in vivo* (Ochong *et al.*, 2003). Based on the literature, it appears that mutations in P*f*CRT play a prominent and definite role in the resistance of CQ and AQ and that the *pfcrt* mutant could be used as a predictor of both CQ and AQ resistance. Another

gene that contributes to CQ resistance is the *pfmdr1* gene. The observations that CQ-resistant parasites accumulate less CQ when compared to CQ-sensitive parasites and that verapamil can modulate this process suggested similarities with the multidrug-resistant (MDR) phenotype of cells. MDR is thought to be caused by an enhanced expression of an ATP-dependent drug transporter known as P-glycoprotein (Riordan *et al.*, 1985; Martin *et al.,* 1987), which is postulated to confer resistance by promoting the efflux of a wide range of cytotoxic drugs out of tumor cells. The MDR gene, *pfmdr1* has been identified in *P. falciparum* (Wilson *et al.*, 1989) and is located on chromosome 5 and codes for a P-glycoprotein homologue 1 (Pgh 1) (Wongsrichanalai *et al.*, 2002). The point mutation of asparagine to tyrosine at codon 86 in the *pfmdr1* gene has been associated with CQ resistance. (Pleeter *et al.*, 2010; Wongsrichanalai *et al.*, 2002; Olliaro, 2001; Ochong *et al.*, 2003).

3. Pheroid Technology

Pheroid technology is a modified fatty-acid based delivery system with the ability to entrap, transport and deliver drugs of markedly different structures. The pheroid delivery system is a colloidal system comprising of unique and stable structures called pheroids, which are evenly distributed within a dispersion medium. The unique composition of the pheroid offers valuable characteristics that may be useful in delivery of anti-malarial compounds. The characteristics include enhanced absorption and/or efficacy of therapeutic compounds, decreased minimal effective drug concentration, decreased cytotoxicity of therapeutic compounds, movement across physical barriers such as the erythrocytic cell membranes and decreased drug resistance. By using pheroid technology, it may be possible to decrease the drug dosage, reduce side effects and shorten exposure to the drug due to shorter treatment time. A number of applications of this technology have been patented in various countries (Saunders *et al.*, 1999; Meyer, 2002; Grobler, 2007; Grobler & Kotzé, 2006; Grobler *et al.*, 2009). Furthermore, the pheroid formulations are easy to prepare, do not use any rigid nanomaterial or supporting polymer matrix, and are more economical than most other drug delivery systems.

3.1 Historical perspective of pheroid technology

Pheroid technology has its origin in Emzaloid™ technology. The latter was unknowingly used to treat and cure the psoriasis of the founder Piet Meyer of MeyerZall (Pty) Ltd. After initial observations that the Emzaloid™-based psoriasis product proved to be more effective than commercially available products for psoriasis, a research programme was initiated in

collaboration with the South African Medical Research Councal (SAMRC) to aid in determining the reason behind the success of their product. The SAMRC concluded that the psoriasis product contained micro-vesicles, which led to the hypothesis that the vesicles constituted a delivery system with the ability to possibly enhance the absorption of active ingredients. This hypothesis was proven to be correct (Saunders *et al.*, 1999; Tzaneva *et al.*, 2003; Goodfield *et al.*, 2004). During 2003, the North-West University (NWU) obtained all intellectual property with regards to the Emazaloid™ technology. The delivery system was subsequently optimized and after being obtained by the North-West University, a solid founding technology for various applications was established (Grobler, 2008).

It is important to note that Emzaloid™ technology was not renamed to pheroid technology and that distinct differences do exist between Emzaloid™ technology and pheroid technology. Firstly, Emzaloid™-based products are manufactured under low pressure gas exposure (80 kPa), for the duration of 4 hours, resulting in under saturation of the formulation with the gas, whereas pheroids are manufactured using a higher pressure gas exposure (200 kPa) for four days, resulting in a nitrous oxide saturated formulation. Secondly, all pheroid-based products contain α-tocopherol (Grobler, 2008). The functions of the different components in pheroid technology will be discussed in section 3.2.2.

3.2   Pheroid types, components, characteristics and functions

3.2.1   Pheroid types

Pheroids can be manipulated in terms of structure, size, morphology and function to suit the solubility characteristics (hydrophilic or hydrophobic nature) of the drug that needs to be entrapped, transported and delivered to the site of action. Additional important factors to bring into consideration when deciding on the type of pheroid to manufacture includes: the rate of delivery as well as the route of administration. Various structures of pheroids can be manufactured, however three main structures exist namely, lipid-bilayer pheroid vesicles, which range in size from 80 – 300 nanometers; pheroid microsponges ranging between 0.5 and 5.0 micrometers and pro-pheroids, which can be regarded as a precursor for pheroid vesicles (Grobler, 2008). The confocal laser scanning micrographs in Figure 3.1 show the structures of lipid bilayer pheroid vesicles and pheroid microsponges.

(a)   (b)
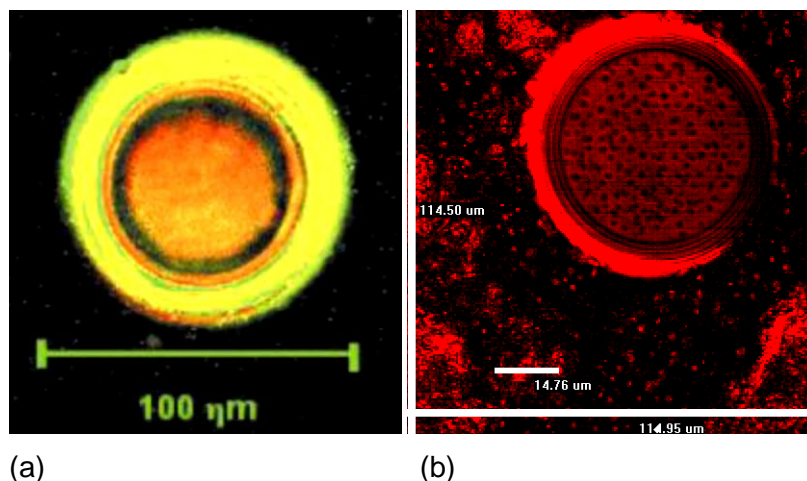
Figure 3.1: Confocal laser scanning micrographs of (a) rifampicin entrapped in a pheroid vesicles. The multiple layers of the multilamellar vesicle is visible in yellow as a result of fluorescent labeling with Nile red, while the red interior fluorescence is that produced by rifampicin and (b) pheroid microsponges of the reservoir type with a mean diameter of 35–40 μm (Reprinted from Grobler, 2008:149 with permission from the author).

3.2.2 Pheroid component characteristics and function

The main components of the pheroids are derived from ethyl esters of essential fatty acids, pegylated ricinoleic acid, α-tocopherol and nitrous oxide saturated water. During this section each component used in the manufacturing of pheroids and pro-pheroids will be discussed in some depth.

3.2.2.1 Essential fatty acid component

The fatty acid component typically used in the manufacturing of pheroids and pro-pheroids consists primarily of ethylated and pegylated polyunsaturated fatty acids or esters thereof including oleic acid, linoleic acid, alpha-linolenic acid. The typical fatty acid distribution of pheriod nanovesicles is as follows:

- $C_{16.0}$: 8.3%;
- $C_{18.0}$: 3.5%;
- $C_{18.1}$ (oleic acid) : 21.7%;
- $C_{18.2}$ (linoleic acid) : 34.8%;
- $C_{18.4}$ (α-linolenic acid) : 28%;
- $> C_{18}$: 1.6% and
- Unknown : 2.1% (Grobler, 2008)

Figure 3.2 illustrated a hypothetical model of a membrane region of a pheroid vesicle, and the manner in which the fatty acid ethyl esters assembles into membrane like structures. The nitrous oxide and α-tocopherol components are not accommodated in the model yet.
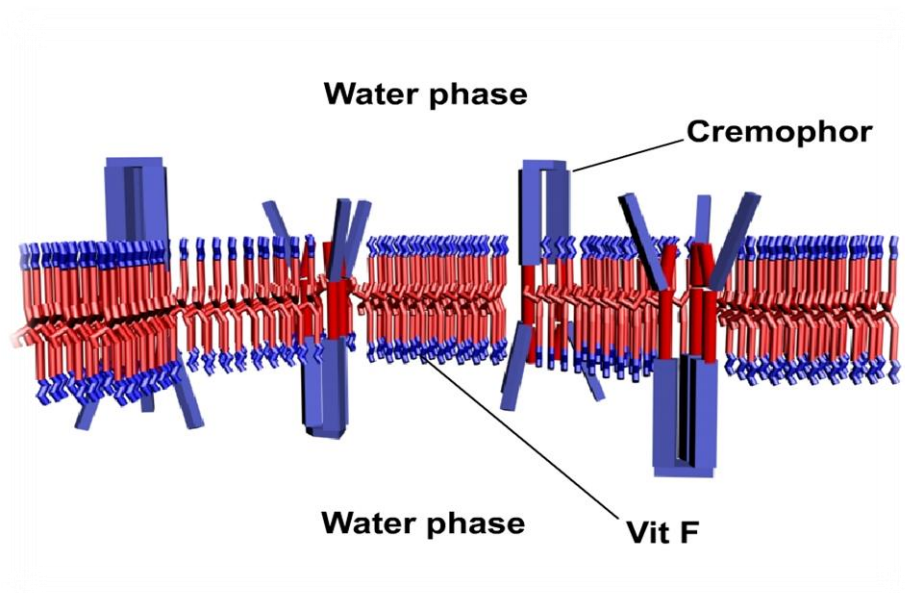
Figure 3.2: A schematic model of the fatty acid components of the generic pheroid vesicle. The blue regions represent the hydrophilic domains, red regions the hydrophobic domains. Each fatty acid ethyl ester is sketched as a red hydrocarbon chain with a blue ethyl ester attached. The hydrocarbon backbone are bent where unsaturated C=C bonds occur. The pegylated ricinoleid acid molecules form the pore structures or channels. The overall structure is referred to as a pro-pheroid. After the application of the drug, and once admixed with nitrous-oxide water, the pro-pheroid reassembles into pheroid vesicles (Reprinted from Grobler, 2008:194 with permission from the Author).

The essential fatty acid content contributes to the inherent therapeutic qualities of pheroids. Functions of this component in pheroids include: maintenance of membrane integrity of cells, modulation of the immune system and energy homeostasis (Grobler, 2008).

3.2.2.2 Nitrous oxide component

Nitrous oxide (N_2O) is the gas known as "laughing gas" and is both water and lipid soluble (Evers *et al.*, 2006). It has been in use for many years as an inhalation anesthetic and analgesic (Berkowitz *et al.,* 1979). Molecular modeling studies indicated that interactions exist between the fatty acids and the nitrous oxide, result in a nitrous oxide essential fatty acid matrix that provides a functional model for transporting hydrophilic as well as

hydrophobic drugs (Grobler, 2008). Nitrous oxide contributes to the miscibility of the essential fatty acids, imparts an ordered structure to the self-assembled vesicle, and ensures its stability. Such vesicles are said to maintain structural integrity over periods of greater than two years at room temperature (Steyn *et al.*, 2011)

3.2.2.3 α-Tocopherol component

Vitamin E is a collective generic name that includes four tocopherols (α-, β-, γ- and δ-) and four tocotrienols (α-, β-, γ- and δ-) of which α-tocopherol is the most important. α-Tocopherol, one of the components of pheroids, is a lipid soluble vitamin, and has been used as a solvent for lipophilic drugs (Salway, 2006; Constantinides *et al.*, 2006). Additional to the role of solubilisation of hydrophobic drugs, α-tocopherol also attributes anti-oxidative qualities to both the drug and fatty acids of the pheroid membranes. α-Tocopherol is classed as a scavenger of lipid peroxyl radicals, which prevents peroxidation of polyunsaturated fatty acids contained in cellular and sub-cellular membrane phospholipids (WHO, 2004).

3.3 Application of Pheroid technology as an anti-malarial drug delivery system

In vitro efficacy and *in vivo* bioavailability and efficacy studies in a murine model have been carried out for anti-malarial compounds incorporated into pheroids at MeyerZall Laboratories (Pty) Ltd., the North-West University (NWU), the University of Cape Town (UCT) and at the Swiss Tropical and Public Health Institute (STPHI). As previously mentioned various applications of pheroid technology have been patented and can be divided into eight classes including: bio-agricultural; central nervous system; nucleic acids; topical; therapeutic proteins; vaccines; anti-inflammatory and anti-infective agents. The anti-infective class can be divided into three sub-classes namely anti-virals; anti-bacterials and anti-protozoans. The studies in this thesis are an expansion of the studies described in the anti-protozoan subclass of existing patent and focus on the quinolines, specifically chloroquine and amodiaquine. In parallel, a number of studies were and are being conducted on other classes of anti-malarials, including the artemisinins (Steyn, 2009; Steyn *et al.*, 2011). The results of previous studies, which are relevant to my studies are summarized in Table 3.1 and Table 3.2.

3.3.1 *In vitro* efficacy studies

Table 3.1: *In vitro* efficacy studies conducted at both, NWU and STPHI

Compound	Institute	Strain	Enhancement factor	Outcome
Chloroquine	NWU[a]	RSA-11	2	For the pheroid-chloroquine formulation: Enhancement of in vitro anti-malarial activity
	STPHI[b]	K1	2	For the pheroid-chloroquine formulation: 1.7 - 2 fold increase in all strains
		NF45	1.9	
		RSA-11	1.7	
Amodiaquine	NWU[a]	RSA-11	1.7	For the pheroid-amodiaquine formulation: Enhancement of in vitro anti-malarial activity

NWU: conducted a standard 48 hour microscopy *in vitro* efficacy study; STPHI: conducted a [3H]-hypoxanthine incorporated *in vitro* efficacy study; RSA-11: chloroquine resistant *P.falciparum* strain; K1: chloroquine resistant *P.falciparum* strain; NF45: drug sensitive isolate.

a: Langley, 2010; b: Jourdan, 2011

3.3.2 *In vivo* bioavailability studies in a murine model

Table 3.2: Summary of pharmacokinetic parameters for chloroquine and amodiaquine

Compound	C_{max} (ng/ml)		T_{max} (h)		$T_{1/2}$ (h)		$AUC_{(0-last)}$ (ng.h/ml)		Outcome
	Ref	Phe	Ref	Phe	Ref	Phe	Ref	Phe	
Chloroquine[a]	701.5±49.6	735.9±43.6	1.5±0.17	1.7±0.15	4.5±0.41	5.43±0.4	4606±316	4971±267	No difference observed
Amodiaquine[a]	581.8±45.44	1015±100.2	1.35±0.43	0.6±0.07	3.88±0.4	3.49±0.3	2410±182	4401±498	For the Pheroid-amodiaquine formulation: 1.8 fold increase in C_{max} and AUC_{0-last}

Murine model: male C57 BL6 mice; Dosage: 20 mg/kg

a:Langley,2010

4. Preclinical pharmacokinetic primate model

The pharmaceutical industry is required to include a non-rodent species in preclinical studies. Recently, Ward and colleagues performed comprehensive meta-analysis studies comparing pharmacokinetics in humans, non-human primates (NHP), dogs and rats during which they concluded that data collected from the NHP were the most predictive of human pharmacokinetics, especially for extrapolation of drug clearance and volume of distribution (Ward & Smith, 2004; Jolivette & Ward, 2005; Ward *et al.*, 2005; Ward *et al.*, 2001; Evans *et al.*, 2006; Tang *et al.*, 2007).

The Vervet monkey has long been among the most important NHP models used for biomedical research, with a PubMed citation record of close to that of rhesus macaque (> 10 000 publications) and much greater than any other NHP (Cynomolgus monkeys (*Macaca fascicularis*) ~ 5000 and all species of baboon (*Papio spp*) ~ 2500). Vervet monkeys have been extensively used in other areas of biomedical research including investigations of:

- Neurobehavioural phenotypes;
- Neurodegeneration;
- Neuroanatomic variability;
- Immune system and infectious disease and
- Metabolic phenotyping.

Vervet monkeys are not commonly used in drug development studies and limited literature is available for oral pharmacokinetics studies involving these monkeys. However, a recent pharmacokinetic study conducted with vervet monkeys (Ward *et al.* 2009) involved an examination of interspecies predictive performance for eight commercially available drugs. Oral pharmacokinetic data were available for these drugs from the literature involving humans as well as cynomolgus and rhesus monkeys. It was concluded that oral bioavailability results obtained from vervet monkeys, provided a similar predictor of human oral bioavailability as did results obtained from cynomolgus or rhesus monkeys (Ward *et al.*, 2009). Furthermore, an oral study by Lee *et al.*, (2006) with chlorzoxazone in vervet monkeys suggested similar oral systemic exposure compared to humans. Therefore, it is concluded that the vervet monkey may be used as a surrogate for cynomolgus or rhesus monkeys in preclinical pharmacokinetic studies (Ward *et al.*, 2008; Ward *et al.*, 2009).

Therefore, for this thesis, the vervet monkey was used as the non-human primate model to investigate the effect of pheroid technology on the oral bioavailability of chloroquine and amodiaquine.

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AN LC-MS/MS METHOD FOR THE DETERMINATION OF CHLOROQUINE IN HUMAN AND MONKEY WHOLE BLOOD AND PLASMA

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Abstract

A sensitive and selective liquid chromatography-tandem mass spectrometry method was developed and validated for the quantification of chloroquine in 50 µL human whole blood and plasma over the concentration range of 4 – 500 ng/mL. Sample preparation was achieved by a liquid-liquid extraction, using a universal Britton Robinson buffer (pH 9) and ethyl acetate containing the internal standard (amodiaquine). Chromatographic separation was achieved on a Luna 5 um PFP (2), 100 A, 50 x 2mm analytical column using acetonitrile and 0.1% formic acid (68:32, v/v) as the mobile phase, at a flow rate of 0.5 mL/min. Chloroquine was quantified in the positive ionization mode with a triple quadrupole mass spectrometer. The mass transitions *m/z* 320.3 → *m/z* 247.1 and *m/z* 356.1 → *m/z* 283.1 were used to measure chloroquine and the internal standard, respectively.

The within-day and between-day precision determinations for chloroquine in whole blood and in plasma, expressed as the percentage coefficient of variation, were lower than 7% and 6% respectively at all test concentrations. Accuracy of the quality controls was between 92.3% and 108.5% for whole blood, and between 97.9% and 101.9% for plasma. No significant matrix effects were observed for the analyte or internal standard. The assay was successfully used to examine the pharmacokinetics of chloroquine in monkey plasma and whole blood as part of a preclinical study.

Keywords: Malaria, Chloroquine, LC-MS/MS, Liquid-liquid extraction, Validation

1. Introduction

Malaria, caused by protozoan parasites belonging to the genus *Plasmodium* remains one of the most widespread diseases and greatest burdens on humanity [1]. In 2010 there was an estimated 655,000 deaths globally of which 91% occured in sub-Saharan Africa. Groups mostly affected include children under the age of 5 and pregnant women [2, 3]. Chloroquine forms part of the 4-aminoquinolines, and has been widely used for the chemoprohylaxis and treatment of malaria, especially in special patient groups such as pregnant woman and children [4,5]. It has a relatively rapid onset of action, it is cost-effective to prepare and well tolerated. Unfortunately, the emergence and rapid spread of chloroquine-resistant strains of *P. falciparum* parasites led to a decline in the clinical use of chloroquine [6]. However, recent evidence from Malawi suggests that the sensitivity to chloroquine may return at least a decade after its withdrawal from first line treatment by the World Health Organization [7]. Hence the possibility of re-emergence of chloroquine as a therapeutic option for treatment of malaria exists.

Several methods have been published for the quantification of chloroquine in biological fluids, with the vast majority based on high performance liquid chromatography (HPLC) with UV or fluorescence detection [8-12]. Other methods that have been used for the quantification of chloroquine include high performance thin layer chomatography (HPTLC) [13], radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA) [14]. However all of these methods lack in sensitivity. The new gold standard for quantification of drugs in biological matrices is liquid chromatography tandem mass spectrometry (LC-MS/MS), owing to the improved sensitivity and selectivity of this analytical teqnique [15].

A number of papers on the quantification of chloroquine by LC-MS/MS have been published [15, 16]. Earlier methods are based on protein precipitation techniques, with the lower limit of quantifiation being around 1.25–2 ng/mL. The disadvantage of these assays is the requirement of large sample volumes (between 0.2 -0.5 mL). The aim of our study was to develop and validate a sensitive and selective LC-MS/MS method using small plasma and whole blood sample volumes to facilitate the quantification of chloroquine in human plasma and whole blood (50 µL). It was essential to establish a method capable of quantifying low as well as high concentrations of chloroquine and at the same time it was expected that this method would be efficient in analyzing large numbers of plasma and whole blood samples obtained during clinical and preclinical pharmacokinetic studies. We describe here a robust LC-MS/MS method coupled with a liquid-liquid extraction procedure for quantifiyng

chloroquine in 50 μL human or monkey plasma and whole blood samples. The method was validated according to the published FDA guidelines [17].

