

**Efficacy enhancement of the antimalarial drugs,
mefloquine and artesunate, with Pheroid™
technology**

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Kol hahatchalot kashot

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ABSTRACT

EFFICACY ENHANCEMENT OF THE ANTIMALARIAL DRUGS, MEFLOQUINE AND ARTESUNATE, WITH PHEROID™ TECHNOLOGY

Malaria is currently one of the most imperative parasitic diseases in developing countries. Artesunate has a short half-life, low aqueous solubility and resultant poor and erratic absorption upon oral administration, which translate to low bioavailability. Mefloquine is eliminated slowly with a terminal elimination half-life of approximately 20 days and has neuropsychiatric side effects. Novel drug delivery systems have been utilised to optimise chemotherapy with currently available antimalarial drugs. Pheroid™ technology is a patented drug delivery system which has the ability to capture, transport and deliver pharmaceutical compounds. Pheroid™ technology may play a key role in ensuring effective delivery and enhanced bioavailability of novel antimalarial drugs. The aim of this study was to evaluate the possible efficacy and bioavailability enhancement of the selected antimalarial drugs, artesunate and mefloquine, in combination with Pheroid™ vesicles.

The *in vitro* efficacy of artesunate and mefloquine, co-formulated in the oil phase of Pheroid™ vesicles and entrapped in Pheroid™ vesicles 24 hours after manufacturing were investigated against a 3D7 chloroquine-sensitive strain of *Plasmodium falciparum*. Parasitemia (%) was quantified with flow cytometry after incubation periods of 48 and 72 hours. Drug sensitivity was expressed as 50% inhibitory concentration (IC₅₀) values. An *in vivo* bioavailability study with artesunate and mefloquine was also conducted in combination with Pheroid™ vesicles, using a mouse model. A sensitive and selective liquid chromatography- tandem mass spectrometry (LC-MS/MS) method was developed to analyse the drug levels. C57 BL6 mice were used during this study. The selected antimalarial drugs were administered at a dose of 20 mg/kg with an oral gavage tube. Blood samples were collected by means of tail bleeding.

The *in vitro* drug sensitivity assays revealed that artesunate, co-formulated in the oil phase of Pheroid™ vesicles and evaluated after a 48 hour incubation period, decreased the IC₅₀ concentration significantly by 90%. Extending the incubation period to 72 hours decreased the IC₅₀ concentration of artesunate, also co-formulated in the oil phase of Pheroid™ vesicles significantly by 72%. No statistically significant differences between the reference and Pheroid™ vesicle groups were achieved when artesunate was entrapped 24 hours after

manufacturing of Pheroid™ vesicles. Mefloquine co-formulated in the oil phase of Pheroid™ vesicles and evaluated after a 48 hour incubation period decreased the IC₅₀ concentration by 36%. Extending the incubation period to 72 hours increased the efficacy of the Pheroid™ vesicles and the IC₅₀ concentration was significantly decreased by 51%. In contrast with the results obtained with artesunate, entrapment of mefloquine in Pheroid™ vesicles 24 hours after manufacturing decreased the IC₅₀ concentration significantly by 66%.

The LC-MS/MS method was found to be sensitive, selective and accurate for the determination of artesunate and its active metabolite, dihydroartemisinin (DHA) in mouse plasma and mefloquine in mouse whole blood. Most of the artesunate plasma concentrations were below the limit of quantification in the reference group and relatively high outliers were observed in some of the samples. The mean artesunate levels of the Pheroid™ vesicle group were lower compared to the reference group, but the variation within the Pheroid™ vesicle group lessened significantly. The mean DHA concentrations of the Pheroid™ vesicle group were significantly higher. DHA obtained a higher peak plasma drug concentration with the Pheroid™ vesicle group (173.0 ng/ml) in relation to the reference group (105.0 ng/ml) and at a much faster time (10 minutes in Pheroid™ vesicles in contrast to 30 minutes of the reference group). Pharmacokinetic models could not be constructed due to blood sampling per animal limitation. The incorporation of mefloquine in Pheroid™ vesicles did not seem to have improved results in relation to the reference group. No statistical significant differences were observed in the pharmacokinetic parameters between the two groups. The relative bioavailability (%) of the Pheroid™ vesicle incorporated mefloquine was 7% less bioavailable than the reference group.

Keywords: Malaria, artesunate, mefloquine, *in vitro* drug sensitivity assays, flow cytometry, bioavailability, LC-MS/MS methods.

UITTREKSEL

EFFEKTIVITEITSVERHOOGING VAN DIE ANTIMALARIA GENEESMIDDELS, MEFLOKIEN EN ARTESUNAAT, MET PHEROID™ TEGNOLOGIE

Malaria is tans een van die belangrikste parasitiese siektes in ontwikkelende lande. Artesunaat het 'n kort half-leeftyd, lae wateroplosbaarheid en gevolglike swak en onreëlmatige absorpsie na orale toediening, wat aanleiding gee tot lae biobeskikbaarheid. Meflokien word stadig geëlimineer met 'n terminale eliminasië half-leeftyd van ongeveer 20 dae en toon sentrale senuweestelsel nuwe-effekte. Nuwe geneesmiddel afleweringstelsels word gebruik om die effektiwiteit van huidige antimalaria geneesmiddels te verbeter. Pheroid™ tegnologie is 'n gepatenteerde geneesmiddel afleweringstelsel wat die vermoë het om aktiewe bestanddele te enkapsuleer, te vervoer en af te lewer. Pheroid™ tegnologie kan dus moontlik 'n belangrike rol speel in effektiewe aflewering en dus verbeterde biobeskikbaarheid van huidige antimalaria geneesmiddels. Die doel van hierdie studie was om die moontlike verhoogde effektiwiteit en biobeskikbaarheid van geselekteerde antimalaria geneesmiddels, naamlik artesunaat en meflokiën, in kombinasie met Pheroid™ vesikels te ondersoek.

Die *in vitro* effektiwiteit van artesunaat en meflokiën, geformuleer in die olie fase van Pheroid™ vesikels en ge-enkapsuleer in Pheroid™ vesikels 24 uur na vervaardiging was getoets op 'n 3D7 chlorokien-sensitiewe vorm van *Plasmodium falciparum*. Parasitemia (%) was geanaliseer met behulp van 'n vloeï-sitometriese metode, na inkubasië periodes van 48 en 72 uur. Geneesmiddel-sensitiwiteit is uitgedruk as 50% inhibisie konsentrasie (IC₅₀). 'n *In vivo* biobeskikbaarheidsstudie was ook met artesunaat en meflokiën in kombinasie met Pheroid™ vesikels uitgevoer deur gebruik te maak van 'n muis model. 'n Sensitiewe en selektiewe vloeïstof chromatografie- tandem massa spektrometrie (LC-MS/MS) metode was ontwikkel om die geneesmiddelvlakke te bepaal. C57 BL6 muis was gebruik gedurende hierdie studie. Die gekose antimalaria geneesmiddels was toegedien teen 'n dosis van 20 mg/kg met behulp van 'n orale maagspoelbuis. Bloedmonsters was geneem deur middel van stert-bloeding.

Die *in vitro* geneesmiddel-sensitiwiteitstoets het getoon dat artesunaat, geformuleer in die olie fase van Pheroid™ vesikels, gemeet na 'n inkubasië periode van 48 uur, die IC₅₀ waarde betekenisvol met 90% verlaag het. Verlenging van die inkubasië periode na 72 uur het die IC₅₀

waarde van artesunaat, ook geformuleer in die olie fase van Pheroid™ vesikels, betekenisvol met 72% verlaag. Daar was egter geen statisties betekenisvolle verskille bereik tussen die verwysings- en Pheroid™ vesikel groepe nadat artesunaat 24 uur na vervaardiging ge-enkapsuleer was in Pheroid™ vesikels nie. Meflokien geformuleer in die olie fase van Pheroid™ vesikels en getoets na 'n 48 uur inkubasie periode het die IC_{50} konsentrasie verlaag met 36%. Verlenging van die inkubasie periode na 72 uur het die effektiwiteit van die Pheroid™ vesikels verhoog en die IC_{50} konsentrasie was betekenisvol verlaag met 51%. In teenstelling met die resultate verkry met artesunaat, het meflokiën ge-enkapsuleer in Pheroid™ vesikels 24 uur na vervaardiging die IC_{50} konsentrasie betekenisvol verlaag het met 66%.

Die LC-MS/MS metode was sensitief, selektief en akkuraat vir die bepaling van artesunaat en sy aktiewe metaboliet dihidroartemisien (DHA) in muis plasma en meflokiën in muis heelbloed. Meeste van die artesunaat plasma-konsentrasies was onder die limiet vir kwantifisering in die verwysingsgroep en relatiewe hoë uitskieters was waargeneem in sommige van die monsters. Die gemiddelde artesunaat-vlakke van die Pheroid™ vesikel groep was laer in vergelyking met die verwysingsgroep, maar die intervariasie van die Pheroid™ vesikel groep was aansienlik minder. Die gemiddelde DHA konsentrasies van die Pheroid™ vesikel groep was aansienlik hoër. DHA het 'n hoër piek plasma-konsentrasie teen 'n vinniger tydperk bereik in die Pheroid™ vesikel groep (173.0 ng/ml na 10 minute) in vergelyking met die verwysingsgroep (105.0 ng/ml na 30 minute). Farmakokinetiese modelle kon egter nie gepas word nie as gevolg van die bloedmonsterneming limiet per muis. Meflokien geïnkorporeer in Pheroid™ vesikels het nie merkwaardig beter resultate in vergelyking met die verwysingsgroep opgelewer nie. Daar was geen statisties betekenisvolle verskille tussen die twee groepe se farmakokinetiese parameters nie. Die relatiewe biobeskikbaarheid (%) van meflokiën geïnkorporeer in Pheroid™ vesikels was 7% minder biobeskikbaar as die verwysingsgroep.