1. Experimental

2.1 Materials and Chemicals

Acetonitrile (LiChrosolv®), formic acid (pro analysis) and water (LiChrosolv®) were purchased from Merck kGaA, Darmstadt, Germany. The chloroquine diphosphate reference standard was supplied by Industrial Analytical (Kyalami, RSA) and amodiaquine hydrochloride (internal standard) supplied by Toronto Research Chemicals (TRC, Ontario, Canada). Drug free heparinised whole blood and plasma was obtained from the Western Province Blood Transfusion Service (Cape Town, South Africa). A Phenomenex Luna, PFP(2) 100A, 50mm×2.0mm column (Phenomenex, USA) was used for retaining chloroquine and the internal standard.

2.2 Chemical structure

The chemical structure of chloroquine is presented in Figure 1.
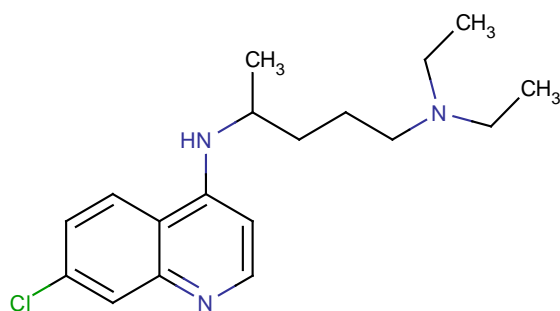
Figure 1: Chemical structure of chloroquine.

2.3 Instrumentation

The mobile phase was delivered with an Agilent 1200 series binary pump and the samples injected with an Agilent 1200 High Performance Autosampler (Agilent, CA, USA). Detection was performed by an AB Sciex API 3200 triple quadrupole mass spectrometer (AB Sciex, Ontario, Canada) fitted with a Turbo V^{TM} ion source.

2.4   Preparation of calibration standards

Calibration standards and quality controls (QC) were prepared in blank human plasma and whole blood, and the preparation procedure performed on ice. Chloroquine stock solutions (SS) were prepared in water at 1000 μg/mL (SS1), 100 μg/mL (SS2), 10 μg/mL (SS3) and 1 μg/mL (SS4). Blank matrix (10 ml each) was spiked with these stock solutions to attain the desired calibration standards; 500, 350, 250, 150, 80.0, 35.0, 16.0 and 4.00 ng/mL. The same methodology was used for the preparation of the high, medium and low QCs (400, 200 and 12 ng/mL), respectively.   The calibration standards and QC standards were briefly vortexed, aliquoted into polypropylene tubes and stored at approximately −80 °C.

2.5   Extraction procedure

An optimized liquid-liquid extraction method was developed for the extraction of chloroquine from human plasma or whole blood. The whole blood/plasma samples were thawed and briefly vortexed.   A universal Britton Robinson buffer (0.1 M) at pH 9 (100 μL) was transferred into clean polypropylene tubes and the whole blood/plasma sample (50 μL) was added to each tube. Ethyl acetate (1000 μL) containing the internal standard, amodiaquine, at 2 ng/mL was added to each of the extraction tubes. The samples were vortexed for 1 minute, centrifuged for 5 minutes at 2000 G and 750 μL of the organic layer was transferred into clean polypropylene tubes. This organic phase was evaporated under nitrogen at 30 °C for 20 minutes. The dried samples were reconstituted with mobile phase (100 μL), which consisted of acetonitrile: 0.1% formic acid dissolved in water (50:50, v/v). The samples were vortexed for 30 seconds and transferred to 96 well polypropylene plates. Two microliters of each sample was injected onto the HPLC column for analysis.

2.6   Liquid chromatography

Chromatography was performed on a Phenomenex Luna, PFP(2) (50mm×2.0mm, 5μm) analytical column. The mobile phase consisted of acetonitrile: 0.1% formic acid dissolved in water (68:32, v/v) and was delivered at a constant flow rate of 0.5 mL/min for 2 minutes. The analytical column was kept in a column compartment at a constant temperature of 35 °C. An Agilent 1200 series autosampler injected 2 μl onto the HPLC column. The injection needle was rinsed with mobile phase (acetonitrile and water with 0.1% formic acid (50:50, v/v)) before each injection for 10 seconds using the flush port wash station. The samples were cooled to 8 °C while awaiting injection. A representative raw chromatogram at the lower limit

of quantification (LLOQ; 4.00 ng/mL) for chloroquine in human plasma and whole blood is presented in Figure 2a and 2b, respectively.

2.7 Mass spectrometry

Electrospray ionisation (ESI) was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 50, 60 and 20 psi, respectively. The heated nebulizer temperature was set at 500 °C. The ionspray voltage was set at 4500 V. The instrument response was optimised for chloroquine and the internal standard by infusing a solution of the compounds dissolved in mobile phase at a constant flow. The pause time was set at 5 ms, the dwell time at 200 ms, and the collision gas (N_2) was set to 6 (arbitrary value).

The AB Sciex API 3200 triple quadrupole mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 320.3 to the product ions at m/z 247.1 and the protonated molecular ion m/z 356.1 to the product ions m/z 283.1 for the chloroquine and internal standard, respectively. The instrument was interfaced with a computer running Analyst version 1.4.2 software.

2.8 Method validation

2.8.1. Calibration standards and quality controls

The calibration curve for chloroquine in plasma and whole blood was validated by analysing quality control samples in each matrix in six fold at high, medium, and low concentrations (400, 200 and 12 ng/mL) over a period of 3 days to determine the intra- and inter-day accuracy and precision. The QC values were interpolated from the calibration curve. The calibration curve contained 8 different concentrations spanning a concentration range of 4 – 500 ng/mL. Individual calibration curves were constructed for each matrix using a weighted quadratic regression (1/concentration) of the peak area ratio of the analyte to its internal standard versus nominal concentration.

2.8.2. Stock solution stability

A stock solution (1 mg/mL) of chloroquine was prepared in water. A portion of the solution (test) was left at room temperature (approximately 21 °C) for 6 hours, and the remainder

kept at -80 °C (reference stock). Both the reference and test stocks were diluted with mobile phase at a high and low concentration (400 ng/mL and 12 ng/mL, respectively). Stability was determined by comparing the peak areas of the reference and test samples.

2.8.3. Freeze and thaw stability

To ascertain freeze-thaw stability, high (400 ng/mL) and low (12 ng/mL) QC standards in plasma and whole blood were frozen, put through three freeze (at -80 °C) and thaw (at room temperature) cycles and analysed against a valid calibration curve.

2.8.4. Benchtop stability

To establish benchtop stability, high (400 ng/mL) and low (12 ng/mL) QC standards in plasma and whole blood were frozen at -80 °C, left at room temperature (approximately 21 degrees Celsius) for 6 hours, and were analysed against a valid calibration curve.

2.8.5. On-instrument stability

A 40 hour on-instrument stability evaluation of chloroquine and the internal standard was performed. Two medium QC standards in plasma were extracted, pooled and analysed over three days. The samples were extracted and analysed on day 1, left on the autosampler and analysed again after 21 and 40 hours.

2.8.6. Matrix effects evaluation

Matrix effects were evaluated for both plasma and whole blood quantitatively. The method described by Matuszewski and co-workers (2003) was followed to evaluate the influence of co-eluting matrix components on chloroquine and the internal standard ionization [18]. Blank plasma and whole blood from ten different sources were extracted. The individual dried samples were reconstituted with mobile phase spiked with the analyte at a high and and low concentration (400 ng/mL and 12 ng/mL, respectively), and at one concentration of the internal standard. The quantitative assessment of matrix effects was obtained by comparing the peak area ratios.

2.8.7. Cross validation between plasma and whole blood

Ten whole blood and ten plasma matrices were spiked at a low concentration and were analysed against a valid calibration curve prepared in whole blood.

2.8.8. Specificity

The high specificity of MS/MS detection prevents the detection of compounds that do not produce the specific parent ion in the Q1 scan and the specific product ion in the Q3 scan. Specificity was assessed by analysing blank plasma and whole blood extracts.

3. Results and Discussion

The liquid-liquid extraction method for chloroquine performed well during validation. The calibration range was validated between 4 ng/mL and 500 ng/mL for both plasma and whole blood. A quadratic regression, with peak area ratio (drug/internal standard) against concentration with 1/concentration (1/x) weighting, was fitted to the calibration curves. The combined accuracy statistics of the quality controls (N=18; high, medium and low) were between 97.7% and 101.9% for the plasma, and 92.3% and 108.5% for the whole blood. The combined precision statistics were below 6% and 7% for plasma and whole blood, respectively. Recovery was reproducible at the high, medium and low levels for both plasma and whole blood.

Storage of the chloroquine stock solution in water at room temperature for 6 hours was examined. The accuracy of the stock solution test samples compared to the reference samples at room temperature was 96.0% (CV% = 3.0, N = 3) and 91.0% (CV% = 4.4, N = 3) for the high and low sample, respectively. Freeze-thaw stability was evaluated over three freeze-thaw cycles on consecutive days at the high and low concentrations. The observed concentrations for both plasma and whole blood were within 4% of the high and 6% of the low quality control concentrations. Chloroquine was found to be stable in both plasma and whole blood through three freeze-thaw cycles. The accuracy of chloroquine benchtop stability in plasma and whole blood over 6 hours at room temperature was 98.4% (CV% = 3.5, N = 6) and 100.0 % (CV% = 3.8, N = 6) at the high QC concentration, and 95.4% (CV% = 2.6, N = 6) and 91.9% (CV% = 9.2, N = 6) at the low QC concentration, respectively, indicating that chloroquine is stable in both plasma and whole blood at room temperature for up to 6 hours (the maximum anticipated time that future study samples will be left thawed until extracted). Lastly, sample extracts of chloroquine and the internal standard are stable

on-instrument at 8 °C for up to 40 hours. The calculated accuracies of the ratios ($N = 5$) were all well within 15% of the reference (ratios on day 1). After 21 hours (day 2) the accuracy was 99.6% and after 40 hours (day 3) the accuracy was 100.8%.

The effect of matrix components was evaluated quantitatively at a relatively high and low concentration using ten different plasma and whole blood sources. The coefficient of variation of the ten peak area ratios (drug/internal standard) was 3.4% at the high level and 2.7% at the low level for plasma, and 3.5% (high) and 3.4% (low) for whole blood (Table 1), indicating that sample analysis from different sources is reliable and reproducible.

Table 1: Assessment of matrix effects of ten different plasma and whole blood matrices

	Plasma		Whole blood	
	High 400 ng/ml	Low 12 ng/ml	High 400 ng/ml	Low 12 ng/ml
Mean ratio of peak areas	140	4.00	152	4.02
SD	4.80	0.108	5.38	0.136
CV %	3.4	2.7	3.5	3.4

In addition, a cross validation between plasma and whole blood was achieved by preparing the low concentration simultaneously in plasma and whole blood. The precision using ten different sources for each matrix was 3.0%. This insignificant difference infers that there is no effect of haemolysis on plasma quantitation.

Due to the high specificity of MS/MS detection, no interfering or late eluting peaks were found when analysing plasma or blank whole blood (Figures 3a and 3b). The lower limit of quantification (LLOQ) for both the plasma and whole blood method, when injecting 2 µl onto the column, is 4 ng/mL (Figures 2a and 2b).
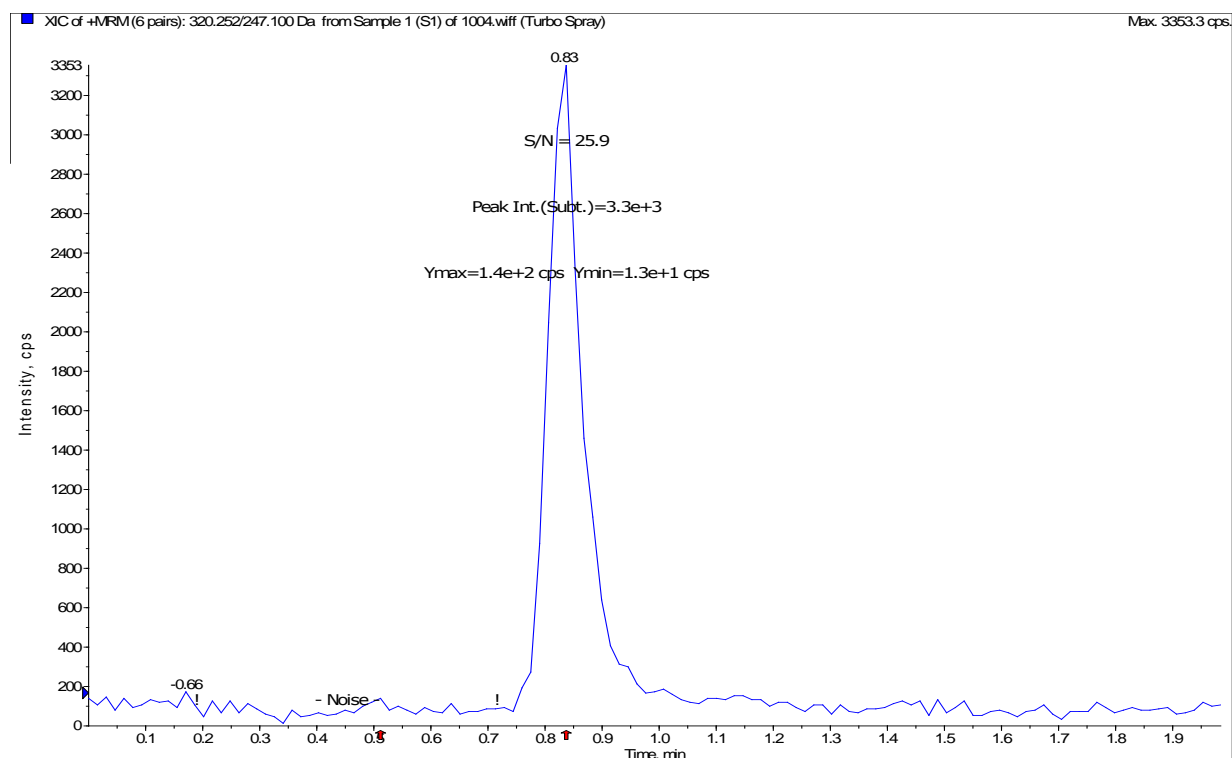
Figure 2a: Chromatogram of chloroquine calibration standard in human plasma at LLOQ (S/N = 25).
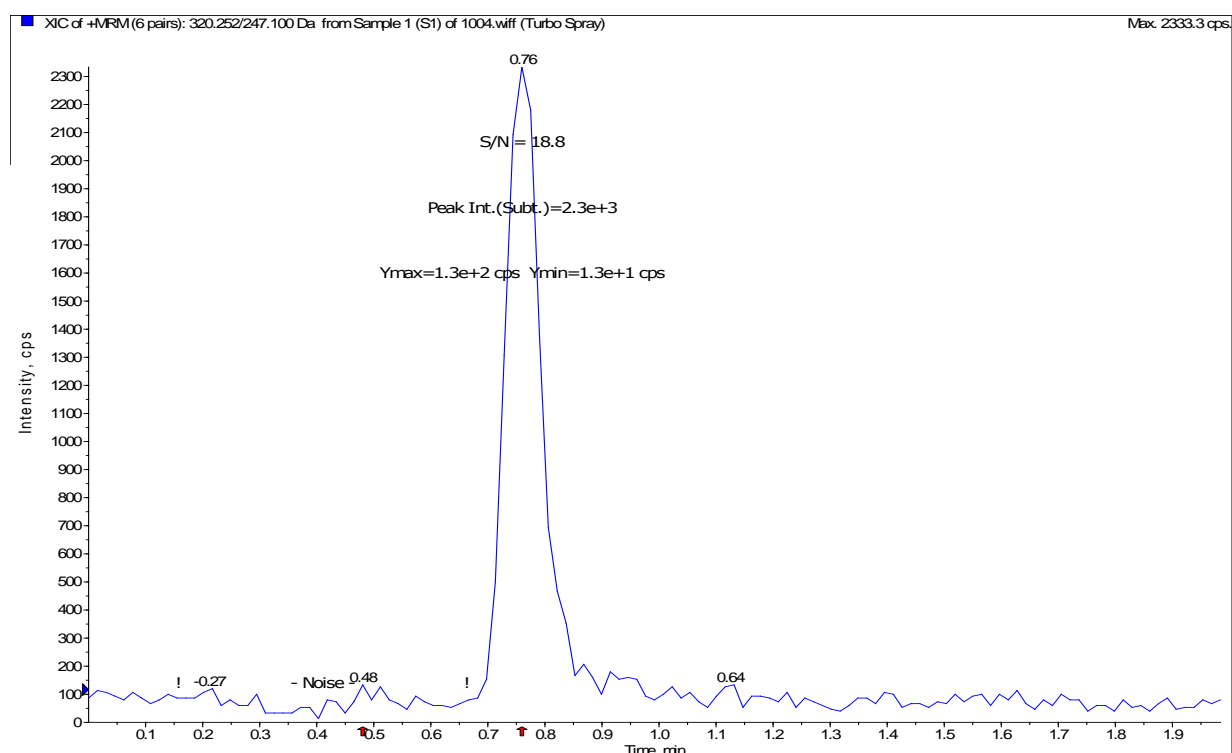
Figure 2b: Chromatogram of chloroquine calibration standard in human whole blood at LLOQ (S/N = 18).
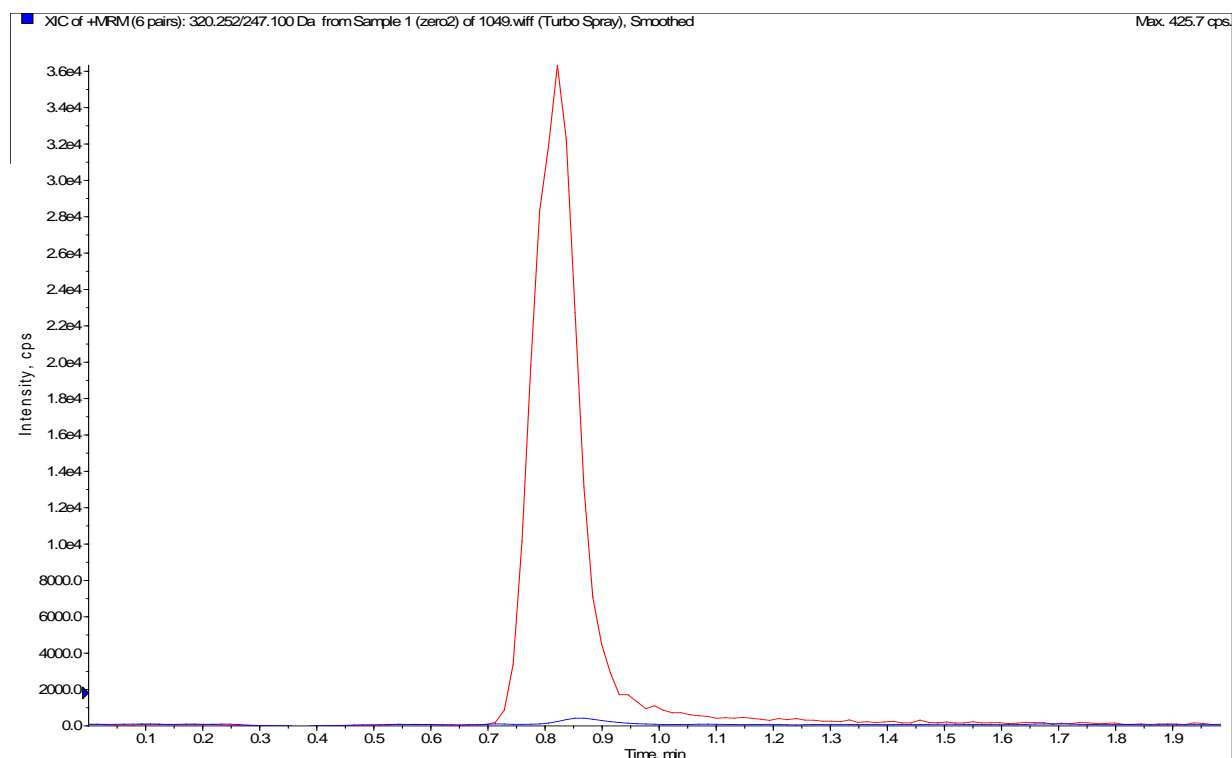
Figure 3a: Chromatogram of a blank plasma sample with amodiaquine internal standard.
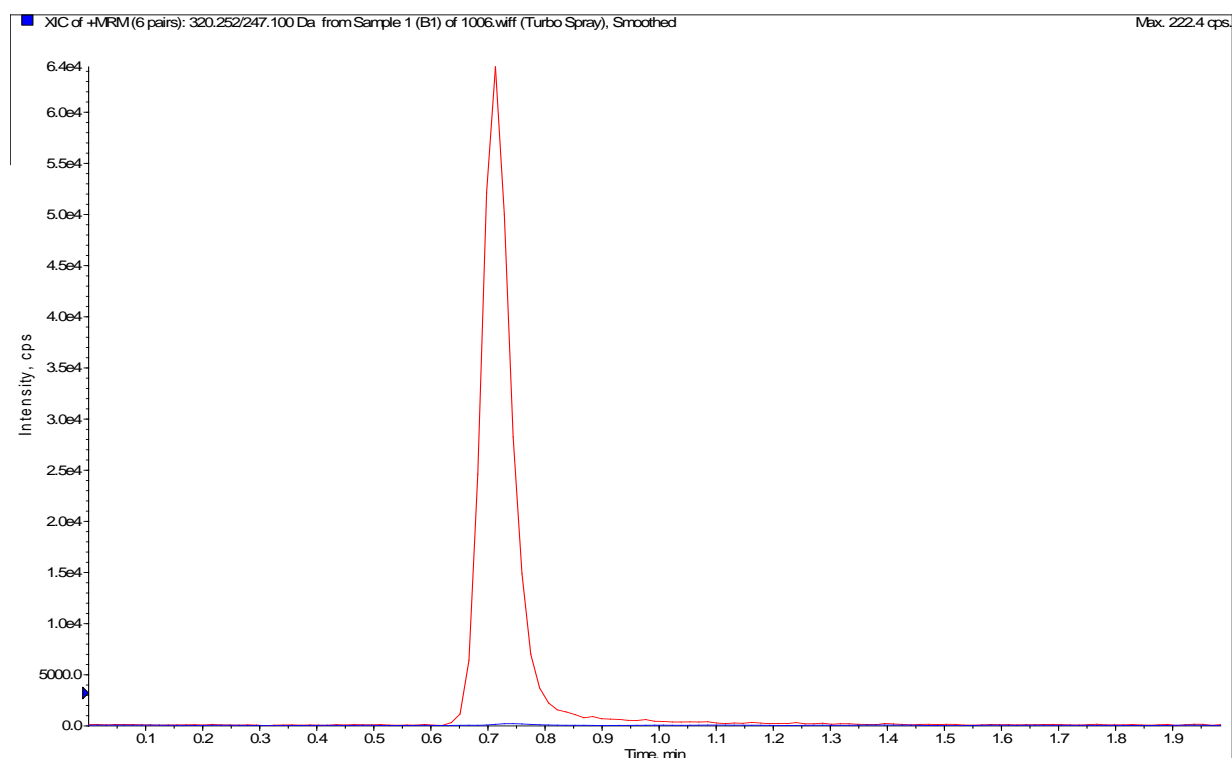
Figure 3b. Chromatogram of a blank whole blood sample with internal standard.