Slutelwoorde: Malaria, artesunaat, meflokiën, *in vitro* geneesmiddel sensitiwiteitstoetse, vloei-sitometrie, biobeskikbaarheid, LC-MS/MS metodes.

INTRODUCTION AND AIM OF STUDY

Nobel Prize winner Sir MacFarlane Burnet said "If we take as our standard of importance the greatest harm to the greatest number, then there is no question that malaria is the most important of all infectious diseases". Malaria is an infectious disease caused by parasites of the *Plasmodium* genus. The parasites are primarily hosted by female *Anopheles* mosquitoes, which act as vectors that transmit the protozoan organisms to humans when feeding. There are four known species that infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*, however, *P. falciparum* can be held liable for the majority of malaria infections (WHO, 2008). Nearly half of the world's population is at risk of contracting malaria. In 2008, 109 countries were endemic for malaria (WHO, 2008). Malaria incidence has increased during the last decade, despite efforts to control the disease. Several reasons for deterioration of malaria control are climate instability, global warming, civil disturbances, increasing travel, infection with human immunodeficiency virus (HIV) as well as insecticide- and multiple drug resistance (Greenwood & Mutabingwa, 2002).

Current effective antimalarial drugs are frequently limited in application for chemotherapy strategies due to poor aqueous solubility, low bioavailability, high toxicity and multiple drug resistance (Santos-Magalhães & Mosqueira, 2009). As an artemisinin derivative, the efficacy of artesunate is impaired by its short plasma half-life, its low aqueous solubility and resultant poor and erratic absorption upon oral administration, as well as a high rate of recrudescence when used alone as monotherapy (Kongpatanakul *et al.*, 2007). The poor solubility and erratic absorption of this drug usually translate to low bioavailability. Mefloquine is eliminated slowly with a terminal elimination half-life of approximately 20 days. Neuropsychiatric toxicity associated with mefloquine has received a great deal of publicity. However, tolerability is improved by splitting administration of the drug into two doses, separated by six to eight hours, or by administering the drug after artesunate in combination treatment (Rosenthal, 2004; Smithuis *et al.*, 2004). Moreover, resistance to mefloquine was reported as early as five years after its introduction as a prophylactic agent in parts of Thailand (Wongsrichanalai *et al.*, 2002). There is thus a growing concern about toxicity and especially resistance emerging towards the drug. Malaria treatment is therefore far from optimal and this can lead to relapse infections and increased drug resistance.

Nanosized lipid-based colloidal drug carriers such as liposomes and nano- and micro-emulsions have received special attention to antimalarial drug delivery with the aim of improving the pharmacokinetic profile of current effective antimalarial drugs (Santos-Magalhães & Mosqueira, 2009). The purpose of a drug delivery system is to control pharmacological parameters such as efficacy and bioavailability. The Pheroid™ drug delivery system may play a key role in ensuring effective delivery and enhanced bioavailability of current antimalarial compounds. Pheroid™ technology is a patented, novel, colloidal type drug delivery system which consists primarily of plant and essential fatty acids that have been emulsified in water and saturated with nitrous oxide (Grobler, 2004). Pheroid™ vesicles are small vesicle like structures with a bilayer membrane and a hydrophilic core (du Plessis *et al.*, 2009). Pheroid™ vesicles have the ability to entrap both hydrophilic and hydrophobic drugs. Drugs can be formulated with Pheroid™ vesicles by either co-formulating the drugs in the oil phase of the Pheroid™ vesicles during manufacturing, or by allowing drug entrapment to take place over a period of 24 hours after manufacturing of the Pheroid™ vesicles.

Malaria *in vitro* drug efficacy assays are a versatile laboratory tool that is regarded as indispensable for drug screening. It is used in malaria experimental studies to determine the efficacy of drugs, drug interactions, to assess the degree of cross resistance between *Plasmodium* species and to compare the response of pre-treatment and post-treatment in clinical isolates. Another important function of *in vitro* drug efficacy assays is to evaluate the effect of drugs on various stages of the erythrocytic cycle of *P. falciparum*. *P. falciparum* completes an erythrocytic cycle in 48 hours *in vivo* and the duration of this cycle is similar *in vitro*. The incubation period for *in vitro* drug efficacy assays varies from 24 to 96 hours, depending on the method of analysis or the drugs being tested. In the standard assay, the incubation period is 48 hours, i.e. within the first erythrocytic cycle. By delaying the incubation period to 72 hours, one can evaluate whether drugs have a delayed inhibitory effect on parasites after the first completed erythrocytic cycle (Basco, 2007).

In vitro studies have greater impact when combined with *in vivo* studies, including pharmacokinetic (bioavailability) studies. Simultaneous analyses of *in vitro* efficacy and *in vivo* bioavailability have the added advantage of establishing a threshold concentration, which can be used in clinical trials (Fidock *et al.*, 2004).

The broad objective of this study was to evaluate the possible efficacy enhancement of the selected antimalarial drugs, artesunate and mefloquine in combination with Pheroid™ technology *in vitro*, as well as the possible enhancement of bioavailability *in vivo*.

The specific aims of this study were:

- analysing the *in vitro* efficacy of artesunate and mefloquine, alone and in combination with Pheroid™ vesicles against a 3D7 chloroquine-sensitive strain of *P. falciparum* via flow cytometry using a standard 48 hour *in vitro* test;
- to evaluate the effect of two different formulation methods with Pheroid™ vesicles, namely co-formulating artesunate and mefloquine in the oil phase of Pheroid™ vesicles during manufacturing, as well as entrapment of the drugs in the Pheroid™ vesicles for 24 hours after manufacturing, utilising *in vitro* drug efficacy assays;
- to evaluate whether the drugs co-formulated with Pheroid™ vesicles or entrapped within the Pheroid™ vesicles for 24 hours have an influence on the stage specificity of the malaria parasite, by performing the *in vitro* efficacy assay with an extended incubation period of 72 hours;
- the development and validation of a sensitive and accurate LC-MS/MS method for evaluation of the levels of artesunate in mouse plasma and mefloquine in mouse whole blood;
- to evaluate the *in vivo* bioavailability of artesunate and mefloquine, alone and in combination with Pheroid™ vesicles in mouse plasma and –whole blood respectively, using pharmacokinetic models.

This dissertation presents a review of the relevant literature regarding malaria (Chapter 1), antimalarial drugs (Chapter 2) and Pheroid™ technology (Chapter 3). Chapter 4 describes the *in vitro* evaluation of the experimental compounds and reports and discusses the results obtained. Chapter 5 describes the *in vivo* evaluation of the experimental compounds and reports and discusses the results obtained. Finally, conclusions are drawn and recommendations are made.

CHAPTER 1

MALARIA, AN ANCIENT DISEASE

1.1 Introduction

Parasitic diseases are of immense global significance as approximately 30% of the world's population is infected with some kind of parasitic pathogen. Moreover, parasitic infections impose a substantial burden of morbidity and mortality around the globe, but especially in developing countries (Edwards & Krishna, 2004). As depicted in Table 1.1, malaria is currently considered the world's most important parasitic infection in humans, being the most widespread illness with almost half of the world's population at risk of contracting this disease (WHO, 2008). The name malaria was derived from the Italian term "*mal'aria*" meaning "bad air", because the disease was thought to be caused by inhaling air from marshy or swampy areas. Malaria was commonly referred to as "swamp fever" (CDC, 2004; Tuteja, 2007).

Table 1.1: Global burden of some of the most important parasitic diseases (TDR: available at www.who.int.tdr/diseases/default.htm).

Disease	Mortality (thousands)	*DALY's (thousands)
Malaria	1272	46,486
Leishmaniasis	51	2090
African trypanosomiasis	48	1525
South American-trypanosomiasis	14	667
Schistosomiasis	15	1702
Lymphatic filariasis	0	5777
Onchocerciasis	0	484

*DALY's: disability adjusted life years (the number of healthy years lost due to premature death and disability).

Before 1940, about two-thirds of the world's population lived in malaria endemic areas. During the 1950's and 1960's, the disease was eradicated from most temperate regions because the insecticide dichloro-diphenyl-trichloroethane (DDT) made it possible to control mosquitoes inexpensively. However, it did not take long for DDT-resistant mosquitoes to emerge and *Plasmodium falciparum*, one of the causative pathogens of malaria, soon became drug resistant. These blows brought a resurgence of malaria during the 1970's. The elimination of malaria from most of Europe and North America and the failure of the global malaria eradication programme led to a loss of interest in malaria for a period of about 25 years, from the early 1970's to the late 1990's. Thus, for many years there was little change in morbidity and mortality of malaria, especially in Africa (Ingraham & Ingraham, 2000; Greenwood & Mutabingwa, 2002). Malaria is as prevalent today as it was early in the 20th century, only its distribution has changed. Malaria today is confined almost exclusively to tropical and sub-tropical countries of the world (CDC, 2004).

Nearly 40% of the world's population is at risk of contracting malaria. In 2008, 109 countries were endemic for malaria, 45 of which were within the World Health Organisation's (WHO) African Region. The latest world malaria report by the WHO estimated 247 million clinical malaria cases worldwide in 2006, of which 91% were due to *P. falciparum*. The majority of cases were in the African region (86%), followed by the South-East Asian (9%) and Eastern Mediterranean (3%) regions (WHO, 2008). In the same year, an estimated 881 000 deaths occurred worldwide due to malaria, 90% of which were in the African region and 4% in each of the South-East Asian and Eastern Mediterranean regions. The risk of death from malaria is thus considerably higher in Africa than other parts of the world. An estimated 85% of all deaths occur in children under five years of age, particularly in the sub-Saharan region of Africa (WHO, 2008).