The signal to noise at LLOQ was well above the minimum international accepted criteria (S/N > 5). The accuracy for the plasma LLOQ (N = 17) was 105.6% (CV% = 9.5) and for the

whole blood LLOQ was 97.6% (CV% = 8.90), well within acceptable limits (accuracy within 80 – 120%, precision below 20%).

A cross validation in monkey whole blood was also performed using whole blood obtained from Vervet monkeys (*Chlorocebus aethiops*) from the animal facility of the Potchefstroom Campus, North-West University, South Africa. Control samples were prepared at high, medium and low concentrations (400, 200 and 12 ng/mL) in monkey whole blood and were analyzed against a valid human whole blood calibration curve and quality controls. The accuracy statistics of the controls when compared with the human whole blood QCs (*N*=6) were between 103.2% and 114.5%, within acceptable limits (accuracy within 85 – 115%).

4. Application to clinical and preclinical pharmacokinetics studies

The validated method performed well during analysis of preclinical samples generated during a preclinical study conducted at the animal facility of the Potchefstroom Campus, North-West University, South Africa. The objective of the study was to determine and compare the pharmacokinetics of formulated and unformulated chloroquine. The assay was applied to determine the levels of chloroquine in vervet monkey plasma and whole blood following oral administration at a dosage 20 mg/kg bodyweight. Initially, samples were analyzed over the concentration range of 4 ng/mL and 500 ng/mL for both plasma and whole blood samples. However, after analysis of the first batch of whole blood samples (n = 5), it was found that levels were far greater in whole blood than in plasma and consequently a broader range with a greater upper limit of quantitation was validated. The remaining whole blood samples (n = 10) were analyzed over the concentration range of 16 ng/mL and 5000 ng/mL. The accuracies for analysis of the first batch of whole blood samples were 104.3%, 106.7% and 99.3% at the low, medium and high QC level, with precision below 5.5%. The accuracies during analysis of the remaining whole blood samples were 98.8%, 102.3% and 101.1% at the low (40 ng/mL), medium (1000 ng/mL) and high (4000 ng/mL) QC levels respectively, with precision less than 7%. The precision (total-assay coefficients of variation; CV%) during plasma sample analysis was less than 5.1% at all three QC levels and the accuracies between 94.6% and 100.5%. A representative mean concentration versus time profile (up to 7 days; n = 7) of unformulated chloroquine in monkey whole blood is presented in Figure 4.
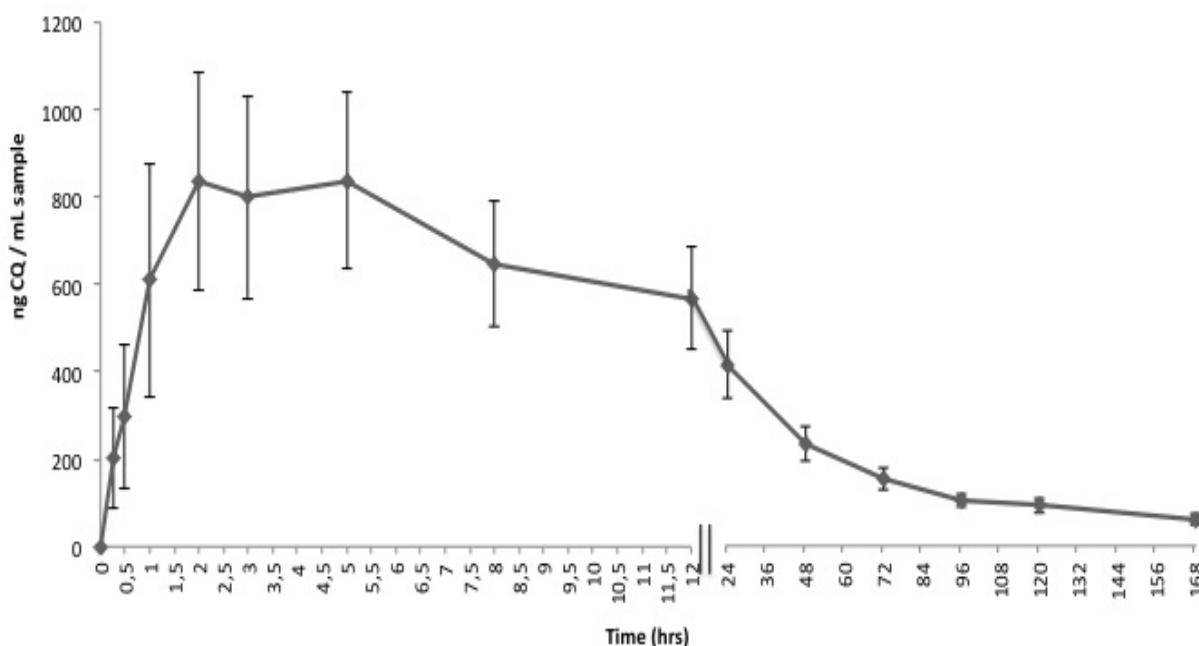
Figure 4: Concentration vs. time profile for chloroquine in monkey whole blood (*N* = 7).

5. Conclusion

A simple liquid-liquid extraction method coupled with LC-MS/MS detection has been developed and fully validated for the quantification of chloroquine present in both plasma and whole blood samples. This method is specific, sensitive and reproducible and has been successfully used to quantify chloroquine in a preclinical pharmacokinetic study in monkeys. A limited number of LC-MS/MS methods to quantify chloroquine have been published [15,16], all of which employ a protein precipitation extraction procedure and require plasma volumes of at least 200 µL. The LC-MS/MS method described here uses a solvent-solvent extraction technique, resulting in a much cleaner extract, and use smaller plasma and whole blood sample volumes (50 µL) and injection volumes (2 µL), with good sensitivity at the LLOQ. The lower plasma or whole blood sample volume is important for preclinical pharmacokinetic studies in monkeys in order to comply with the National Code for animal use in research, education, diagnosis and testing of related substances in South-Africa.

6. Acknowledgements

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THE EFFECT OF PHEROID® TECHNOLOGY ON THE BIOAVAILABILITY OF CHLOROQUINE IN PRIMATES

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Running title: Application of Pheroid® technology to malarial drugs

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Abstract

Malaria remains a potentially life threatening disease. The first reports of increased tolerance of the malaria parasite to artemisinins have alarmed the malaria community. This has rendered even more urgent the tasks both of improving existing drug treatment regimens so that these can be used more effectively, and of developing new drugs for malaria. One approach to enhancing the efficacy of existing drugs is to develop formulations that may enhance bioavailability and suppress possible toxic side effects, in particular when dealing with dual or fixed drug combinations. As a start to this proposed work, we describe here the application of Pheroid® technology that has the ability to entrap, transport and deliver therapeutically-useful compounds, to the anti-malarial drug chloroquine (CQ). Oral pharmacokinetic studies were conducted for CQ in Pheroid® vesicle formulations. Formulated CQ was administered orally at 20 mg/kg to vervet monkeys (*Chlorocebus aethiops*). Blood samples were collected from the femoral vein. The concentrations of CQ in both blood and prepared plasma samples were determined with a LC-MS/MS method. The C_{max} obtained for whole blood was 1039 ± 251.04 ng/mL for the unformulated reference sample of CQ and 1753.6 ± 382.8 ng/mL for the Pheroid® formulation. The AUC_{0-inf} was 37365 ± 6383 ng.h/mL (reference) and 52047 ± 11210 ng.h/mL (Pheroid®). The results indicate that the Pheroid® delivery system enhances the absorption of chloroquine. Pheroid® is a registered trademark but for ease of reading will be referred to as pheroid(s) throughout the rest of the article.

Introduction

Malaria remains one of the greatest burdens on humanity and is the fifth leading cause of death worldwide, with almost half the world's population at risk (1). Annual global deaths are estimated at approximately 655,000, of which 91% occur in Sub-Saharan Africa. Groups mostly affected include children under the age of 5 and pregnant women (2, 3). Malaria is caused by protozoan parasites of the genus *Plasmodium.* While five species cause malaria, *Plasmodium falciparum* is the most virulent. It is the intra-erythrocytic stages of the malaria parasite that are responsible for the clinical manifestations of the disease (4, 5). Efforts to control the disease have taken on an increased sense of urgency, given the increasing resistance of *P falciparum* to anti-malarial drugs. Recently a delay in the malaria parasite's response to artemisinin therapy was reported (6, 7). Such increased tolerance, reported initially in the Cambodia-Thailand region, where resistance was first reported for chloroquine in 1957 (8), is now being reported increasingly in Southeast Asia (7). Should this tolerance manifest itself in treatment failure, there will be little or no options for backup therapy (8).

The 4-aminoquinoline chloroquine (CQ) (Figure 1) was introduced 50 years ago as an alternative to quinine (9).
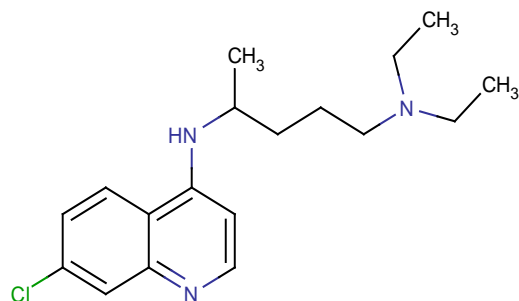
Figure 1: structure of CQ.

CQ exerts its anti-malarial activity by acting primarily on the late ring forms and mature trophozoite stages in the intra-erythrocytic life cycle of the parasite through accumulation within the acidic digestive vacuole (DV) where it inhibits the polymerization of the heme-Fe(III) to hemozoin(10, 11, 12, 13). Heme-Fe(III) is toxic to the malaria parasite (14, 4, 9).

The emergence and spread of CQ-resistant strains of *P. falciparum* parasites has resulted in a decline in the clinical use of CQ (15). Nevertheless CQ has many attractive features. It has a relatively rapid onset of action, it is economical to prepare and is well tolerated. It is also useful in the treatment of *P. vivax* and special patient groups such as pregnant woman

and children (16, 1). In humans, the oral bioavailability of CQ is ~ 80%. The drug has a large volume of distribution of > 100 L/kg and a half-life of 1-2 months. At the designated dose of 10-20 mg/kg CQ is an extremely safe drug. However, acute CQ toxicity may be associated with rapid administration of high doses of CQ via the parenteral route. Side effects when CQ is taken orally include GI upset, headache, visual disturbance and urticaria. Pruritus may also occur (17).

CQ-resistant parasites accumulate significantly less CQ in the DV than CQ-sensitive parasites (18). The decrease in accumulation is believed to be due to polymorphisms in the *P. falciparum* CQ resistant transporter gene (*PfCRT*) on chromosome 7, which effects CQ transport across the digestive vacuolar membrane of the parasite (4, 19). It is predicted that the efficacy of CQ may return at least a decade after its withdrawal from use, suggesting the possible re-emergence of CQ as an important therapeutic option for treatment of malaria (20, 21, 22).

Published pharmacokinetic studies of CQ in preclinical primate models are relatively scarce (23, 24, 25). In general, pharmacokinetic studies conducted with non-human primates are limited by the high cost of cynomolgus or rhesus monkeys since both species are endangered. The African green or vervet monkey (*Chlorocebus aethiops*) has been identified as a suitable alternative for drug development studies (26, 27) and is the model that was used in the current study.

The ability of pheroid technology to entrap, transport and deliver drugs of markedly different structures to ensure effective delivery and enhanced bioavailability of therapeutic compounds has been demonstrated (28, 29, 30) and a number of applications of this technology have been patented (31, 32, 33) in various countries. Both *in vitro* and *in vivo* studies have shown that the use of pheroid technology may improve the therapeutic efficacy and/or bioavailability of active pharmaceutical ingredients (28, 31-33, 29, 30). Since *in vitro* studies suggested that the therapeutic efficacy of anti-infective compounds can be enhanced by entrapment in pheroid, the aim of this study was to investigate the impact of pheroid technology on the bioavailability and intra-erythrocytic levels of CQ in a non-human primate model using the African green or vervet monkeys (*Chlorocebus aethiops*). A decrease in the bioavailability by entrapment of CQ in pheroid would have negated the observed increased efficacy.

The main components of the pheroid formulation are essential fatty acid ethyl esters (collectively known as vitamin F ethyl ester), pegylated ricinoleic acid (Cremophor), α-

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tocopherol, and nitrous oxide-saturated water (31-33). This combination can be formulated to produce carriers in the form of lipid-bilayer vesicles (pheroid vesicles), microsponges (pheroid microsponges) or pro-pheroid, depending on the ratios of components and method of production (28). The nitrous oxide imparts an ordered structure to the self-assembled carriers and ensures its stability. Such carriers maintain structural integrity over periods of greater than two years at room temperature (30). The formulations are easy to prepare, do not use any rigid nanomaterial or supporting polymer matrix, and are more economical than most other delivery systems (31-33). The technology is well established; there are currently five pheroid technology-derived topical products and one agricultural product on the market in a number of countries.

2. Materials and Methods

2.1 Materials

Chloroquine as the diphosphate salt was purchased from IPCA Laboratories Limited, Mumbai, India. Vitamin F ethyl ester was obtained from Chemimpo (South Africa) and cremaphor® EL was obtained from BASF (South Africa). Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and dl-α-tocopherol where purchased from Chempure (South Africa). Purified water was obtained from SABAX (Johannesburg, South Africa). Amodiaquine hydrochloride was obtained from TRC (Canada).

2.2 Reference formulations

The oral reference formulation was prepared from CQ diphosphate (1.6125 g) in DI water (50 mL).

2.3 Pheroid vesicle formulations

Pheroid vesicles were produced by firstly preparing the water phase, which consisted of saturating purified water with nitrous oxide (N_2O) under pressure (200 kPA). The oil phase was prepared by mixing and heating the Vitamin F ethyl ester (2.8 g), Chremphor EL (1.0 g), BHA (0.02 g), BHT (0.2 g) and dl-α-tocopherol to 70 °C. The oil phase was then added to the water phase while homogenizing with a Heidolph Diax 600 homogenizer (Labotec, South Africa) at 2041 G until the temperature has decreased to ≤ 40 °C. The mixture was shaken using a GLF shaker (DJB Labcare, England) until it has reached room temperature (20-25 °C). The described process resulted in the formation of pheroid vesicles. CQ was

spontaneously entrapped in the pheroid vesicles by the addition of chloroquine diphosphate (1.6125 g) to pheroid vesicle formulation (50 mL). The pheroid vesicles formulation containing CQ were shaken over night to ensure optimal entrapment.

The reference formulation and the pheroid vesicle formulation were prepared one day before commencing the study.   All formulations were kept in amber glass bottles at room temperature.

2.4 Animals

Healthy male and female vervet monkeys (*Chlorocebus aethiops*) weighing between 2.2 and 7.0 kg were utilized. All studies and procedures was conducted with prior approval of the Ethics Committee of the North-West University (approval number of NWU-00027-10-A5) in accordance with the National Code for animal use in research, education, diagnosis and testing of drugs and related substances in South Africa (based on the 'Guide for care and use of laboratory animals';NIH85-23, revised 1985).

The monkeys were maintained at the animal facility of Potchefstroom Campus, North West University and were individually housed in 89 x 88 x 187 squeeze cages in rooms with controlled temperature (21 ± 2°C) and relative humidity (55 ± 5%) and 12 hour light/dark cycles.   Their diet consisted of standard laboratory food, fresh fruits, vegetables and peanuts. Water was available *ad libitum*. The monkeys were environmentally enriched so as to ensure a minimal stress environment. Environmental enrichment was achieved by reproducing some of the main features of their natural habitat and to create opportunities for captive animals to develop such skills as they may need in the wild (34).

2.5 Oral bioavailability studies

For the assessment of oral absorption, the bioavailability of CQ was evaluated in plasma and whole blood samples after administration of the reference formulation and test formulation in a randomized single-blind parallel group study.

A study was conducted in plasma in seven monkeys to determine the bioavailability of CQ in plasma. The monkeys were randomly divided into two groups with one group receiving the reference formulation (n = 3) and the other group receiving the test formulation (n = 4). For the reference group, 20 mg/kg of CQ in water was administered; for the test groups, a similar dosage of 20 mg/kg CQ entrapped in pheroid vesicles was administered. The

volume per administration was 1mL/kg bodyweight. The monkeys were anaesthetized with ketamine hydrochloride (10mg/kg) to enable handling and blood sampling. The femoral triangle was then cleaned with alcohol and shaved if required. Monkeys were kept under continuous sedation for the first 3 hours of the study and were monitored at all times for distress or other signs of adverse drug reactions. Blood samples were collected at a range of time intervals and centrifuged at 0 °C for 7 minutes at 1000 G to determine the CQ plasma profile. The plasma was then transferred to cryovial tubes, snap frozen and transported overnight on dry ice to the University of Cape Town for analysis.

For the determination of the CQ whole blood profile the same protocol was followed as described above with the following exceptions. Fifteen monkeys, randomly divided into a reference group (n = 7) and test group (n = 8) were utilized and the blood samples were collected pre-dose and at 0.25, 0.5, 1, 2, 3, 5, 8, 12, 24, 48, 72, 96, 120 and 168 hours post-dose.

2.6 Sample analysis

Chloroquine concentrations were determined using a validated LC-MS/MS assay method developed in the Division of Clinical Pharmacology, University of Cape Town. An optimized liquid-liquid extraction method was developed for the extraction of CQ from 50 µL monkey whole blood or 50 µL monkey plasma, using a universal Britton Robinson buffer (pH 9) and ethyl acetate containing the internal standard. The organic phase was evaporated under reduced pressure by means of a rotary evaporator at 30 °C for 20 minutes. The dried samples were reconstituted with mobile phase consisting of equal volumes of acetonitrile and 0.1% formic acid, and 2 µL was injected onto the analytical column kept at 35 °C. Chromatographic separation was achieved on a Luna 5 µm PFP(2), 100 A, 50 mm × 2 mm analytical column (Phenomenex, USA) using acetonitrile and 0.1% formic acid (80:20, v/v) delivered at a constant flow rate of 0.5 mL/min. An AB Sciex API 3200 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 320.2 to the product ions at m/z 247.0 for CQ, and the protonated molecular ions at m/z 356.2 to the product ions m/z 282.9 for the internal standard. Initially the assay was validated over the concentration range of 4 ng/mL to 500 ng/mL for both plasma and whole blood samples. However, after analysis of the first batch of whole blood samples (n = 5) it was observed that the levels were far greater in whole blood than in plasma and consequently a new, broader range (16 ng/mL to 5000 ng/mL) was validated for analysis of the remaining whole blood samples (n = 10). The accuracy and precision for both assays were well within internationally accepted criteria.