Although most deaths from malaria arise in Africa, evidence suggests that the actual number of clinical episodes of *P. falciparum* malaria is higher than that widely quoted and that morbidity due to malaria in Asia has been greatly underestimated, including that due to *Plasmodium vivax* infections (Hay *et al.*, 2004). The actual number of clinical cases of malaria and its impact is probably underestimated by current surveillance approaches (Fevre & Barnish, 1999; Snow *et al.*, 2005). One reason for this statement is that, in countries where malaria infection is frequent, residents often recognise the symptoms as malaria and treat themselves without seeking diagnostic confirmation (CDC, 2006). The effect of malaria extends far beyond these direct measures of mortality and morbidity. The current social, economic and medical impact of malaria is immense, as this massive burden of disease is borne disproportionately by some of

the poorest countries in the world and is in itself an obstacle to economic growth (Sachs & Malaney, 2002).

In addition to the actual numbers affected by malaria being underestimated, malaria incidence has increased during the last decade, despite efforts to control the disease. Several reasons for deterioration of malaria control are climate instability and global warming, civil disturbances, increasing travel, infections with human immunodeficiency virus (HIV) and insecticide resistance (Greenwood & Mutabingwa, 2002). However, the main cause of the worsened malaria situation recorded in recent years has been the spread of drug resistant *Plasmodium* parasites (White *et al.*, 1999).

This chapter provides an overview of the epidemiology of malaria and the *Plasmodium spp.* life cycle. The clinical symptoms and pathogenesis of the disease are described and finally, the methods for diagnosis and the basic trends for the treatment of malaria are discussed.

1.2 Epidemiology of malaria

The main driving force behind the prevention of malaria has been the science of epidemiology combined with public health programs. Epidemiology is the study of when and where diseases occur and how they are transmitted. Public health programs develop and implement ways to prevent and control disease. Epidemiology thus generates the information needed to carry out effective public health programs (Ingraham & Ingraham, 2000).

1.2.1 Insect vectors and malaria-causing pathogens

In 1880, Charles Louis Alphonse Laveran, a French army surgeon, was the first to notice parasites in the blood of a patient suffering from malaria. In 1897, Sir Ronald Ross, a British officer in the Indian Medical Service, discovered that mosquitoes transmitted malaria. The Italian professor Giovanni Batista Grassi subsequently showed that human malaria could only be transmitted by *Anopheles* mosquitoes (CDC, 2004). Of the approximately 400 *Anopheles* species throughout the world, about 60 are malaria vectors under natural conditions, of which 30 are of major importance (Tuteja, 2007). Female *Anopheles* mosquitoes take blood meals to carry out egg production and such blood meals are the link between the human and mosquito hosts in the parasite's life cycle. In contrast with the human host, the mosquito vector does not suffer noticeably from the presence of the parasites (CDC, 2004).

The malaria parasite is a *Plasmodian* protozoan species, which evolved over time, differentiating into four distinct species important to man - *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* (CDC, 2004). These species differ morphologically, immunologically, in their geographic distribution, in their relapse patterns and in their drug responses (Tuteja, 2007). The least common malaria parasite is *P. ovale*, which is almost exclusively restricted to West Africa, while *P. malariae* is found worldwide, mainly in Africa, but also with relatively low frequency. The most prevalent malaria parasite is *P. vivax*, which rarely causes death, but contributes substantially to the disease burden of malaria (CDC, 2004; Tuteja, 2007). *P. falciparum* is the predominant specie in most endemic countries and is found particularly in sub-Saharan Africa. *P. falciparum* is the causative agent of severe, potentially fatal malaria and accounts for the preponderance of global morbidity and mortality, causing an estimated 2,7 million deaths annually, of which the primary victims are young children in Africa (WHO 2008).

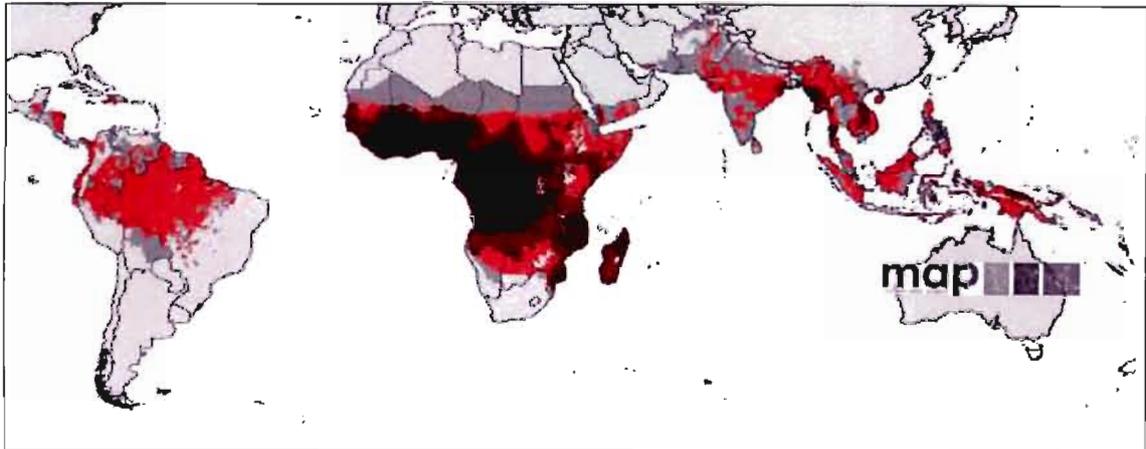
1.2.2 Incidence and geographic distribution of malaria

It has been reported that malaria directly causes about 1 million deaths per year or 3000 deaths a day and that most of these deaths occur in African children. Of the 500 million clinical attacks of malaria that occur every year, 2-3 million are categorised as severe attacks (CDC, 2007; WHO, 2008).

Climate is a key determinant in the geographic distribution and the seasonality of malaria, as it can influence all three components of the *Plasmodium* life cycle, namely *Anopheles* mosquitoes, humans and *Plasmodium* parasites. Malaria today is confined almost exclusively to tropical and subtropical countries where climatic factors such as temperature, humidity and rainfall are ideal for the survival and multiplication of *Anopheles* mosquitoes. Temperature is particularly critical for malaria parasites to complete their growth cycle or extrinsic incubation period in the mosquitoes. Warmer ambient temperatures shorten the duration of this extrinsic cycle, thus increasing the chances of transmission (CDC, 2004).

Malaria has a worldwide distribution with many areas of the tropics endemic for the disease. A global malaria distribution map is shown in Figure 1.1. The highest transmission is found in sub-Saharan Africa (WHO, 2008). There are however major differences in the prevalence of malaria between countries in Africa, between districts in the same country and even between villages situated only a mile or two apart. Consideration of the whole of tropical Africa as an area of hyper-endemic malaria transmission is however a simplification of a very complex

epidemiological situation (Greenwood & Mutabingwa, 2002). Interventions to control malaria are discussed in Section 1.6.



Light grey: no risk

Medium grey: unstable risk

Light red: low risk

Medium red: intermediate risk

Dark red: high risk

Figure 1.1: Global malaria endemicity (Modified from MAP: available at www.map.ox.ac.uk).

1.3 The *Plasmodium spp.* life cycle

Knowledge of the life cycle of *Plasmodium* is a key to understanding the clinical manifestations, treatment and research on malaria (Daily, 2006). *Plasmodium* is an obligate endoparasite and has a complex life cycle that requires specialised protein expression for survival in both the insect and human hosts. These proteins are required for both intracellular and extracellular survival, the invasion of a variety of cell types and the evasion of host immune responses (Tuteja, 2007). Sexual reproduction, called sporogony, occurs in female *Anopheles* mosquitoes and asexual reproduction, called schizogony, takes place in the liver and red blood cells of the human host (Ingraham & Ingraham, 2000). The multifaceted life cycle of *Plasmodium spp.* is presented in Figure 1.2.

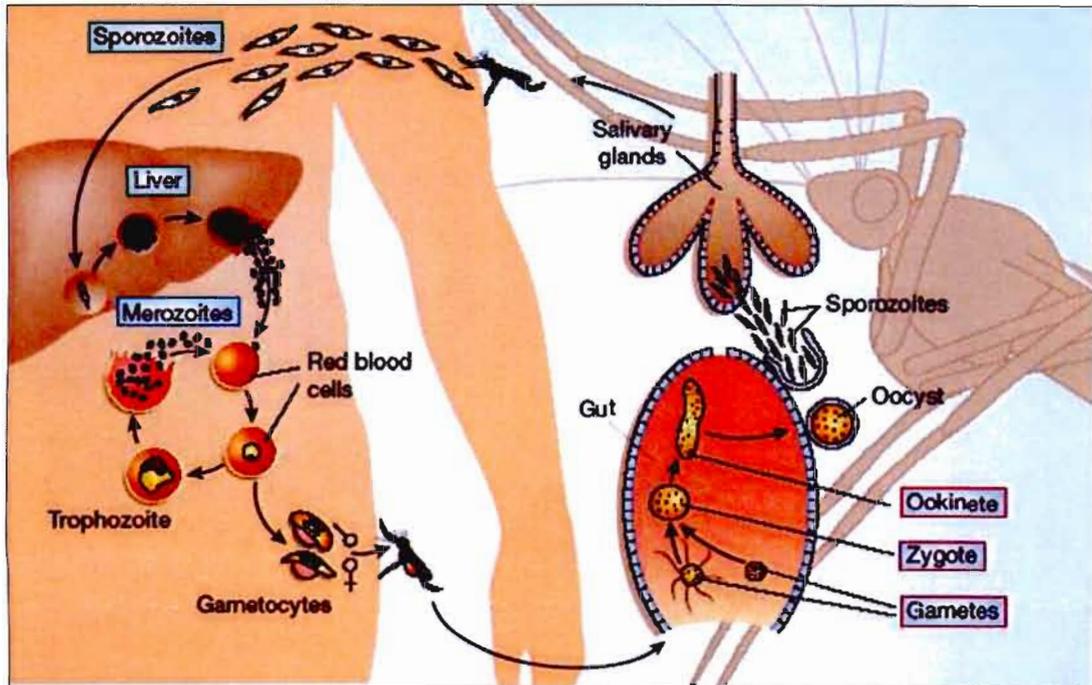


Figure 1.2: A schematic presentation of the *Plasmodium spp.* life cycle (Ménard, 2005).