2.7 Statistical analysis

The experimental data was evaluated in terms of drug concentration versus time. The following parameters were calculated by using the SAS statistical computer package (35):

- The peak drug concentration (C_{max}) in ng/mL
- Time to peak concentration (T_{max}) in h
- Apparent elimination half-life ($t_{1/2}$) in h
- Area under the concentration-time curve between time 0 and the time of the last sample collected (AUC_{0-last}) in ng.h/mL.
- Area under the concentration-time curve between time 0 to infinity (AUC_{0-inf}) in ng.h/mL

Descriptive statistics are reported for each characteristic in both pheroid and reference groups as mean ± SEM together with its 95% confidence intervals. To compare the means of the groups, the nonparametric Mann-Whitney test was used. The computer package Statistica (36) was used to obtain these results.

The area under the blood concentration curve up to the last time point (AUC_{last}) was calculated by using the trapezoidal rule. The area under the curve extrapolated to infinity (AUC_{inf}) was determined by adding C_{last} / k_e to AUC_{last}, where C_{last} is the concentration of the last time point and k_e the elimination rate constant. The half-life is calculated as $t_{1/2} = \ln(2)/k_e$.

3. Results

3.1 Pheroid vesicle manufacturing

The size of the pheroid vesicles was determined with the aid of a Malvern Mastersizer. The particle size distribution defines the size range of 10% (d(0.1)), 50 % (d(0.5)) and 90% (d(0.9)) of particle in sample. Figures 2 and 3 illustrate the particle size distribution prior to and after entrapment of CQ.

d(0.1): 0.267 um   d(0.5): 1.242 um   d(0.9): 3.277 um
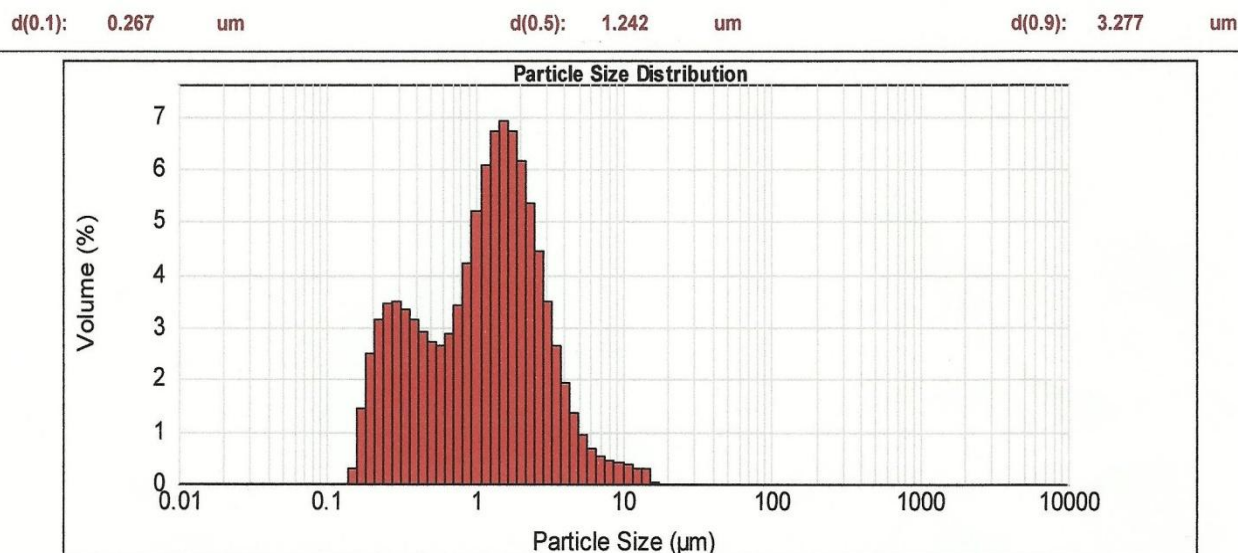
Figure 2: Particle size distribution prior to spontaneous entrapment of CQ in pheroid vesicles.

d(0.1): 0.287 um   d(0.5): 1.348 um   d(0.9): 3.394 um
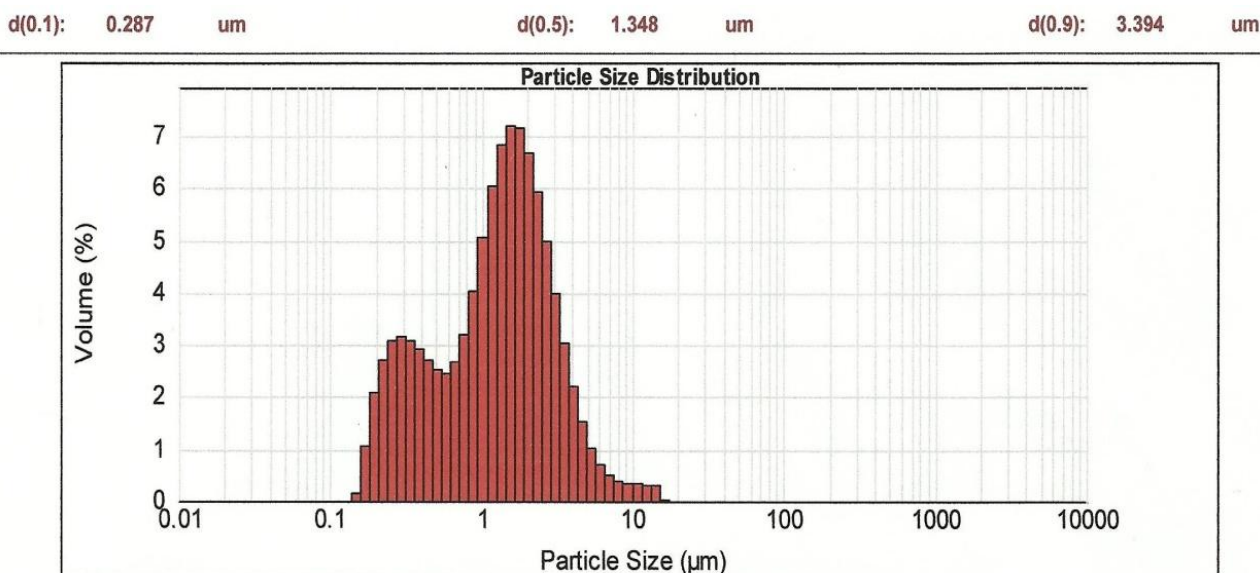
Figure 3: Particle size distribution after entrapment of CQ in pheroid vesicles.

3.2 LC-MS/MS assay

The assay was validated for both monkey plasma and whole blood. The accuracy and precision of the assay during validation were well within internationally accepted criteria (37,38). The limit of quantification for CQ was 16 ng/mL for the whole blood samples and 4 ng/mL for the plasma samples. Quadratic (1/x, weighting) regression analysis was used to construct calibration curves. The assay also performed well during study sample analyses. The calibration standards and quality control (QC) standards were analyzed in duplicate in each study sample batch. During inter-batch analysis of the plasma samples, the

accuracies for the assay were 94.6%, 100.5% and 99.4% at the low, medium and high QC levels respectively. The precision (expressed as % coefficient of variation) was less than 5.1% at all three QC levels. The accuracies for analysis of the first batch of whole blood samples were 104.3%, 106.7% and 99.3% at the low, medium and high QC level, with precision less than 5.5% at the three levels. The accuracies during inter-batch analysis of the remaining whole blood samples were 98.8%, 102.3% and 101.1% at the low, medium and high QC level respectively, with precision less than 7% at all three levels.

3.3 Oral bioavaolability studies

The plasma pharmacokinetic data obtained for both the reference formulation and pheroid vesicle formulation after a single oral (20 mg/kg) administration are given in Table 1.

Table 1: Summary of the pharmacokinetic parameters of CQ in plasma (reference n = 3, test n = 4)

	C_{max} (ng/mL)		T_{max} (h)		AUC_{0-last} (ng.h/mL)	
	Ref	**Phe**	**Ref**	**Phe**	**Ref**	**Phe**
Mean	110.87	100.78	1.17	4.8	515	493
Lower 95 % CI	0	0	0.26	1.2	0	0
Upper 95 % CI	238.37	259.77	2.6	11.55	1132	1321
SEM	29.64	49.96	0.3	1.8	143	260

Ref = Reference formulation
Phe = Pheroid formulation

These results were unexpected. The mean maximum plasma concentration (C_{max}) of CQ was relatively low: 110.87 ± 29.64 ng/mL and 100.78 ± 49.96 ng/mL for the reference and pheroid formulation respectively. The time to peak CQ concentrations in the plasma (T_{max}) were 4.8 ± 1.8 h for the pheroid formulation and 1.17 ± 0.33 h for the reference formulation, indicating a substantial increase of T_{max} by pheroid technology. No statistically significant AUC_{0-last} differences were observed.

The CQ drug profiles obtained from whole blood was substantially different from that obtained for plasma. The pharmacokinetic data after a single oral dose of 20 mg/kg were calculated for both the reference formulation and pheroid vesicle formulation in whole blood and are given in Table 2.

Table 2: Summary of the pharmacokinetic parameters of CQ in whole blood (reference n = 7, test n = 8)

	C_{max} (ng/mL)		T_{max} (h)		$t_{1/2}$ (h)		$AUC_{0\text{-last}}$ (ng.h/mL)		$AUC_{0\text{-inf}}$ (ng.h/mL)	
	Ref	**Phe**	**Ref**	**Phe**	**Ref**	**Phe**	**Ref**	**Phe**	**Ref**	**Phe**
Mean	1039	1753.6	4.6	4.5	18.09	16.24	33409	45580	37365	52047
Lower 95 % CI	424.7	848.4	2.0	0.0	13.92	12.45	18332	25306	21746	25539
Upper 95 % CI	1653.3	2658.8	7.1	11.1	22.27	20.03	48487	65855	52984	78556
SEM	251	382.8	1.0	2.8	4.53	4.51	6162	8574	6383	11210
Mann-Whitney T-test	0.06		0.075		0.23		0.20		0.20	

Ref = Reference formulation
Phe = Pheroid formulation

The results obtained from whole blood is comparable to that described for man in the literature and are graphically presented in Figure 4 as the mean whole blood concentration versus time during 168 hours after oral administration of CQ for the reference formulation and the pheroid vesicle formulation. Each curve shows an absorption phase followed by an elimination phase. The CQ in the pheroid vesicle formulation improved C_{max} and AUC. The mean maximum whole blood concentration (C_{max}) of CQ was increased significantly on a 10% level of significance by the pheroid vesicles, ie. a C_{max} of 1753.6 ± 382.8 ng/mL vs that of 1039 ± 251.04 ng/mL for the reference formulation. The observed maximum peak whole blood concentration was reached at approximately 4.5 hours in both the reference and pheroid vesicle formulations. The apparent half life ($t_{1/2}$) was 18.09 ± 4.53 hours for the reference and 16.24 ± 4.51 hours for the pheroid vesicle formulation, representing approximately a 2 hour time difference between the two formulations. $AUC_{0\text{-last}}$ represents the entire amount of CQ absorbed during 168 hours into the systemic circulation after a single dose of CQ as either the reference or pheroid vesicle formulation. The mean $AUC_{0\text{-last}}$ obtained for the reference formulation was 33409 ± 6162 ng.h/mL and for the pheroid formulation 45580 ± 8574 ng.h/mL. The $AUC_{0\text{-inf}}$ values quantified for the reference formulation and pheroid vesicle formulation were 37365 ± 6383 ng.h/mL and 52047 ± 11210 ng.h/mL respectively. Unlike the results obtained in plasma samples, both $AUC_{0\text{-last}}$ and $AUC_{0\text{-inf}}$ from whole blood samples were greatly increased in the pheroid group.
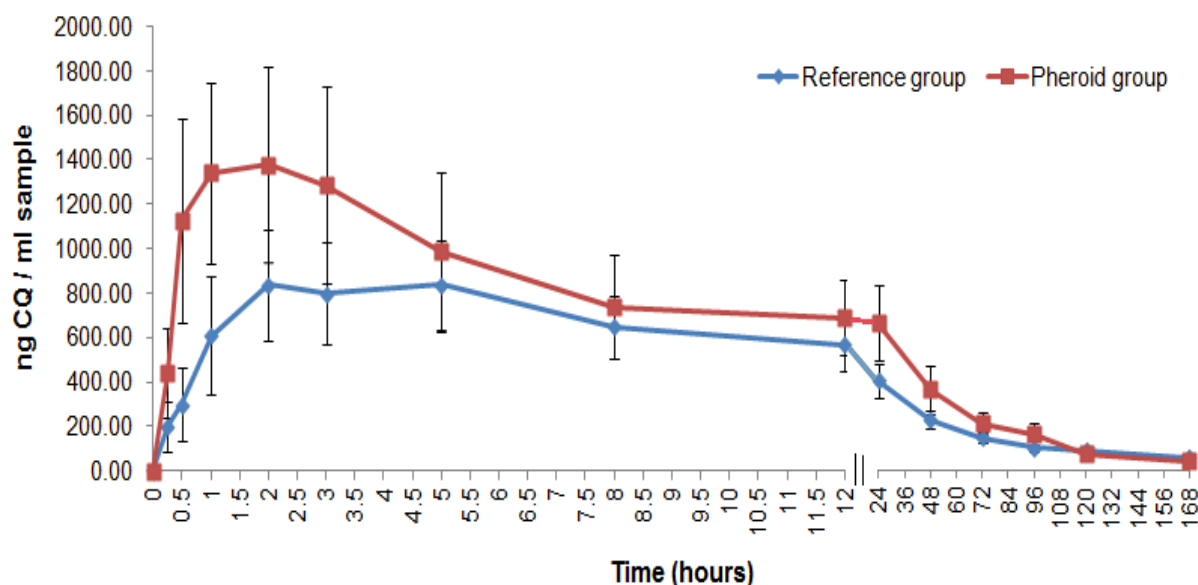
Figure 4: Mean whole blood concentration versus time graph of oral CQ reference and pheroid vesicle formulations.

4. Discussion

The results of the bioavailability studies in plasma after oral administration of CQ reference and pheroid formulation indicate a C_{max} of 100.78 ± 49.96 and 110.87 ± 29.64 ng/mL respectively. Furthermore, the reference formulation returned a much lower C_{max} value when compared to the C_{max} of 320.83 ± 22.25 ng/mL reported in a comparable study using rhesus monkeys (25). In contrast, the results of studies performed using whole blood showed substantial improvements in bioavailability as a result of entrapment of CQ in a pheroid formulation. Administration of the test formulation containing CQ entrapped in pheroid vesicles resulted in increases of the C_{max}, AUC_{0-last} and AUC_{0-inf} by 68%, 36% and 99% respectively when compared to the reference group.

The disease-generating phase of the malaria parasite arises during the intra-erythrocytic life cycle. Previous studies have indicated that CQ accumulates in erythrocytes (39, 40). The current study in non-human primates is the first study to compare the concentration of CQ in whole blood and plasma after an oral administration of 20 mg/kg CQ. The results indicates that the concentrations of CQ in plasma were lower than that observed in whole blood, i.e. at the C_{max} there were 17.4 times more CQ in the whole blood than that in the plasma for the test formulation, while there is only 9.3 times more CQ in the whole blood for the reference formulation. Similarly, when one is looking at the total measure absorption (AUC_{0-last}), there was 92 times more CQ in the whole blood than in the plasma for the test formulation and 64

96

times for the reference formulation. For the reference formulation, this indicates that there is a general enrichment of CQ in whole blood, which correlates well with other findings (39, 41) and supports the idea that pharmacokinetic studies for CQ should be conducted using whole blood. Furthermore pheroid technology contributes to this enrichment in fact the whole blood content of CQ is nearly double for the test formulation is both cases. In Table 3 the pharmacokinetic data obtained for CQ in non-human primates in the current study compared with literature data are shown.

Table 3: Pharmacokinetic data for CQ in non-human primates

Species	Matrix	N	Drug	Route	Dose	C_{max}	T_{max}	AUC (0-last)	Reference
					(mg/kg)	(ng/mL)	(h)	(ng.h/mL)	
Rhesus monkey	WB	4	CQP	im	4	560	3	-[a]	23
Vervet monkey	**WB**	**7**	**CQP**	**po**	**20**	**1039**	**4.6**	**33409**	Current study
Vervet monkey	**WB**	**8**	**CQ-pheroid**	**po**	**20**	**1753.6**	**4.5**	**45580**	Current study
Rhesus monkey	P	4	CQP	im	4	183.3	3	-[a]	23
Rhesus monkey	P	2	CQ	im	3.7	262	3	-[a]	24
Rhesus monkey	P	6	CQP	po	20	320.8	2	-[a]	25
Vervet monkey	**P**	**3**	**CQP**	**po**	**20**	**110.87**	**1.17**	**515.47**	Current study
Vervet monkey	**P**	**4**	**CQ-pheroid**	**po**	**20**	**100.78**	**4.8**	**493**	Current study

a = no data available; CQP =Chloroquine diphosphate; WB = Whole blood; P = Plasma

As seen from the table, the C_{max} values obtained in plasma for the reference formulation orally administered at 20 mg/kg in the current study are substantially lower than the much higher C_{max} of 320.83 ± 22.25 ng/mL reported for plasma obtained from rhesus monkeys (25). This result is rather different to that (110.9 ng/mL) we report here. The difference may be due to the analytical procedure used in each case. The CQ concentrations in the relevant rhesus monkey study (25) were determined by an analytical method based on fluorometric detection after solvent extraction. According to authors this technique does not differentiate between CQ and its metabolites (41,42), and hence may provide artificially high concentrations of CQ. With the LC-MS/MS method used in the current study, only CQ levels were determined.

The whole blood pharmacokinetic data obtained in the current study is nearly double that observed in one of the earlier studies (23). The C_{max} of 1039 ± 251 ng/mL in the current study compares with that of 560 ng/mL observed earlier. It is once again important to note that CQ was administered intramuscularly at 4 mg/kg (23) in comparison with the 20 mg/kg oral administration used in the current study. Our whole blood pharmacokinetic data shows a better correlation with that obtained from humans (43). A C_{max} of 838 ng/mL (95% confidence interval of 656-1587 ng/mL) after an oral dose of 25 mg/kg CQ in whole blood was reported. These results support the use of the Vervet monkey (*Chlorocebus aethiops*) as an alternative preclinical non-human primate model for predicting pharmacokinetic parameters in humans (44, 45).

In line with the ability of pheroid technology to enhance absorption of orally-administered anti-infective drugs (28, 30) and topical applications (46), pheroid offers the potential for exploitation as an anti-malarial drug delivery system. The method of formulation and the type of pheroid plays a role in the extent of absorption and the rate and measure of transport of the pheroid-drug formulation into the intracellular domain (28). However, the precise manner in which the pheroid is taken up by the cells needs to be elucidated.

5. Conclusion

The results provide evidence that pheroid technology has the ability to enhance the intracellular absorption of chloroquine. From a drug delivery standpoint, the CQ-pheroid formulation rendered major improvements in C_{max} and AUC of CQ in whole blood. This is of potential significance in relation to enhancing efficacy of CQ against intra-erythrocytic stages of the malaria parasite, there exists the possibility that the pheroid-CQ formulation may act to inhibit incipient access of CQ to the *Pf*mdr transporter.

6. Acknowledgements

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THE *IN VITRO* METABOLISM AND *IN VIVO* PHARMACOKINETICS OF PRO-PHEROID® FORMULATED AMODIAQUINE

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c) The number of text pages, number of tables, figures, and references, and the number of words in the *Abstract, Introduction,* and *Discussion* (each item should be placed on a separate line).

d) A list of nonstandard abbreviations used in the paper.

Abstract: The abstract should concisely present the hypothesis being tested, general methods, results, and conclusions. Abstracts of more than 250 words will not be accepted. The abstract must be a single paragraph.

Introduction: This section must contain a clear statement of the aims of the work or of the hypotheses being tested. A brief account of the relevant background that supports the rationale of the study should also be given. The length of the *Introduction* should not exceed 750 words.

Materials and Methods: This section should contain explicit, concise descriptions of all new methods or procedures employed. Whereas modifications of previously published methods must be described, commonly used procedures require only a citation of the original source. Descriptions of methods must be sufficient to enable the reader to judge the accuracy, reproducibility, and reliability of the experiment(s). The name and location (city and state or country) of commercial suppliers of chemicals, reagents, and equipment must be given. Sources of compounds, reagents, and equipment not available commercially should be identified by name and affiliation here or in the *Acknowledgments* section.

Results: Contained in this section are the experimental data, with no discussion of their

significance. Results are typically presented in figures or tables, with no duplication of information in the text. If a table or figure includes less than four values, the data should be presented in the text rather than as a separate table or figure. Magnitudes of variables reported should be expressed in numerals. Sufficient data should be presented to allow for judgment of the variability and reliability of the results.

Figure captions: Figures are numbered consecutively with Arabic numerals and listed in order rather than one per page. Legends must provide sufficient explanation for the reader to understand the figure independent of the text.

Tables: Each table must be double-spaced and begin on a separate page, each page numbered continuous with the rest of the manuscript. Tables are numbered consecutively with Arabic numerals. A brief descriptive title is provided at the top of each table. Footnotes to tables are referenced by italicized, lower case, superscript letters and defined beneath the table.

Discussion: Conclusions drawn from the results presented are included in this section. Whereas speculative discussion is allowed, it must be identified as such and be based on the data presented. The *Discussion* must be as concise as possible and should not exceed 1,500 words.

Acknowledgments: The *Acknowledgments* section is placed at the end of the text. Personal assistance is noted here. Financial support is acknowledged as an unnumbered footnote to the title.

References: References are cited in the text by giving the first author's name (or the first and second if they are the only authors) and the year of publication. In the reference list, the references should be arranged alphabetically by author and not numbered. The names of all authors should be given in the reference list. Journal titles should be abbreviated as given in the Medline abbreviation list. The total number of references is limited to 60 for manuscripts submitted after November 30, 2011. References to personal communications, unpublished observations, and papers submitted for publication are given in parentheses at the appropriate location in the text, not in the list of references.

Abstract

Amodiaquine (AQ) is used in artemisinin based combination therapy for uncomplicated *Plasmodium falciparum* malaria. The use of Pheroid® technology improves the pharmacokinetics of some drugs by improving their absorption and bioavailability. In this study we evaluate the effect of Pheroid® technology on the *in vitro* and *in vivo* drug metabolism and pharmacokinetics (DMPK) of AQ. Oral pharmacokinetic studies were conducted in two groups of vervet monkeys (*Chlorocebus aethiops*), with the reference group receiving capsules containing AQ and the test group receiving capsules containing a pro-Pheroid® formulation of AQ. AQ was administered at 60 mg/kg. Blood concentrations of AQ and *N*-desethylamodiaquine (DEAQ) were monitored from 0.5 to 168 hours. AQ and pro-Pheroid® formulated AQ were incubated *in vitro* with human and monkey liver (HLM and MLM) and intestinal (HIM and MIM) microsomes and recombinant cytochrome P450 enzymes. The studies confirmed the rapid metabolism of AQ to the main metabolite DEAQ in monkeys. The pharmacokinetic parameters varied substantially among the monkeys, with no statistical difference between the test and reference formulation. DEAQ pharmacokinetics was similar except that the AUC in the reference was significantly higher than that in the test formulation. For HLM, MLM and CYP2C8, the test formulation showed significantly longer AQ clearance and slower formation of DEAQ. However, the effect was reversed in MIM. Pheroid® technology did not enhance the absorption and bioavailability of AQ or DEAQ. Pheroid® is a registered trademark but for ease of reading will be referred to as pheroid(s) throughout the rest of the article.

1. Introduction

Amodiaquine is a Mannich base 4-aminoquinoline that is structurally related to chloroquine, with the exception that the aminoalkyl side chain is rigidified by partial incorporation into an aromatic ring (O'Neill *et al.*, 1998; Schlitzer, 2007). During the 1970s it was withdrawn from chemoprophylactic use due to its toxic effects that included agranulocytosis and hepatitis (Neftel *et al.*, 1986). Despite these concerns, and in view of the widespread resistance to chloroquine and fansidar (pyrimethamine and sulfadoxine), amodiaquine is currently recommended by the WHO as a partner drug in artemisinin based combination therapy (ACT) of uncomplicated malaria (German & Aweeka, 2008; WHO, 2010). In one of the currently used ACTs, amodiaquine is combined with artesunate (WHO, 2010). However, this ACT does appear to be the least effective of the ACTs currently in use (White *et al.,* 2010).

The pharmacokinetic-pharmacodynamic (PK/PD) rationale of ACTs is to combine the very potent but short half-life artemisinin derivatives such as artemether, artesunate or dihydroartemisinin with a longer half-life partner drug such as amodiaquine, lumefantrine, mefloquine or piperaquine. Whilst the artemisinin derivatives cause rapid reduction in parasitemia, they tend to be associated with high recrudescence if used alone (WHO, 2010). Partnering them with long half-life anti-malarial partner drugs ensures total clearance of parasitemia (WHO, 2010; Bosman & Mendis, 2007).

Amodiaquine is rapidly metabolized *in vivo* to its major metabolite, *N*-desethylamodiaquine (DEAQ) (Figure 1) that has 3-fold less anti-malarial potency than AQ. AQ has a half-life of approximately 4 hours, whereas that of DEAQ is approximately 211 hours. This effectively renders AQ a prodrug of DEAQ, which then acts as the long half-life anti-malarial (Jullien *et al.*, 2010). Due to structural similarities between AQ and CQ, their pharmacokinetic properties in humans may be expected to be similar. However, it turns out that AQ has a smaller volume of distribution (17 – 34 L/kg), a shorter terminal elimination half-life (1 to 3 weeks) and, along with DEAQ, is over 90% bound to plasma proteins (Krishna & White, 1996).
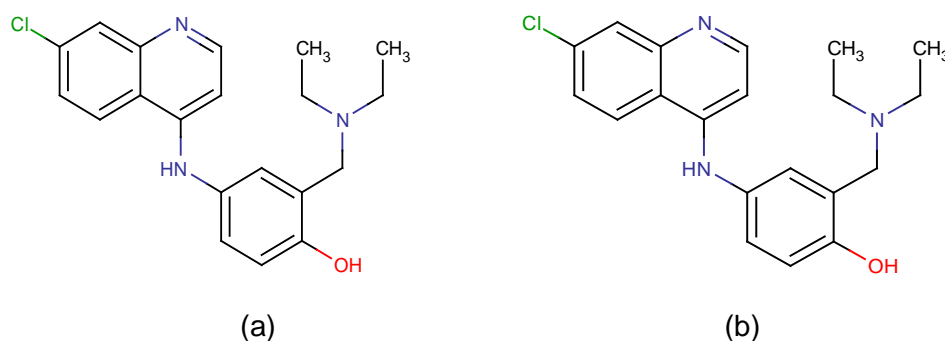
Figure 1: structures of (a) amodiaquine (AQ) and (b) N-desethylamodiaquine (DEAQ).

I*n vitro* studies of AQ with human liver microsomes (HLM) and recombinant human cytochrome P450 (rCYPs) shows that CYP2C8 is responsible for the rapid hepatic metabolism of AQ to DEAQ (Li *et al.*, 2002; Li *et al.*, 2003). The liver toxicity of AQ is associated with its biotransformation to a quinone imine (Schlitzer, 2007). AQ is also metabolised by extra-hepatic CYPs 1A1 and 1B1 to a reactive aldehyde metabolite (Johansson *et al.*, 2009; Li *et al.*, 2003) that may be a possible cause of agranulocytosis (Johansson *et al.*, 2009; Schlitzer, 2007).

Very few pharmacokinetic studies of AQ using primate models have been carried out (Ramanathan-Girish *et al.*, 2004). In general, pharmacokinetic studies conducted with non-human primates are limited by the high cost of cynomolgus or rhesus monkeys since both species are endangered. The African green or vervet monkey (*Chlorocebus aethiops*) has been identified as a suitable alternative for drug development studies (Ward *et al.*, 2008; Ward *et al.*, 2009) and is the model chosen for the current study.

There are relatively few new anti-malarial drugs being produced (Schlitzer *et al.*, 2012), largely because market forces discourage major pharmaceutical companies from investing in the discovery of drugs for neglected diseases (Chatelain & Loset, 2011; Hotez & Pecoul, 2010). Therefore, efforts are being made to protect current anti-malarial drugs from their pharmacokinetic and safety liabilities and to minimize development of drug resistance. Thus, formulations designed to improve PK/PD profiles by increasing the bioavailability or lengthening the duration of drug exposure are being developed (Crowley & Martini, 2004). In addition, the design and synthesis of analogues of current drugs aimed at overcoming adverse PK, toxic properties, and potential for resistance are also generating useful leads.

Pheroid technology forms the basis of a colloidal type drug delivery system that has the ability to entrap, transport and deliver drugs of markedly different structures (Grobler, 2008;

Saunders *et al*., 1999; Steyn *et al*., 2011). *In vitro* and *in vivo* (rodent) studies suggested that the therapeutic *in vitro* efficacy and *in vivo* bioavailability of amodiaquine can be enhanced by pheroid technology (Langley, 2010). The aim of this study was to investigate the impact of pheroid technology on the *in vivo* bioavailability of amodiaquine in vervet monkeys (*Chlorocebus aethiops*). We also conducted *in vitro* metabolism studies of formulated and unformulated AQ with human and monkey liver and intestinal microsomes, and with recombinant human CYPs to investigate the effect of the delivery system on the different enzymes.

2. Materials and Methods

2.1 In vivo pharmacokinetics

2.1.1 Materials

Amodiaquine hydrochloride was purchased from DB Fine Chemicals (South Africa). Vitamin F ethyl ester was obtained from Chemimpo (South Africa) and Cremaphor® EL was obtained from BASF (South Africa).   Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), polyethylene glycol (PEG 400) and dl-α-tocopherol were purchased from Chempure (South Africa). Purified water was obtained from SABAX (Johannesburg, South Africa). Capsules were purchased from CapsuGel (Durban, South Africa). The internal standards, amodiaquine-d10 and *N*-desethylamodiaquine-d5 and the *N*-desethylamodiaquine reference standard were obtained from Toronto Research Centre (Canada).

2.1.2 Reference standard

For the reference, unformulated AQ hydrochloride was encapsulated in capsules.

2.1.3 Pro-pheroid formulations

The pro-pheroid formulation was prepared by mixing and then heating the Vitamin F ethyl ester (24.38 g), Chremphor EL (10.16 g), PEG 400 (2.5 g), BHA (0.005 g) and BHT (0.005 g) to 70 °C. Dl-α-tocopherol (0.5 g) was then added. Amodiaquine (12.45 g) was added to the mixture. The mixture was gassed with nitrous oxide (N_2O) under pressure (200 kPA). This formulation does not contain a water phase and is referred to as pro-pheroid. Once admixed with 0.1 N HCl the pro-pheroid reassembles into discrete pheroid vesicles, whose

size was determined with the aid of a Malvern Mastersizer Micro 2000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The pro-pheroid formulation was kept in an amber glass bottle at room temperature. The capsules for the reference and test group were prepared one day in advance of treatment.

2.1.4 Animals

Healthy male and female vervet monkeys (*Chlorocebus aetiops*) weighing between 2.2 and 7.0 kg were used. All studies and procedures were conducted with prior approval from the Ethics Committee of the North-West University (approval number of NWU-00027-10-A5) in accordance with the National Code for animal use in research, education, diagnosis and testing of drugs and related substances in South Africa (based on the 'Guide for care and use of laboratory animals'; NIH85-23, revised 1985).

The monkeys were maintained at the animal facility of the Potchefstroom Campus, North-West University, and were individually housed in 89 x 88 x 187 cm squeeze cages in rooms in which the temperature was kept at 21 ± 2 °C and the relative humidity at 55 ± 5%, with 12 hour light/dark cycles. Their diet consisted of standard laboratory food, fresh fruits, vegetables and peanuts. Water was available *ad libitum*. Environmental enrichment for the monkeys was achieved by reproducing some of the main features of their natural habitat and creating opportunities for captive animals to develop such skills as they may need in the wild (Terao, 2005).

2.1.5 Oral bioavailability study

For the assessment of oral absorption, the bioavailability of AQ and DEAQ was evaluated in whole blood samples after administration of the reference standard and test formulation in a randomized single-blind parallel group study.

A study was conducted in fourteen monkeys, randomly divided into two groups with the reference group (n = 6) receiving AQ hydrochloride capsules and the test group (n = 8) receiving encapsulated pro-pheroid formulation containing the same dosage, namely 60 mg/kg AQ. Capsules were prepared for each monkey in accordance with their weight. The monkeys were anaesthetized with ketamine hydrochloride (10 mg/kg) to enable handling and blood sampling. Drug administration was accomplished by placing a single capsule at the back of the tongue and holding the mouth shut while externally massaging the throat to ensure swallowing. The femoral triangle was then cleaned with alcohol and shaved if

required. The monkeys were kept under continuous sedation for the first 3 hours of the study and were monitored at all times for distress or other signs of adverse drug reactions. Blood samples were collected pre-dose and at 0.5, 1.5, 2.5, 3.5, 5, 8, 12, 24, 48, 72, 96, 120 and 168 hours post-dose. The collected blood was then transferred to cryovial tubes, snap frozen and transported overnight on dry ice to the University of Cape Town for analysis.

2.1.6 Sample analysis

The concentrations of AQ and DEAQ were determined using a validated quantitative LC-MS/MS assay method developed in the Division of Clinical Pharmacology, University of Cape Town. An optimized liquid-liquid extraction method was developed for the extraction of AQ and DEAQ from 20 µL monkey whole blood samples, using an universal Britton Robinson buffer (pH 8) containing the internal standards (amodiaquine-d10 and *N*-desethylamodiaquine-d5) and ethyl acetate. The organic phase was evaporated under nitrogen at 30 °C for 20 minutes. The dried samples were reconstituted with mobile phase and 2 µL per sample was injected onto the analytical column at 35 °C.

Chromatography was performed on a Luna 5 µm PFP(2), 100 A, 50 mm $\times$ 2 mm analytical column (Phenomenex, USA) using acetonitrile (ACN) and 0.1% formic acid (85:15, v/v) as mobile phase at a flow rate of 0.5 mL/min. An AB Sciex API 4000 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 356.2 to the product ions at m/z 283.0 for AQ, the protonated molecular ions at m/z 328.1 to the product ions m/z 282.9 for DEAQ, the protonated molecular ions at m/z 366.2 to the product ions m/z 283.1 for the deuterated AQ internal standard, and the protonated molecular ions at m/z 333.2 to the product ions m/z 283.0 for the deuterated DEAQ internal standard. The assay was validated over a range of 3.91 ng/mL to 4000 ng/mL for AQ and 9.77 ng/mL to 10 000 ng/mL for DEAQ. The precision and accuracy were within 15% of the nominal value; well within internationally accepted criteria. The reliability of the assay was monitored at three levels with quality control samples.

2.1.7 Statistical analysis

The experimental data were evaluated in terms of drug concentration versus time. The following parameters were calculated from the blood drug levels obtained:

- The peak drug concentration (C_{max}) in ng/mL
- Time to peak concentration (T_{max}) in h
- Apparent elimination half-life ($t_{1/2}$) in h
- Area under the concentration-time curve between time 0 and the time of the last sample collected ($AUC_{0\text{-last}}$) in ng.h/mL.
- Area under the concentration-time curve between time 0 to infinity ($AUC_{0\text{-inf}}$) in ng.h/mL

Descriptive statistics are reported for each characteristic in both reference and pro-pheroid groups as mean ± SEM together with its 95% confidence intervals. To compare the means of the groups, the nonparametric Mann-Whitney test was used. The computer package Statistica (StatSoft, 2011; SAS, 2003) was used to obtain these results.

The area under the blood concentration curve up to the last time point (AUC_{last}) was calculated by using the trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{\inf}$) was determined by adding C_{last} / k_e to AUC_{last}, where C_{last} is the concentration of the last time point and k_e the elimination rate constant. The half-life is calculated as $t_{1/2} = \ln(2)/k_e$ (StatSoft, 2011).

2.2 In vitro metabolism

2.2.1 Materials

Recombinant CYPs 1A1, 1B1 and 2C8 were purchased from CYPEX (Dundee, UK). Human liver microsomes (HLM), human intestinal microsomes (HIM), monkey intestinal microsomes (MIM) and monkey liver microsomes (MLM) were obtained from Xenotech (Kansas, USA). All other reagents were of bioanalytical grade.

2.2.2 Reference formulation

The reference formulation for the *in vitro* metabolism studies was prepared with AQ dihydrochloride (0.0465 g) in deionised water (100 mL). The end concentration of AQ in the reference formulation for the *in vitro* metabolism studies was 1mM. Deionised water was used for dilutions.

2.2.3 Pro-pheroid formulations

The pro-pheroid formulation was prepared as described above, with the concentration of AQ entrapped in the pro-pheroid formulation being 1 mM.   A blank pro-pheroid formulation, containing no entrapped active (AQ), was also prepared and used for dilution of the study formulations.

The reference and pro-pheroid formulations were kept in amber glass bottles at room temperature.

2.2.4 Metabolic stability

Reference and pro-pheroid formulations (1 µM) were incubated with enzyme (0.5 mg/mL) and 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH. An aliquot was taken at various time points (0, 5, 10, 20, 30 and 45 minutes) and the reaction was stopped by the addition of ice cold acetonitrile. The samples were then stored at 4 °C to allow protein precipitation. Extraction was carried out twice with acetonitrile. The acetonitrile extracts were combined and submitted to centrifugation at 10 000 G for 15 minutes at 4 °C. The supernatant was collected and dried under a gentle stream of nitrogen. The residue was reconstituted in mobile phase and qualitatively analysed by LC-MS.

2.2.5 Enzyme inhibition

Reference and pro-pheroid formulations (2 µM) were incubated with enzyme (0.5 mg/mL (microsomes) or 10 pmol/mL (rCYPs)) and 0.1 M phosphate buffer (pH 7.4) and inhibitor (5 µM). The reaction was initiated by the addition of NADPH, and stopped at zero and 30 minutes with the addition of ice cold acetonitrile. The zero time point represented the unmetabolised drug and the 30 minute time point the remaining drug after oxidative metabolism. Extraction was carried out twice with acetonitrile, the extracts were combined and centrifuged at 10 000 G for 15 minutes at 4 °C. The supernatant was collected and dried under a gentle stream of nitrogen. The residue was reconstituted in mobile phase and analysed by LC-MS.

2.2.6 Sample analysis

The qualitative analysis of AQ and DEAQ were performed on a Waters Quattro mass spectrometer (Waters, Milford, MA, USA) with ESI interface. Chromatographic separation was performed on an Agilent 1100 system (Agilent Technologies, USA) connected to a CTC HTS PAL autosampler, using an Atlantis T3 C18 column (2.1 x 30 mm, 3µm). The mobile phase consisted of water and acetonitrile (50:50, v/v) acidified with 0.1% formic acid at a flow rate of 0.2 mL/min.

2.2.6.1 Determination of intrinsic clearance

Intrinsic clearance (CL_{int}) studies were performed for AQ with HLM, HIM, MLM and MIM using the substrate depletion approach (Houston, 1994; Obach *et al.*, 1999), where drug concentrations determined after different times of incubations with microsomes were expressed as percentage of drug remaining, using the t = 0 minutes concentration value as 100%. The fundamental basis of this approach lies in the derivation of the integrated Michaelis-Menten equation (Segel, 1975):

$$V_M \times dt = \frac{-K_{Mapp} + [S]}{[S]} \times d\,S \qquad \text{Equation 1}$$

Over one $t_{\frac{1}{2}}$ (i.e. when [S] =0.5[S]$_{t=0}$ the following equation applies:

$$\frac{V_M \times t_{\frac{1}{2}}}{K_{Mapp}} = Ln2 + \frac{0.5[S]_{t=o}}{K_{Mapp}} \qquad \text{Equation 2}$$

A necessary assumption in this approach, which is included in the experimental design, is that the substrate concentration used is below the K_{Mapp} value (in this case 1 µM versus the K_M of 2.4 µM), such that:

$$\frac{0.5[S]}{K_{Mapp}} \ll Ln2 \qquad \text{Equation 3}$$

The *in vitro* $t_{\frac{1}{2}}$ is incorporated into the following equation:

$$CL_{int} = \frac{Ln2 \times volume\ of\ incubation(mL)}{t_{\frac{1}{2}}\ (min) \times Protein\ or\ enzyme\ amount} \qquad \text{Equation 4}$$

2.2.6.2 In vitro-in vivo correlations

The CL_{int} in the incubations is expressed as µL/min/mg (microsomes). The CL_{int} can be scaled to the apparent clearance for the whole liver (whole liver CL_{int}). The following scaling factors were used: microsomal protein yield per gram of liver (MPGL): human, 39.8 mg/g (Hakooz *et al.,* 2006); monkey, 45 mg/g (Laufer *et al.*, 2009); and liver weight used for human and monkey were 1680 g and 150 g respectively (Davies & Morris, 1993). Using these factors the equation is:

$$Whole\ liver\ CL_{int} = \frac{in\ vitro\ CL_{int}\ \times MPGL\ (mg) \times liver\ (g)}{Body\ weight\ (kg)} \qquad \text{Equation 5}$$

The *in vivo* hepatic clearance (CL_H) due to oxidative metabolism was estimated according to the 'well-stirred liver' model (Laufer *et al.*, 2008). The *in vivo* hepatic clearance (CL_H), is expressed as:

$$In\ vivo\ CL_H\ = \frac{Q_H \times whole\ liver\ CL_{int}}{Q_H + whole\ liver\ CL_{int}} \qquad \text{Equation 6}$$

where Q_H is the hepatic blood flow. The values used for hepatic blood flow (Q_H) were as follows: human 21 mL/min/kg and monkey 43.6 mL/min/kg (Davies & Morrris, 1993).