1.3.1 Exo-erythrocytic or tissue schizogony (initial asexual replication of the parasite in the human host's liver)

During a blood meal, a *Plasmodium*-infected female *Anopheles* mosquito inoculates the sporozoite form of the protozoan from its salivary glands into the human host's bloodstream. Sporozoites migrate to the liver where they infect liver cells (hepatocytes), undergo asexual replication and mature into schizonts, which subsequently rupture from the hepatocytes, releasing another morphological form of the parasite (merozoites) into the bloodstream (Ingraham & Ingraham, 2000; CDC, 2006).

1.3.2 Erythrocytic schizogony (asexual multiplication of the parasite in the human host's red blood cells)

Merozoites infect red blood cells and rapidly replicate into early ring stage trophozoites and finally into schizonts. The morphological differences between these specific merozoite phases are shown in Figure 1.3. Mature schizonts rupture from the erythrocytes, releasing even more merozoites to infect yet more red blood cells. This asexual cycle in the blood, from the invasion of red blood cells by merozoites until schizont rupture takes 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* infections and 72 hours for *P. malariae* infection. A small proportion of the merozoites in the erythrocytes eventually differentiate to produce micro- (male) and macro-

(female) gametocytes. These gametocytes are ingested by a female *Anopheles* mosquito when feeding, where the next phase of the parasite's life cycle begins (Ingraham & Ingraham, 2000; CDC, 2006).

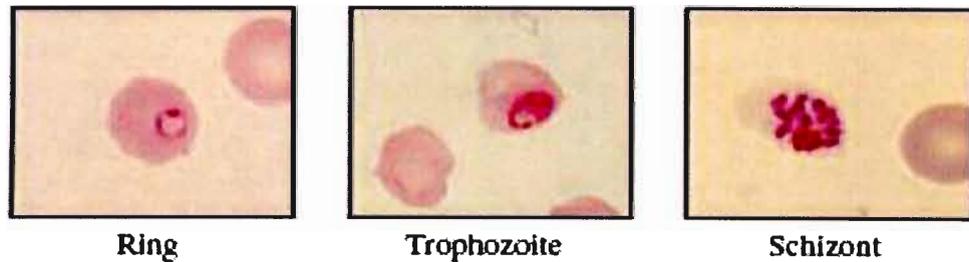


Figure 1.3: Different merozoite phases of *Plasmodium* spp. (Tuteja, 2007).

1.3.3 Sporogony (the parasites' sexual multiplication in the mosquito)

In the mosquito's digestive system, the gametocytes undergo gametogenesis to produce micro- and macrogametes. These gametes fuse, undergo fertilisation to form a zygote which transforms into an ookinete, which penetrates the midgut wall of the mosquito and develops into an oocyst. Sporogony within the oocyst produces numerous sporozoites. The oocyst grows, ruptures and releases numerous sporozoites which migrate to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host during a blood meal perpetuates the malaria life cycle. The mosquito thus acts as a vector carrying the disease from one human to another (Ingraham & Ingraham, 2000; CDC, 2006).

All four species of malaria parasites have similar life cycles, except that *P. vivax* and *P. ovale* can remain dormant in the liver as hypnozoites for months or even years, causing relapse malaria long after the original illness seems to be over. *P. falciparum* and *P. malariae* do not cause relapsing malaria, although *P. malariae* produces long-lasting chronic infections and if left untreated, can persist asymptotically in the human host for years (Tuteja, 2007).

1.4 Pathogenesis and clinical features of malaria

1.4.1 Incubation period

Following the infective bite of a female *Anopheles* mosquito, a period of time (the incubation period) goes by before the first symptoms appear, which is specific to the four species of

Plasmodium (Ingraham & Ingraham, 2000). The incubation period is variable, but in most cases it varies from 7 to 30 days. Symptoms generally occur within six weeks after leaving an endemic area in more than 90% of *P. falciparum* infections and within one year in *P. vivax* infections (Pasvol, 2005).

1.4.2 The clinical disease

The typical clinical symptomology and severe disease pathology associated with malaria is caused by the asexual erythrocytic or blood stage parasites, which invade and destroy red blood cells, localise in tissues and organs via cyto-adherence and induce the release of many pro-inflammatory cytokines, particularly tumor necrosis factor α (TNF- α) (Newton & Krishna, 1998). A wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death may result with infection of malaria parasites. The severity of the symptoms depends on several factors, such as the species (type) of infecting parasite and the individual's acquired immunity and genetic background (CDC, 2006). Malaria disease can be categorised as uncomplicated or severe (complicated). *P. falciparum*, unlike the other species, can achieve very high bloodstream parasitemia levels, which result in severe disease (Daily, 2006).