3. Results

3.1 Pro-pheroid manufacturing

The size distribution of the pro-pheroid formulation was determined with the aid of a Malvern Mastersizer. The particle size distribution defines the size range of 10% (d(0.1)), 50 % (d(0.5)) and 90% (d(0.9)) of particles in the sample and should comply to set size parameters. Figure 2 illustrates the particle size distribution of the pro-pheroid formulation.
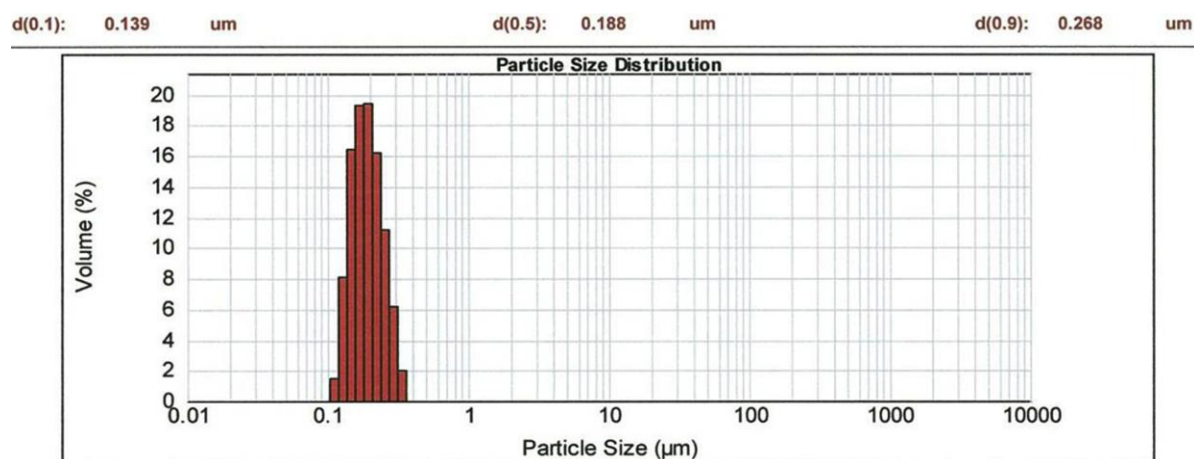
Figure 2: Particle size distribution of pre-entrapped AQ in pheroid vesicles. The initial formulation used was a pro-pheroid formulation. After the application of the drug, and once admixed with 0.1 N HCl, the pro-pheroid reassembles into pheroid vesicles containing AQ, of which the size distribution is then determined with the aid of the Malvern Mastersizer. Upon contact with the gastric fluid, the same process occurs.

3.2 In vivo pharmacokinetic study

3.2.1 LC-MS/MS assay

The bioanalytical assay was validated for monkey whole blood. The accuracy and precision of the assay during validation were within internationally accepted criteria (FDA, 2001; EMA, 2011). The assay also held up well during study sample analyses. The limit of quantification for AQ and DEAQ was 3.91 ng/mL and 9.77 ng/mL, respectively. Quadratic regressions (1/x weighting) were used to construct calibration curves. The calibration standards and quality control (QC) standards were analyzed in duplicate in each study sample batch. The precision (total-assay coefficients of variation; CV%) during sample analysis was less than 8% at high, medium and low (3000, 1500 and 11.7 ng/mL, respectively) QC levels for AQ, and was less than 10% at high, medium and low (8000, 4000 and 31.3 ng/mL, respectively) QC levels for DEAQ.

3.2.2 Oral bioavailibility study

The pharmacokinetic parameters of all the monkeys (n=14), after a single oral dose of 60 mg/kg of AQ were calculated for both the reference (n=6) and test group (n=8). The data are presented in Table 1.

Table 1: Summary of the pharmacokinetic parameters of AQ and DEAQ in whole blood (reference n = 6, test n = 8)

	C_{max} (ng/ml)		T_{max} (h)		$t_{1/2}$ (h)		AUC_{0-last} (ng.h/ml)		AUC_{0-inf} (ng.h/ml)	
	Ref	Phe	Ref	Phe	Ref	Phe	Ref	Phe	Ref	Phe
Amodiaquine										
Mean	841,1	480,4	12,3	6,1	14,8	21,8	9417,6	5126,5	9431,3	5183,0
Lower 95 % CI	-421,0	182,0	2,8	3,1	9,2	4,1	-1372,3	2900,5	-1383,6	3039,6
Upper 95 % CI	2103,1	778,8	21,9	9,0	20,3	39,6	20207,4	7352,5	20246,2	7326,3
SEM	491,0	126,2	3,7	1,3	1,8	7,5	4197,4	941,4	4207,2	906,4
p-value MW test	0,5		0,1		0,4		0,2		0,2	
Desethylamodiaquine										
Mean	3754,0	2790,1	14,0	9,4	57,6	55,6	164680,8	109875,3	194180,0	131734,3
Lower 95 % CI	1153,5	1268.3	2,1	3,7	46,8	38,7	129559,0	61659,1	152267,1	74625,9
Upper 95 % CI	6354,5	4311,9	25,9	15,2	68,5	72,4	199802,6	158091,6	236092,9	188842,6
SEM	936,6	643,6	4,3	2,4	3,9	7,1	12649,9	20390,7	15095,9	24151,1
p-value MW test	0,5		0,3		0,5		**0,03**		**0,04**	

*Ref = Reference group; **Phe** = pro-pheroid/Test group; **MW** = Mann Whitney test*

The incorporation of AQ in the pro-pheroid formulation (test group) resulted in lower concentrations of AQ in the systemic circulation compared to that observed in the reference group. At the mean maximum whole blood concentration (C_{max}) in the test group there was 43% less AQ in the systemic circulation compared to that of the reference group. Similarly, for the total drug exposure over 168 hours (AUC_{0-last}), the amount of AQ in the test group was 46% lower than that of the reference group. The AUC_{0-inf} values quantified for the reference and test groups had a 56% lower AQ concentrations in the systemic circulation of the test group compared to the reference group. The observed maximum peak whole blood concentration for the test group was reached at half the time of the reference group. The apparent half life ($t_{1/2}$) of the test group was increased approximately 0.7 fold compared to the reference group.

The mean peak whole blood concentration (C_{max}) of the main metabolite (DEAQ) was 26% lower in the test group compared to the reference group. Similarly, the mean AUC_{0-last} and AUC_{0-inf} obtained for the test group was 32% lower than that of the reference group. The values obtained for the AUC_{0-last} and AUC_{0-inf} was statistically significant ($p < 0.05$). The difference in apparent half life ($t_{1/2}$) between the reference and test group was very small,

representing approximately a 2 hour time difference between the two groups. Table 2 illustrates the pharmacokinetic data obtained for AQ and DEAQ in non-human primates in this study compared with literature data.

Table 2: Means of pharmacokinetic parameters for AQ and DEAQ in whole blood of non-human primates

Species	N	Drug	Dose	C_{max}	T_{max}	$T_{1/2}$	AUC (0-last)	AUC (0-inf)	CL
			(mg/kg)	(ng/ml)	(h)	(h)	(ng.h/ml)	(ng.h/ml)	(L/kg/h)
Amodiaquine									
Cynomolgus monkey[b]	2	AQ-13	20	356,3	7,9	36,3	12438,7	-[a]	0,3
Cynomolgus monkey[b]	2	AQ-13	100	5694,4	1,9	38,2	124422,6	-[a]	0,35
Vervet monkey	**6**	**AQ**	**60**	**841,1**	**12,3**	**14,8**	**9417,6**	**9431,3**	**-[a]**
Vervet monkey	**8**	**Phe AQ**	**60**	**480,4**	**6,1**	**21,8**	**5126,5**	**5183,0**	**-[a]**
Desethylamodiaquine									
Cynomolgus monkey[b]	2	AQ-13	20	1441,4	12,0	21,5	43935,9	-[a]	-[a]
Cynomolgus monkey[b]	2	AQ-13	100	5516,5	48,0	57,8	697670,8	-[a]	-[a]
Vervet monkey	**6**	**AQ**	**60**	**3754,0**	**14,0**	**57,6**	**164680,8**	**194180,0**	**-[a]**
Vervet monkey	**8**	**Phe AQ**	**60**	**2790,0**	**9,4**	**55,6**	**109875,3**	**131734,3**	**-[a]**

*a = not determined; **Phe AQ** = AQ in the pro-pheroid formulation; **b** = Ramanathan-Girish et al., 2004.*

3.3 In vitro metabolism studies

3.3.1 Metabolic stability and inhibition

In an effort to evaluate the effect of pheroid technology on the metabolism of AQ in humans and monkeys, the *in vitro* clearance of AQ and the formation of the DEAQ was investigated in systems comprising human liver microsomes (HLM), human intestinal microsomes (HIM), monkey liver microsomes (MLM) and monkey intestinal microsomes (MIM). Studies were performed to determine the *in vitro* intrinsic clearance (CL_{int}) of the two formulations by measuring the depletion of AQ over time from an initial substrate concentration of 1 µM.
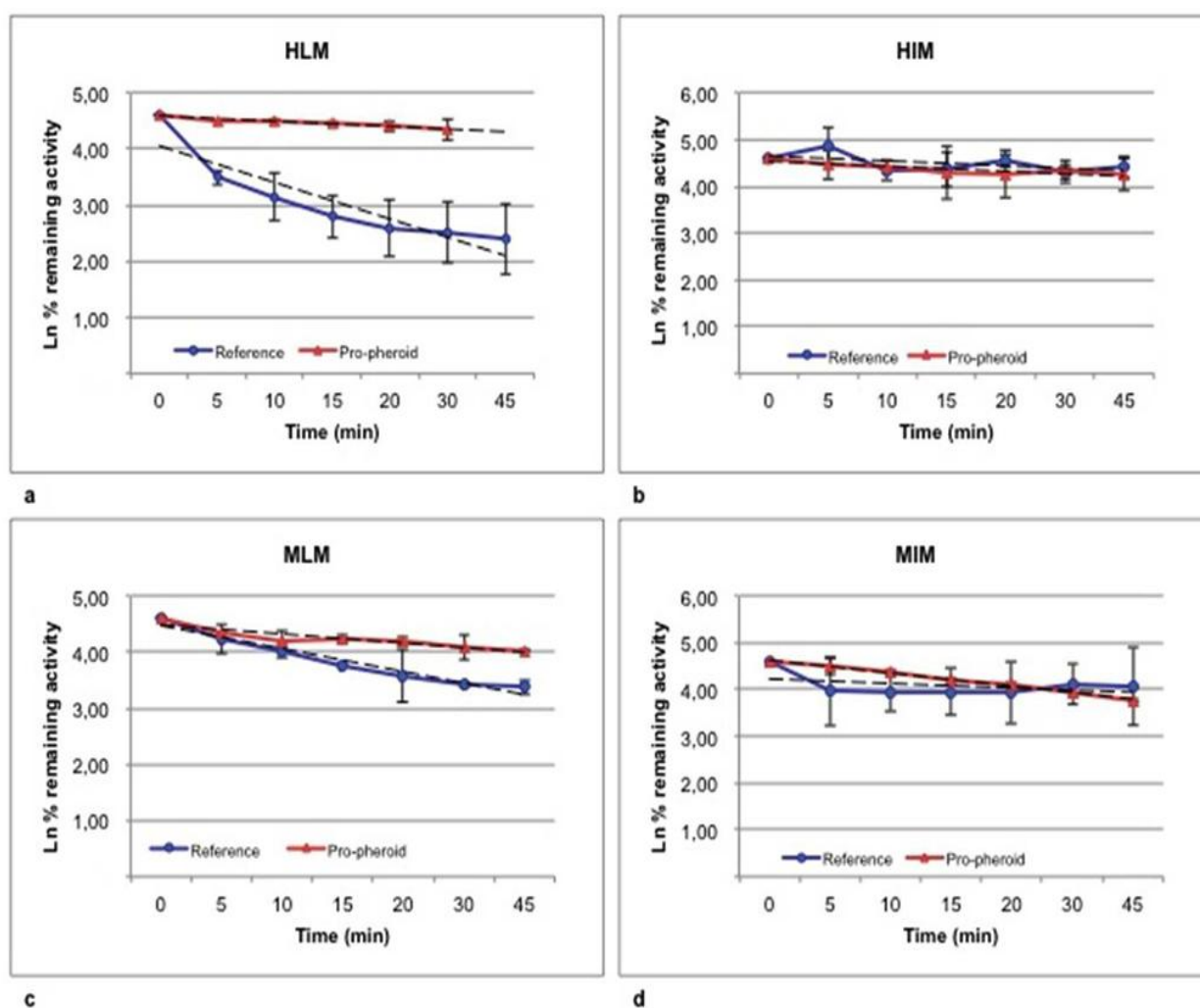
Figure 3: Metabolic stability of AQ reference standard and pro-pheroid formulation in (a) HLM, (b) HIM, (c) MLM and (d) MIM over 45 minutes. The dashed line represents the trendlines.

The metabolic *in vitro* half life ($t_{1/2}$) was calculated using the slope of the linear regression from natural logarithmic percentage substrate remaining versus incubation time (Figures 3a-d). The *in vitro* half life ($t_{1/2}$) was used to determine the intrinsic clearance (CL_{int}) of AQ in both formulations. The results are given in Table 3.

Table 3: Calculated intrinsic clearance (CL_{int}) of the AQ reference and AQ incorporated in pro-pheroid using the $t_{1/2}$ in HLM, HIM, MLM and MIM and *in vivo* estimated hepatic clearance (CL_H) for HLM and MLM

Compound	*In vitro* CL_{int} (µL/min/mg)				Estimated *in vivo* CL_H (L/kg/h)	
	HLM	MLM	HIM	MIM	HLM	MLM
AQ reference	41	30	6	5	0,82	1,26
AQ in pro-pheroid formulation	8	11	5	16	0,34	0,66

In Figures 4a-d, the percentages of AQ remaining following incubation in the different enzyme systems in the absence and presence of specific inhibitors are given. Previous studies have shown that CYP2C8 is the main hepatic CYP isoform responsible for the oxidation of AQ to DEAQ in the liver. Quercetin is a specific inhibitor for CYP2C8 (Li *et al.*, 2003). Further, it has been established that extra-hepatic CYP1A1 and CYP1B1 also play a role in the oxidation of AQ, and that α-naphthoflavone is a specific inhibitor for CYP1A1 (Li *et al.*, 2002; Li *et al.*, 2003; Johansson *et al.*, 2009; Martignoni *et al.*, 2006). In the absence of specific inhibitors the results suggest the possibility that pheroid technology has the ability to protect AQ from oxidative metabolism in HLM, HIM, MLM and to a lesser extent in MIM. The presence of quercetin in the HLM and MLM incubations failed to inhibit the oxidation of AQ in the reference formulations. The incubation of AQ incorporated in the pro-pheroid formulation in the presence of quercetin, showed inhibition of AQ in the HLM. The results of incubations of HIM and MIM in the presence of α-naphthoflavone are not given, because very little metabolism took place during these incubations; the effect of the inhibitor is minimal in these enzyme systems.
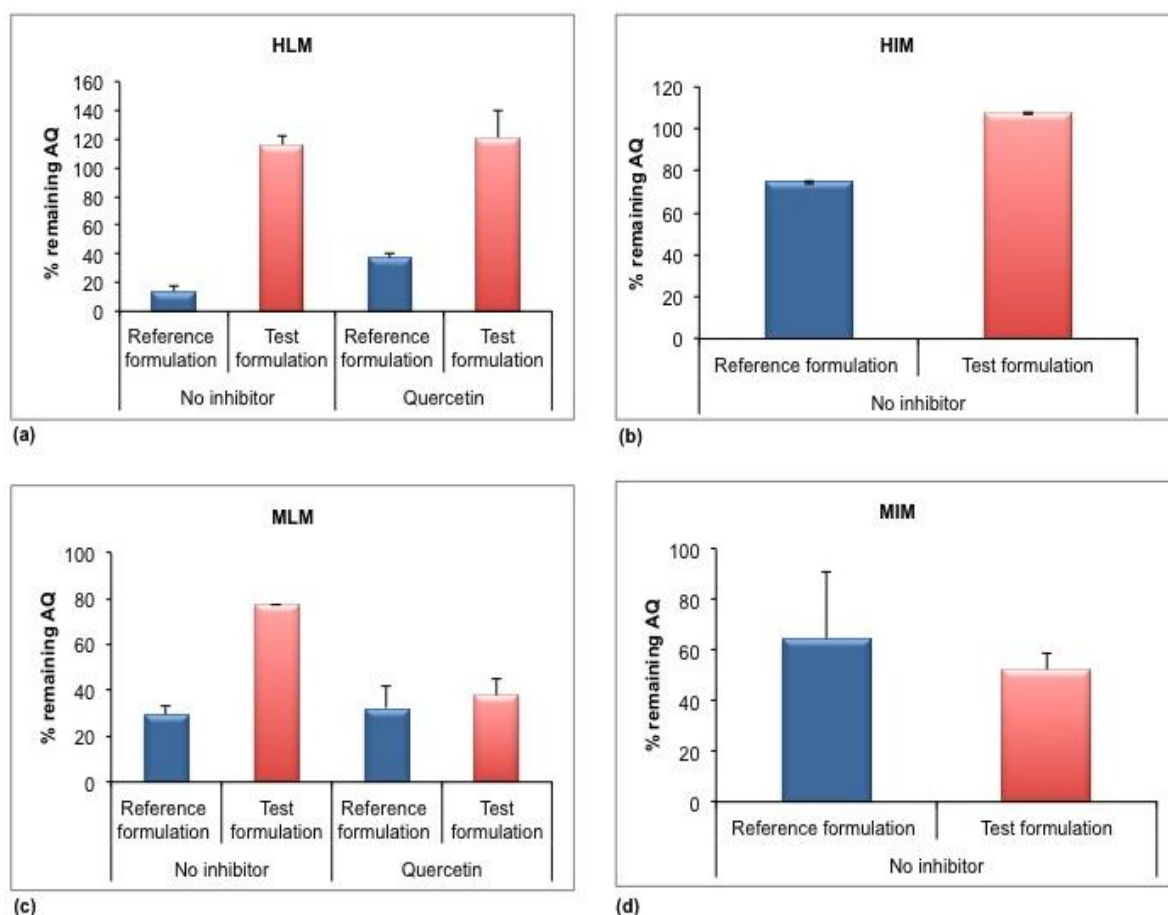
Figure 4: Effect of pheroid technology on AQ clearance in the presence and absence of selective inhibitors in (a) HLM, (b) HIM, (c) MLM and (d) MIM.

Figures 5a-d show the percentage of DEAQ formed following incubation in the different enzyme systems in the absence and presence of specific inhibitors. In the absence of specific inhibitors, DEAQ was formed in HLM, HIM, MLM and MIM following incubations with the reference and test formulations. However, HLM incubation with the pro-pheroid formulation resulted in formation of decreased levels of DEAQ compared to the other enzyme systems. In the presence of specific inhibitors of the CYP isoforms involved in the oxidative metabolism of AQ, the following observations were made: quercetin inhibited the formation of DEAQ in both the reference and pro-pheroid formulations in HLM; quercetin inhibited the formation of DEAQ in the reference formulation in MLM, but not in the pro-pheroid formulation.
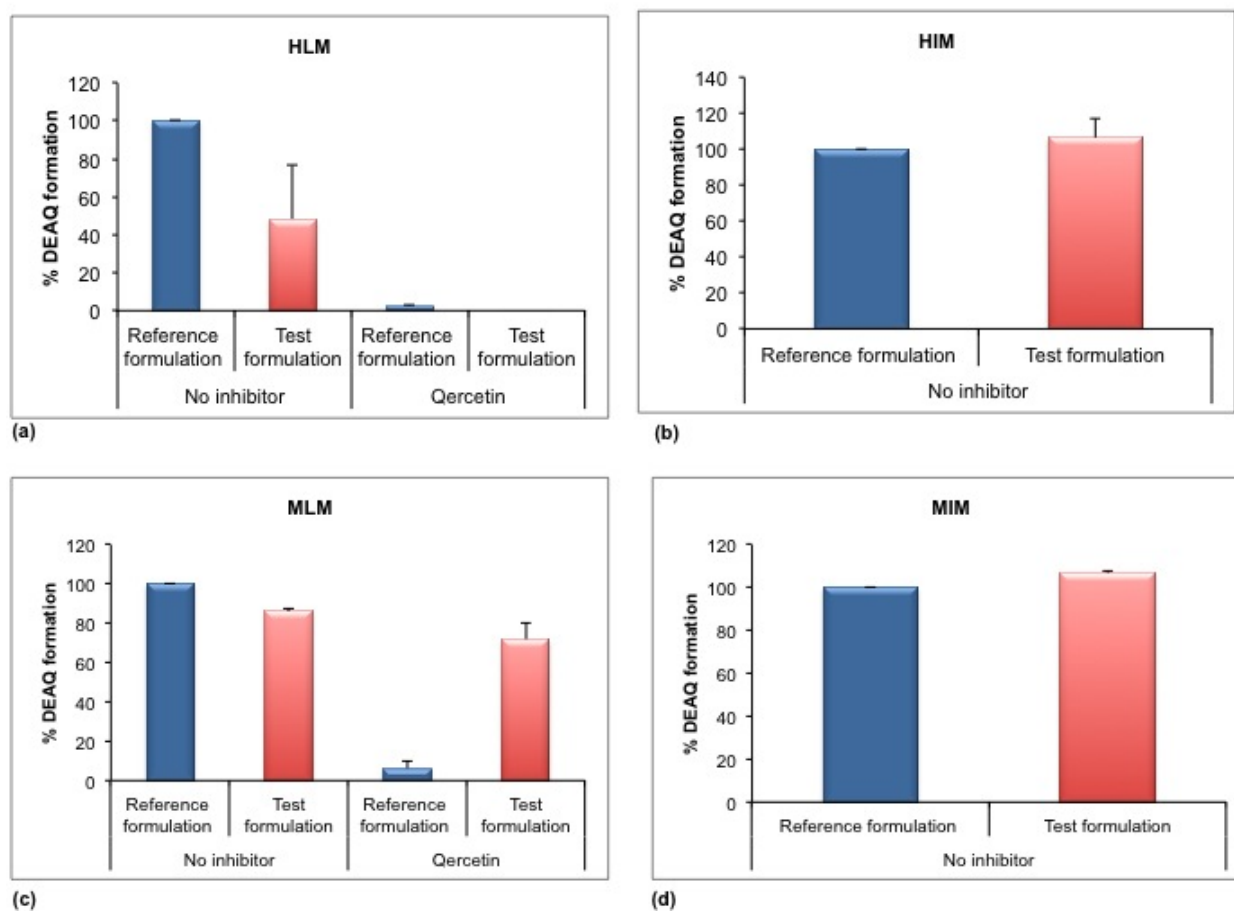
Figure 5: Effect of pheroid technology on DEAQ formation in the presence and absence of selective inhibitors in (a) HLM, (b) HIM, (c) MLM and (d) MIM.

In addition, as illustrated in Figure 6, the pro-pheroid formulation protected AQ from oxidative metabolism in human recombinant CYP1A1, CYP1B1 and CYP2C8. The recombinant CYP2C8 correlated well with the results obtained from HLM and MLM incubations with the reference formulation.

Recombinant CYP enzymes
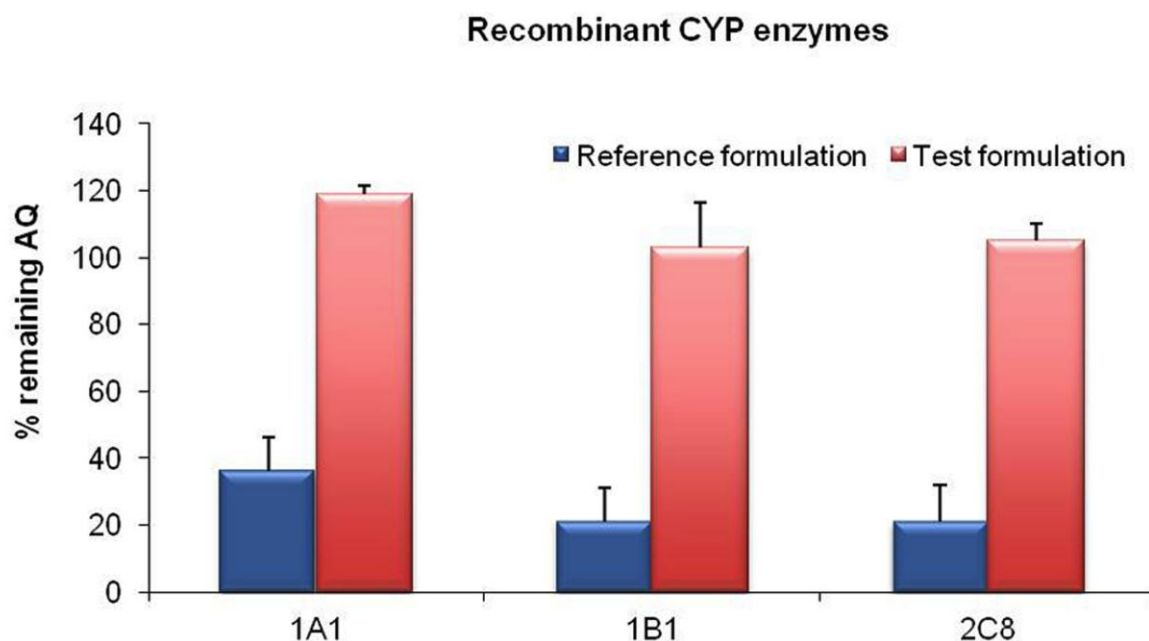
Figure 6: Effect of pheroid technology on AQ clearance in different recombinant enzyme systems.

4. Discussion

The incorporation of AQ into pro-pheroids gave unexpected results in the vervet primate model. It is clear from Table 1 that lower concentrations of AQ were present in the systemic circulation when compared to the reference standard. However, with HLM, MLM and CYP2C8 *in vitro* the pro-pheroid formulation tended to suppress the metabolism of AQ to its major metabolite DEAQ. The results of the MIM showed a reverse effect (Table 3).

After a single 60 mg/kg oral dose of AQ in the reference and test formulations, C_{max} of AQ was observed earlier than the C_{max} of the metabolite (DEAQ) for the reference group, which is consistent with a lag time associated with the formation of DEAQ (Table 1). Table 2 illustrated the pharmacokinetic data obtained for AQ and DEAQ in non-human primates in the current study compares well with literature data. The C_{max} and $AUC_{(o\text{-last})}$ values obtained for AQ and DEAQ in the reference group are comparable with the C_{max} and $AUC_{(o\text{-last})}$ values obtained in a previous study using cynomolgus monkeys (Ramanathan-Girish *et al.*, 2004). Pharmacokinetics parameters for AQ and DEAQ show great inter-individual variation (Pussard *et al.*, 1987; White *et al.*, 1987). The pharmacokinetic parameters evaluated in the current study also showed great inter-individual variation. The cause of these inter-individual variations is unclear, but may be due in part to variability in the capacity of the enzymes to metabolize AQ.

It is evident from Table 1 that the concentrations of AQ and DEAQ after oral administration of the pro-pheroid formulation were lower than those observed for the reference standard. Though not statistically significant, the following trends were observed. For the ratios of the means $(C_{max})_{DEAQ}:(C_{max})_{AQ}$ and $(AUC_{0-last})_{DEAQ}:(AUC_{0-last})_{AQ}$ for both the reference standard and pro-pheroid formulation, it is apparent that at C_{max} there is 5.8 times more DEAQ in the whole blood after administration of the pro-pheroid formulation, while there is 4.6 times more DEAQ in the whole blood after administration of the reference standard. Similarly, for the mean total measured absorption (AUC_{0-last}), there is 25.4 times more DEAQ in the whole blood for the pro-pheroid formulation, compared to 17.5 times more DEAQ for the reference standard. The cause of the lower AQ levels in the test group is unclear, but may be due in part to an increased metabolic turnover of AQ after the administration of the pro-pheroid formulation or decreased absorption.

Limited literature is available for species differences in intestinal metabolic activities of other CYP isoforms, i.e. CYP2C8, which is involved in the biotransformation of AQ to DEAQ. However a recent study indicated that there was a higher intestinal metabolic activity for CYP2C8 and CYP4F in cynomolgus monkeys compared to that in humans (Nishimuta *et al.,* 2011). It is important to note that cynomolgus monkey CYP2C8 cDNA has 95% homology with human CYP2C8 cDNA (Komori *et al.*, 1992; Uno *et al.*, 2006). Furthermore, the CYP2C8 cDNA of the vervet monkey is very similar to cynomolgus CYP2C8 cDNA (Uno *et al.*, 2011). CYP4F plays an important role in the metabolism of polyunsaturated fatty acids (PUFA) (Fer *et al.*, 2008), one of the main components of the pro-pheroid formulation. Therefore we presume that when the pro-pheroid enters the intestine of the monkey. The increased intestinal metabolic activity of CYP4F isoforms results in rapid metabolism of the pro-pheroid. Consequently AQ is rapidly released and metabolized by CYP2C8 that is also present in the intestine of the monkey. This presumption is partly supported by the *in vitro* metabolic stability data presented here, in which a higher CL_{int} for the pro-pheroid formulation in the MIM were observed compared to the reference, HIM and MLM (Table 3).

Interestingly, the $t_{1/2}$ of AQ in the pro-pheroid formulation is increased: $t_{1/2}$ for the parent compound (AQ) in the reference standard is 14.77 ± 1.82 hours, whilst for the pro-pheroid formulation, $t_{1/2}$ is approximately 21.84 ± 7.51 hours. This suggests that when AQ is incorporated in the pro-pheroid formulation, there is a longer duration of exposure in the systemic circulation compared with that of the reference standard. Further, this result correlates well with results of the *in vitro* studies on the metabolic clearance of AQ, in which the pro-pheroid formulation displays reduced clearance of AQ in HLM and MLM (Figures 4a

and 4b).   The formulation was also demonstrated to protect AQ from microsomal metabolism in recombinant enzymes CYP2C8, 1A1 and 1B1 (Figure 6).

An unexpected observation was made during the *in vitro* studies. We expected to see decreased levels of DEAQ formed in the HLM, HIM and MLM studies, given that the pro-pheroid formulation seemed to protect AQ from depletion *in vitro*. Despite the latter, it seems that DEAQ is still formed. A possible reason may be the intercession of autoxidation of AQ. Previous studies have indicated that autoxidation of AQ occurs in a solution at physiological pH under air (Maggs *et al.*, 1988; Harrison *et al.*, 1992). It is apparent that AQ is labilized by the presence of the aminophenol moiety that is susceptible to autoxidation.

5. Conclusion

The results of the *in vivo* pharmacokinetic and *in vitro* metabolism studies confirm the rapid metabolism of AQ to the main metabolite DEAQ in monkeys. The results of the *in vivo* PK studies indicated higher levels of AQ and DEAQ in the reference group compared to the test group, with the exception of the extended half-life of the formulated drug. The *in vitro* results suggest that pheroid technology has the ability to protect AQ from microsomal metabolism, however a increase in the bioavailibility was not observed in our *in vivo* model.

6. Acknowledgements

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CHAPTER 6

SUMMARY AND FUTURE PROSPECTS

The WHO recommends the use of artemisinin combination therapy (ACT) for the treatment of uncomplicated *P.falciparum* malaria (WHO, 2010). In spite of the apparent utility of combination therapies in suppressing emergence of resistance (White, 2004), it is particularly alarming to note that ACTs are becoming less potent for the treatment of malaria in Cambodia. The time given for fixed doses of an ACT to clear parasites from the blood is increasing. This has been shown to be the result of increasing tolerance of the malaria parasite to the artemisinins, the core component of ACTs (Cheeseman *et al.*, 2012; Noedel *et al.*, 2009). The spread of artemisinin-resistant parasites will have devastating consequences for malaria control. Therefore, the importance of the search for new treatment modalities that may overcome resistance, such as the development of novel drugs, and improvements in the use of existing drug regimens to prolong the lifespan of these regimens. Alternative drug delivery technologies such as pheroid technology have the potential to enhance both the effectiveness of delivery and increase the bioavailability of existing anti-malarial drugs. The aim of this study was to evaluate the effectiveness of formulating the well-known anti-malarial drugs amodiaquine (AQ) and chloroquine (CQ) by application of pheroid technology. Pheroids are lipophilic vesicles that have demonstrated capacity to enhance bioavailability and efficacy of other known drugs (Steyn *et al.*, 2010). CQ and AQ were each evaluated in a reference formulation and in pheroid formulations. *In vivo* pharmacokinetic studies were conducted for reference and pheroid formulations of CQ, and *in vitro* and *in vivo* drug metabolism and pharmacokinetic (DMPK) studies were conducted for AQ.

CQ, a 4-aminoquinoline, has been the mainstay for anti-malarial therapy and was widely used before the emergence of resistant *P. falciparum* in 1957 (Trape *et al.*, 1998; O'Neil *et al.*, 2012; Kaur *et al.*, 2009). However, because CQ usage has been withheld for such a long time, it is now predicted that the efficacy of CQ may return, suggesting the possible re-introduction of CQ as an important therapeutic option in combination therapies for the treatment of malaria (Kublin *et al.*, 2003; Laufer *et al.*, 2006). AQ, also a 4-aminoquinoline drug, is used as a partner drug with artesunate in one of currently used ACTs. However, this ACT does appear to be the least effective of ACTs currently in use (White *et al.*, 2010). Both CQ and AQ exert their mode of action during the intra-erythrocytic stages of the parasites life cycle, by interfering with the sequestration of heme-Fe^{3+} (ferriprotoporphyrin IX), which is produced during the digestion of hemoglobin (Kumar *et al.*, 2007; Klonis *et al.*, 2007). These drugs bind to heme-Fe (III) to prevent the formation of hemozoin (Müller & Hyde, 2010; Fitch, 2004; Egan *et al.*, 2006; Stiebler *et al.*, 2010). However a recent alternative hypothesis involving drug-assisted transport of heme-Fe(III) out of the DV into

the cytosol may more realistically account for drug action of each of CQ and AQ (Haynes *et al.*, 2012).

For any drug to be effective, it has to reach its site of action in quantities that are at least sufficient to exert a therapeutic effect. *In vivo* availability of any active pharmaceutical ingredient (API), that is, the drug, within a formulation can be greatly influenced by the formulation. The latter plays an important role in ensuring the stability of the API as well as the points of absorption and the absorption rates. The pheroid formulation primarily consists of ethyl esters of essential fatty acids and nitrous oxide water and has the ability to entrap, transport and deliver hydrophilic and hydrophobic drugs of markedly different pharmacological categories (Meyer 2002, Grobler, 2008; Steyn *et al.*, 2011; Grobler, 2007; Grobler & Kotze, 2006; Grobler *et al.*, 2009). Formulations used without a water phase for oral administration are referred to as a pro-pheroid formulation. After the application of the drug, and once admixed with nitrous oxide water, the pro-pheroid reassembles into pheroid vesicles. A similar process has been shown to occur in gastric fluid *in vitro* (Grobler, 2008). The resulting vesicles can be manipulated in various ways to suit the solubility characteristics of the drug molecules, which need to be delivered.

Chapter 4 describes the oral pharmacokinetic studies conducted in vervet monkeys (*Chlorocebus aethiops*). Two formulations were prepared with CQ, a reference formulation of CQ in water, and also a pheroid vesicle formulation. Monkeys were randomly divided into two groups with one group receiving the reference formulation and the other group receiving the pheriod vesicle formulation. The monkeys were anaesthetized with ketamine hydrochloride to enable handling and blood sampling. The formulations were administered at a dose of 20 mg/kg via oral gavage. Blood samples were collected by means of percutaneous venipuncture of the femoral vein at a range of pre-determined time intervals. The concentration of CQ in both whole blood and plasma samples were determined with a LC-MS/MS method.

A sensitive, selective and reproducible LC-MS/MS method was developed and validated in the Division of Clinical Pharmacology, University of Cape Town, for the quantification of CQ in 50 μL vervet monkey whole blood and plasma and is described in chapter 3. The accuracy and precision of the assay during validation were well within internationally accepted criteria (EMA, 2011). The LC-MS/MS method described here involves the use of a solvent-solvent extraction technique, resulting in a much cleaner extract. Compared to previous LC-MS/MS methods used to quantify CQ (Singal *et al.*, 2007; Hodel *et al.*, 2009) the method described here uses smaller whole blood and plasma sample volumes and

injection volumes (2 µL). The smaller whole blood or plasma sample volume is important for preclinical pharmacokinetic studies in monkeys in order to comply with the National Code for animal use in research, education, diagnosis and testing of related substances in South Africa.

The bioavailability studies in plasma after oral administration of each of the CQ reference and pheroid formulation, resulted in a C_{max} of 100.78 ± 49.96 and of 110.87 ± 29.64 ng/mL, respectively. In contrast, the result of entrapment of CQ in a pheroid formulation indicated a substantial improvement in bioavailability in the whole blood. The C_{max} obtained for whole blood was 1039 ± 251.04 ng/mL for the unformulated reference sample of CQ and 1753.6 ± 382.8 ng/mL for the pheroid formulation of CQ. The $AUC_{0\text{-inf}}$ was 37365 ± 6383 ng.h/mL (reference) and 52047 ± 11210 ng.h/mL (pheroid). The results show an enhanced bioavailability of CQ after administration of the pheroid formulation. Overall, the general enhancement of CQ levels in the whole blood correlates well with other findings (Gustafsson *et al.*, 1983; Bergqvist & Domeij-Nyberg, 1983) and supports the idea that pharmacokinetic studies for CQ should be conducted using whole blood. Use of pheroid formulations further enhances CQ levels in whole blood; in fact the whole blood content of CQ was nearly double for the test formulation when compared to the reference formulation in both cases.

In general it can be concluded that pharmacokinetic data obtained for CQ delivered compelling evidence in favour of the ability of pheroid technology to enhance the bioavailability of CQ. Studies rendered major improvements in the observed C_{max} and AUC of CQ in whole blood. The pheroid vesicle formulation had the ability to enhance intracellular absorption of CQ. It is important to keep in mind that the disease-generating phase of the malaria parasite arises during the intra-erythrocytic life cycle. Thus the use of pheroid formulations is of potential importance in relation to the enhancement of efficacy of CQ against the intra-erythrocytic stages of the malaria parasite. The use of CQ in pheroid formulations may prove to be an essential component in anti-malarial combination therapy regimens.

The entrapment of AQ in a pro-pheroid formulation did not produce such promising results. Oral pharmacokinetic studies were conducted in two groups of vervet monkeys, with the reference group receiving capsules containing the hydrochloride salt of AQ and the test group receiving capsules containing a pro-pheroid formulation of AQ. Blood concentrations of AQ and *N*-desethylamodiaquine (DEAQ) were monitored over 13 time points. In

addition, AQ and pro-pheroid formulations of AQ were incubated *in vitro* with human and monkey liver (HLM and MLM) and intestinal (HIM and MIM) microsomes and recombinant cytochrome P450 enzymes.

Both the *in vitro* and *in vivo* studies confirmed the rapid metabolism of AQ to the main metabolite DEAQ in monkeys. The pharmacokinetic parameters varied greatly. Parameters for AQ and DEAQ in the test (pro-pheroid) group were lower compared to those observed in the reference group. During incubations with HLM, MLM and CYP2C8, the pro-pheroid formulation showed significantly longer AQ clearance and slower formation of DEAQ. However, the effect was reversed in MIM. Though not statistically significant, the following trends were observed, at C_{max} there was 5.8 times more DEAQ present in the systemic circulation compared to 4.6 times more in the reference group. The same was true for the AUC_{0-last}. These findings were indicative of an increase in the metabolism of AQ after the administration of the pro-pheroid formulation and could possibly explain the lower levels of AQ for the test group. A recent study indicated a higher intestinal metabolic activity for CYP2C8 and CYP4F in cynomolgus monkeys compared to that in humans (Nishimuta *et al.,* 2011). We therefore speculate that when the pheroid vesicles is absorbed in the intestine of the monkey the increased intestinal metabolic activity of CYP4F isoforms, which is responsible for the metabolism of polyunsaturated fatty acids (Fer *et al.*, 2008) results in rapid metabolism of the pheroid vesicles. Consequently the AQ is rapidly released and metabolized by CYP2C8 that is also present in the intestine of the monkey. This speculation was partly supported by the *in vitro* metabolic stability data presented in this thesis, in which a higher CL_{int} for the pro-pheroid formulation in the MIM was observed compared to the reference, HIM and MLM.

The pheroid formulation did not enhance the absorption and bioavailability of AQ or DEAQ. This is contrary to the observed effects of pheroid technology on the PK of other drugs such as artemisone and CQ where the formulation increases AUC and prolongs the drug half-life.

Recommendations to consider for future studies on CQ, AQ and pheroid technology may include the following:

- Complete metabolic studies should be conducted to determine the involvement of the CYP P450 enzymes, especially the CYP 4F family in the metabolism of the pheroid delivery system. The involvement of mitochondrial metabolism should also be examined.
- The stability profile of AQ and other compounds supceptible to oxidation, entrapped in pheroid formulations should be investigated, as it is apparent that AQ and DEAQ

are susceptible to autoxidation due to the presence of the 4-hydoxyanilino aromatic moiety in AQ. It will be important to establish if the components of the pheroid formulation may possibly influence this process.

- DNA integrity (Comet assay) studies should be conducted on all pheroid formulations.

The efficacy both *in vitro* and *in vivo* of chloroquine in combination with other anti-malarial drugs should be evaluated in combination with pheroid formulations, as these combinations may prove to be of great value in the near future.

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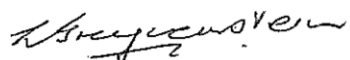
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PROOF OF EDITING

This is to certify that the language editing of this thesis by Ms L Gibhard was done by Prof L A Greyvenstein.

Prof L A Greyvenstein was a member of the South African Translators Institute, membership number: 1001691. She completed her primary secondary and tertiary education, including a doctoral thesis, in English. She has done the English language editing of many proposals, dissertations, theses and scientific articles

Lesley Ann Greyvenstein (Prof)

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Klerksdorp

2572

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RESPONSE: ETHICS APPLICATION

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
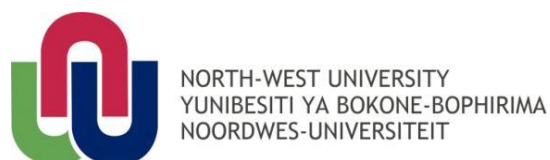
Private Bag X6001, Potchefstroom
South Africa 2520

Tel: (018) 299-4900
Faks: (018) 299-4910
Web: http://www.nwu.ac.za

ETHICS APPROVAL OF PROJECT

Ethics Committee
Tel   +27 18 299 4850
Fax   +27 18 293 5329
Email Ethics@nwu.ac.za

2010-07-15

This is to certify that the next project was approved by the NWU Ethics Committee:

Project title *Investigation into the comparative bioavailability and efficacy of Pheroid™-entrapped anti-infective agents in non-human primates*

Project leader: Dr. AF Grobler

Ethics number: **NWU-00027-10-A5**

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Expiry date: 2015/07/11

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

The formal Ethics approval certificate will be sent to you as soon as possible.

Yours sincerely

Me.Marietjie Halgryn
NWU Ethics Secretariate

CERTIFICATE OF ANALYSIS OF CQ

ċa **Ipca Laboratories Limited**

FACTORY & OFFICE : 89 A-B/90/91. INDUSTRIAL ESTATE. POLOGROUND, INDORE - 452 003 (M.P.)

Tel.	: (0731) 2421163 / 24211
	2422081
Telefax	: (91-731) 2422082
E-mail	: lpcalli@ipca.co.in

QUALITY DIVISION
CERTIFICATE OF ANALYSIS

NAME OF THE PRODUCT : CHLOROQUINE PHOSPHATE BP

BATCH NO.	: 8234C1RJB	A.R. NO.	: B/81600 – J
BATCH SIZE	: 776.00 Kgs.	DT. OF RECEIPT	: 20/11/2008
MFG. DATE	: NOV. 2008	DT. OF ANALYSIS	: 20/11/2008
EXP. DATE	: OCT. 2013		

PROTOCOL OF TESTS	SPECIFICATIONS		RESULTS
CHARACTERS	A white or almost white, crystalline powder, hygroscopic, freely soluble in water, very slightly soluble in alcohol and in methanol.		Conforms
MELTING POINT	Between 193°C and 198°C.		197°C
IDENTIFICATION	A) Conforms by UV.		Conforms
	B) Infrared absorption spectrum of sample and standard are concordant.		Conforms
	C) The picrate derivative melts at 206°C to 209ºC.		208°C
	D) Gives reaction (b) of Phosphates.		Conforms
APPEARANCE OF SOLUTION	A 10% solution is clear and not more intensely coloured than reference solution BY_5 or GY_5.		Conforms
pH	pH of a 10% w/v solution is 3.8 to 4.3.		4.21
RELATED SUBSTANCES (By TLC)	Any Related Substance	: NMT 1.0%	< 1.0%
	NMT one Related Substance	: NMT 0.5%	< 0.5%
HEAVY METALS	NMT 20 ppm		< 20ppm
LOSS ON DRYING (at 105°C)	NMT 2.0% w/w		1.18%
ASSAY	98.5% -101.0% (on dried basis)		99.54%

REMARKS : The sample **CONFORMS** with respect to **BP** specification.

PB 2621

Pooje

(ANALYST)

Date : 24/11/2008

REPRESENTED BY
D B Fine Chemicals (Pty) Limited
P.O Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS
1995 2005

(MANAGER QUALITY CONTROL)

Date : 24/11/2008

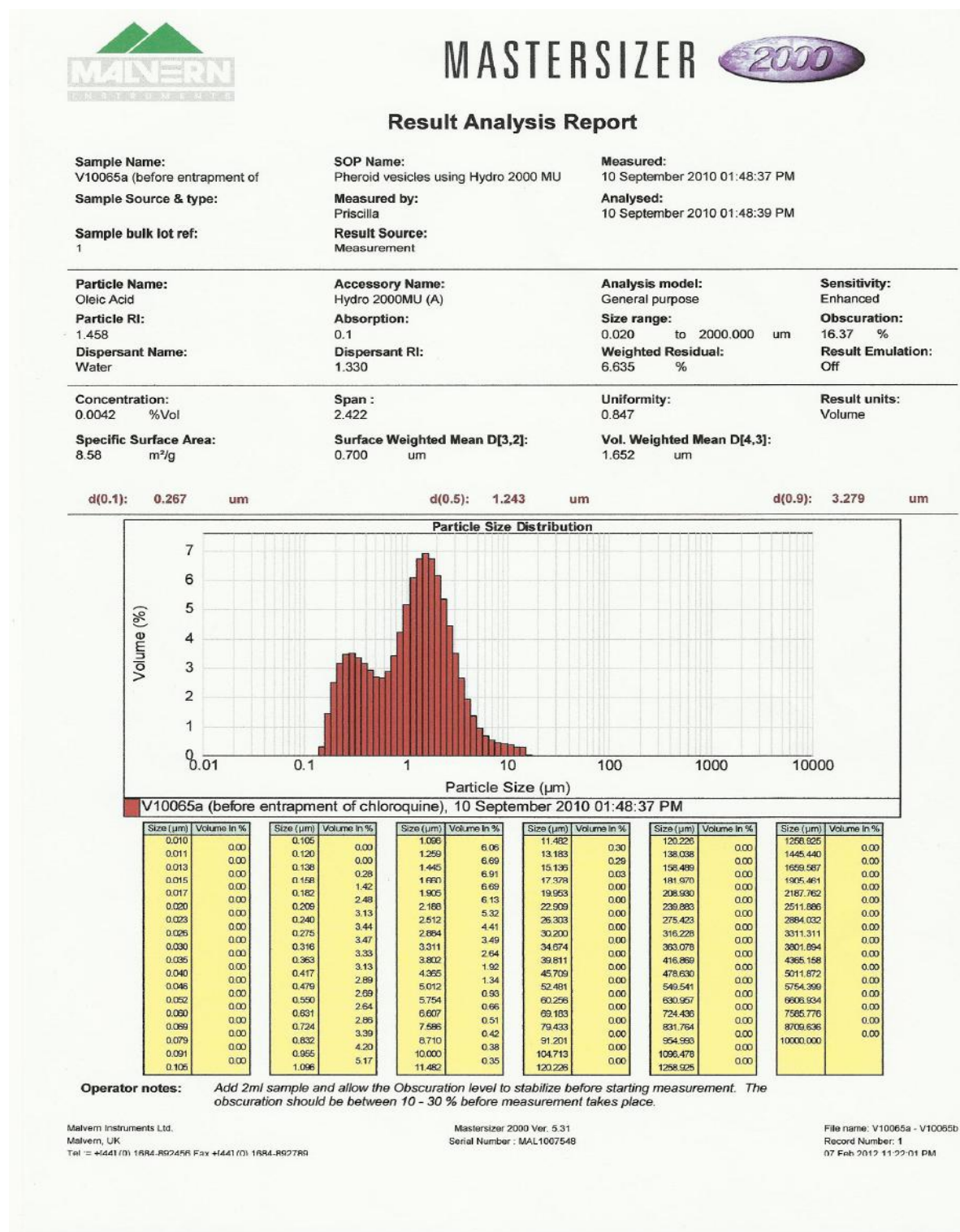Regd. Off. : 48, Kandivli Industrial Estate, Kandivli (West), Mumbai - 400 067. Phone : 6647 4444
Corporate Office : 142-AB. Kandivli Industrial Estate, Kandivli (West). Mumbai-400 067   Phone : 6647 4747
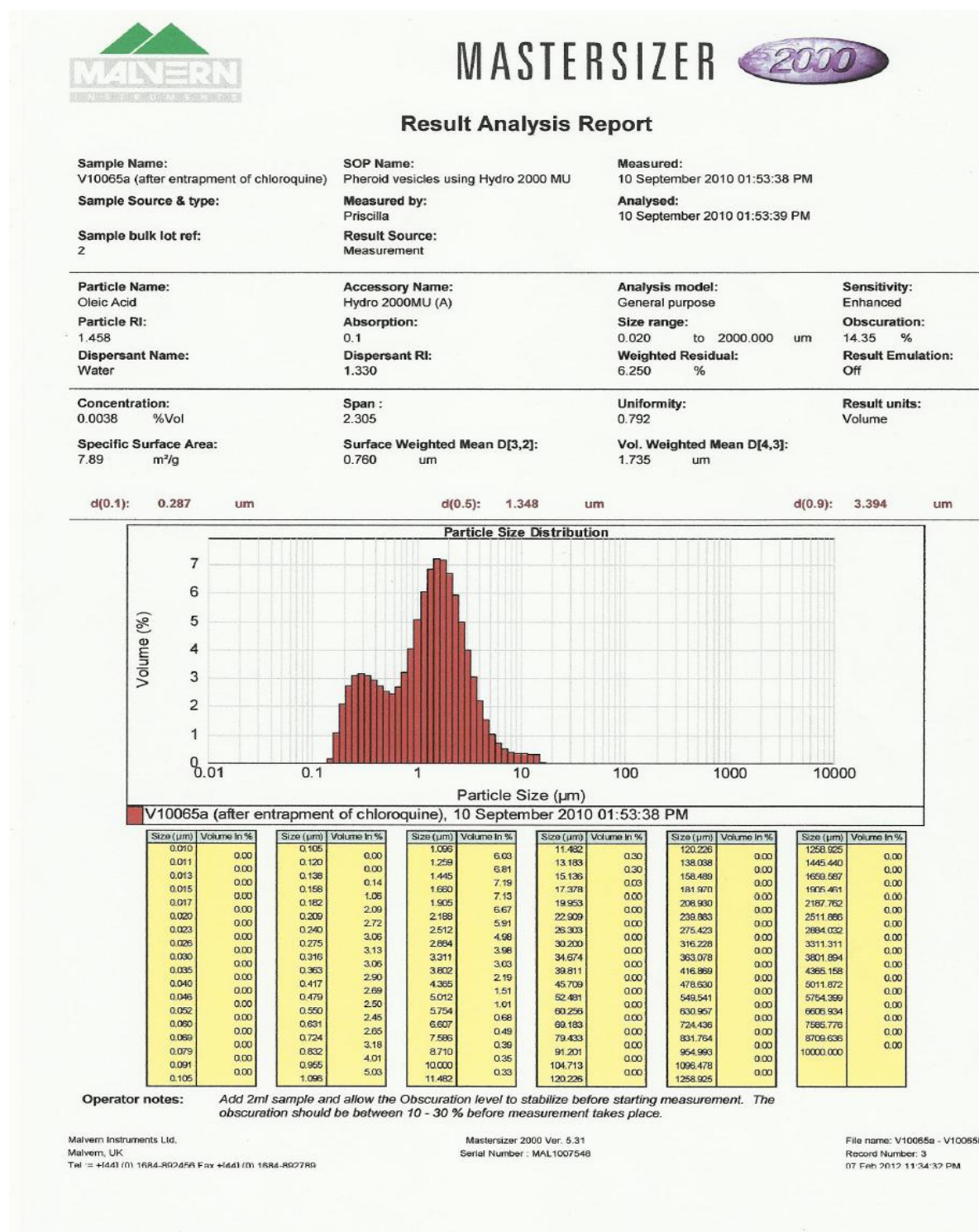
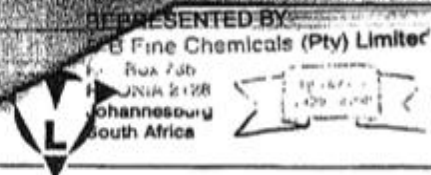**MALVERN MASTERSIZER REPORTS FOR CHLOROQUINE
ENTRAPPED IN PHEROID VESICLES**

Particle size distribution before entrapment of chloroquine

Particle size distribution before entrapment of chloroquine

Particle size distribution after spontaneous entrapment of chloroquine

MASTERSIZER 2000

Result Analysis Report

Sample Name: V10065a (after entrapment of chloroquine)	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 10 September 2010 01:53:38 PM
Sample Source & type:	**Measured by:** Priscilla	**Analysed:** 10 September 2010 01:53:39 PM
Sample bulk lot ref: 2	**Result Source:** Measurement	

Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 14.35 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 6.250 %	**Result Emulation:** Off

Concentration: 0.0038 %Vol	**Span:** 2.305	**Uniformity:** 0.792	**Result units:** Volume
Specific Surface Area: 7.89 m²/g	**Surface Weighted Mean D[3,2]:** 0.760 um	**Vol. Weighted Mean D[4,3]:** 1.735 um	

d(0.1): 0.287 um   **d(0.5):** 1.348 um   **d(0.9):** 3.394 um

Particle Size Distribution

V10065a (after entrapment of chloroquine), 10 September 2010 01:53:38 PM

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	6.03	11.482	0.30	120.226	0.00	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	6.81	13.183	0.30	138.038	0.00	1445.440	0.00
0.013	0.00	0.138	0.14	1.445	7.19	15.136	0.03	158.489	0.00	1659.587	0.00
0.015	0.00	0.158	1.06	1.660	7.13	17.378	0.00	181.970	0.00	1905.461	0.00
0.017	0.00	0.182	2.09	1.905	6.67	19.953	0.00	208.930	0.00	2187.762	0.00
0.020	0.00	0.209	2.72	2.188	5.91	22.909	0.00	239.883	0.00	2511.886	0.00
0.023	0.00	0.240	3.06	2.512	4.98	26.303	0.00	275.423	0.00	2884.032	0.00
0.026	0.00	0.275	3.13	2.884	3.98	30.200	0.00	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	3.06	3.311	3.03	34.674	0.00	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	2.90	3.802	2.19	39.811	0.00	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	2.69	4.365	1.51	45.709	0.00	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	2.50	5.012	1.01	52.481	0.00	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	2.45	5.754	0.68	60.256	0.00	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	2.65	6.607	0.49	69.183	0.00	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	3.18	7.586	0.39	79.433	0.00	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	4.01	8.710	0.35	91.201	0.00	954.993	0.00	10000.000	
0.091	0.00	0.955	5.03	10.000	0.33	104.713	0.00	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place.*

Malvern Instruments Ltd.
Malvern, UK
Tel := +(44) (0) 1684-892456 Fax +(44) (0) 1684-892789

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: V10065a - V10065b
Record Number: 3
07 Feb 2012 11:34:32 PM

CERTIFICATE OF ANALYSIS OF AQ

REPRESENTED BY
B Fine Chemicals (Pty) Limited
Box 786
UNIA 2128
ohannesburg
South Africa

Vital Laboratories Private Limited

PLANT : Plot No. 1710 & A1/2208, GIDC Estate,
3rd Phase, VAPI - 396 195. Gujarat, India
Tele : +91-260-2420154, 2424744
Fax No. : +91-260-2401497
E-mail: vitallab@vitallab.com website: www.vitallab.com

CERTIFICATE OF ANALYSIS

AMODIAQUINE HYDROCHLORIDE USP

Manufacturer Name	: Self	A. R. No.	: AAMQ/003/09
License No.	: G/1664	Mfg. Date	: Feb. – 2009
Batch No.	: AMQ0902003	Exp. Date	: Jan. – 2014
Batch Size	: 2.0 Kgs.	Date of Analysis	: 20/02/2009
Date of Receipt	: 19/02/2009	Date of Report	: 20/02/2009

Sr. No.	Test	Result	Standard
1.	Description	Yellow, crystalline powder. Is odorless and has bitter taste.	Yellow, crystalline powder. Is odorless and has bitter taste.
2.	Solubility	Passes	Soluble in water, sparingly soluble in alcohol; very slightly soluble in benzene, in chloroform and in ether.
3.	Identification	Complies Complies Complies	(A) : IR (B) : UV Spectrum (C) : Chloride test
4.	Completeness of solution	Passes	A solution of 200 mg in 10 ml water is clear.
5.	Chromatography purity by TLC.	Passes	As per USP
6.	Water	8.47 %	7.0% to 9.0%
7.	Residue on ignition	0.05 %	NMT 0.2 %
8.	Assay (on dried basis.)	100.53 %	97.0 % to 103.0 %
9.	Residual solvent Ethyl alcohol	145.67 ppm	NMT. 5000 ppm.

Remarks: The above sample complies as per USP specification.

Analyst By. _____   Checked By. _____   Approved By. _____

Corp. Office :- "VENKATESH" Mahant Road Extn, Uttkarsh Mandal Circle, Vile Parle (E), MUMBAI - 400 057. (INDIA)
Ph. : +91-22-26183641 / 26136946 32483114 / 5 Fax : +91-22 - 26136945

**MALVERN MASTERSIZER REPORT FOR PRE-ENTRAPPED AQ IN
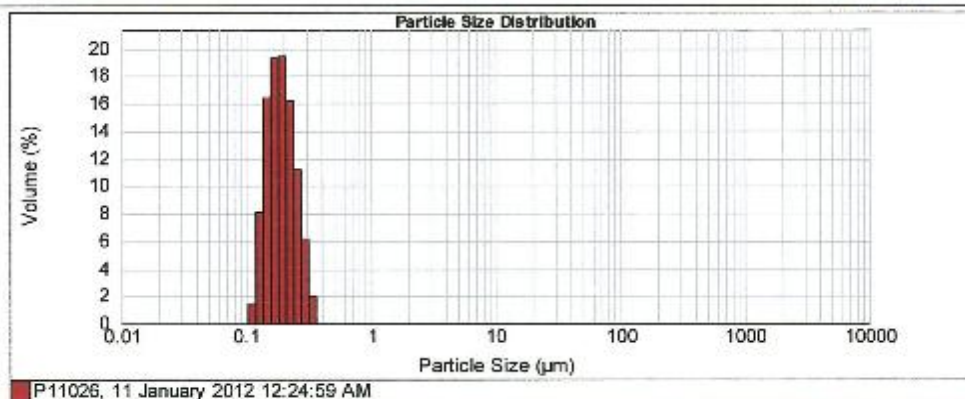PRO - PHEROIDS**

MASTERSIZER 2000

Result Analysis Report

Sample Name: P11026	**SOP Name:** Pharoid vesicles using Hydro 2000 MU	**Measured:** 11 January 2012 12:24:59 AM
Sample Source & type:	**Measured by:** Maggie	**Analysed:** 11 January 2012 12:25.00 AM
Sample bulk lot ref: 1	**Result Source:** Measurement	

Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 6.22 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 23.771 %	**Result Emulation:** Off

Concentration: 0.0013 %Vol	**Span :** 0.685	**Uniformity:** 0.214	**Result units:** Volume
Specific Surface Area: 32.5 m²/g	**Surface Weighted Mean D[3,2]:** 0.185 um	**Vol. Weighted Mean D[4,3]:** 0.196 um	

d(0.1): 0.139 um   **d(0.5):** 0.188 um   **d(0.9):** 0.268 um

Particle Size Distribution

■ P11026, 11 January 2012 12:24:59 AM

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place.*

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.40
Serial Number : MAL1007548

File name: P11026
Record number: 1
11 Jan 2012 12:27:25 AM