When the parasite develops in the erythrocyte, numerous known and unknown malarial antigens and waste substances such as hemozoin pigment accumulate in the infected red blood cell. Lysis of infected red blood cells releases these substances into the blood stream together with invasive merozoites. The stimulation of macrophages, T-cells and other immunostimulatory cells by hemozoin and other toxic factors such as glucose phosphate isomerase (GPI) to produce cytokines and TNF- α causes fever, rigors and chills and influence other severe pathophysiology associated with malaria (Ingraham & Ingraham, 2000).

1.4.3 Uncomplicated malaria

Fever is the most characteristic symptom of malaria (Pasvol, 2005). In uncomplicated malaria, patients typically experience flu-like symptoms such as fever, chills, sweats, headaches, muscle pains, diarrhea, vomiting and general malaise (Miller *et al.*, 2002). These early signs and symptoms of malaria tend to be non-specific, as these symptoms may also be attributed to influenza, a cold, or other common infections, especially in countries where malaria is not suspected. Conversely, in countries where malaria infection is frequent, residents often recognise the symptoms as malaria and treat themselves without seeking diagnostic confirmation, commonly referred to as "presumptive treatment" (CDC, 2006).

As the disease progresses, some patients may develop the classic malaria intermitted fever paroxysms with bouts of illness alternating with symptom-free periods. The classical paroxysm begins abruptly with an initial cold stage, with dramatic rigors during which the patient shakes visibly. This leads to a hot stage in which the patient has a temperature of more than 40°C, may be restless and excitable and may vomit or convulse. Finally, a sweating stage develops when the fever abates and the patient falls asleep. This paroxysm may last 6 to 10 hours and is followed by a prolonged asymptomatic period (Pasvol, 2005). This periodicity is however rarely seen and is most likely to occur if the infection is left untreated (Ashley *et al.*, 2006).

1.4.4. Severe malaria

Mass destruction of red blood cells has other numerous damaging consequences as seen in severe malaria. Severe malaria occurs when *P. falciparum* infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism (Ingraham & Ingraham, 2000). The two most frequent presentations of severe malaria are severe anemia and cerebral malaria, but respiratory distress is the most dangerous, especially in combination with other syndromes (Schellenberg *et al.*, 1999). Severe malarial anemia also consists of a group of conditions with different causes, namely direct destruction of parasitised red blood cells, indirect destruction of non-parasitised red blood cells by immune mechanisms and bone-marrow suppression, associated with imbalances in cytokine concentrations (Ekvall, 2003). Metabolic derangement, including acidosis, hypoglycemia and sub-clinical convulsions have been observed in many cases of severe malaria (Silamut *et al.*, 1999; Dondorp *et al.*, 2004). In children, increasing evidence has shown that tissue hypo-perfusion (decreased blood circulation) has a central role in disease severity (Maitland & Newton, 2005). All of these manifestations are associated with poor prognosis.

Malaria is especially dangerous to pregnant women and small children and in endemic countries it is an important determinant of prenatal mortality (Van Geertruyden *et al.*, 2004). Parasite sequestration (cyto-adherence) in the placenta is a key feature of infection with *P. falciparum* during pregnancy and is associated with severe adverse outcomes for both mother and baby, such as premature delivery, low birth weight and increased mortality in the newborn (Beeson *et al.*, 2001).

Severe malaria occurs most often in persons who have little or no immunity to the malaria parasite, such as residents of areas with low or no malaria transmission, young children and pregnant women in areas with high transmission, travelers to malaria endemic areas and people with an underlying chronic illness such as acquired immunodeficiency syndrome (AIDS) (CDC,

2006). Severe malaria is a medical emergency and should be treated urgently and aggressively.

1.5 Diagnosis of malaria

Malaria is diagnosed using a combination of clinical observations, case history and laboratory diagnostic tests (Bell *et al.*, 2006).

1.5.1 Clinical diagnosis

People infected with malaria parasites typically experience a combination of the following clinical symptoms: chills, sweats, headaches, nausea and vomiting, body aches and general malaise. Physical findings may include elevated temperature, perspiration, weakness and an enlarged spleen. In *P. falciparum* infection, additional findings may include mild jaundice, enlargement of the liver and increased respiratory rate (Miller *et al.*, 2002). However, these symptoms and physical findings are not specific and are also found in other diseases such as flu and common viral infections (CDC, 2006). Clinical diagnosis of malaria has been repeatedly shown to be unreliable (Chandramohan *et al.*, 2001). Thus, malaria needs to be confirmed by a laboratory test demonstrating the malaria parasites or their components.

1.5.2 Microscopy

The gold standard for malaria diagnosis is simple light microscopy. Examination of Giemsa stained blood smears by a skilled microscopist allows for identification of asexual forms of *Plasmodium* within red blood cells (Ashley *et al.*, 2006). An illustration of such a blood smear is shown in Figure 1.4.

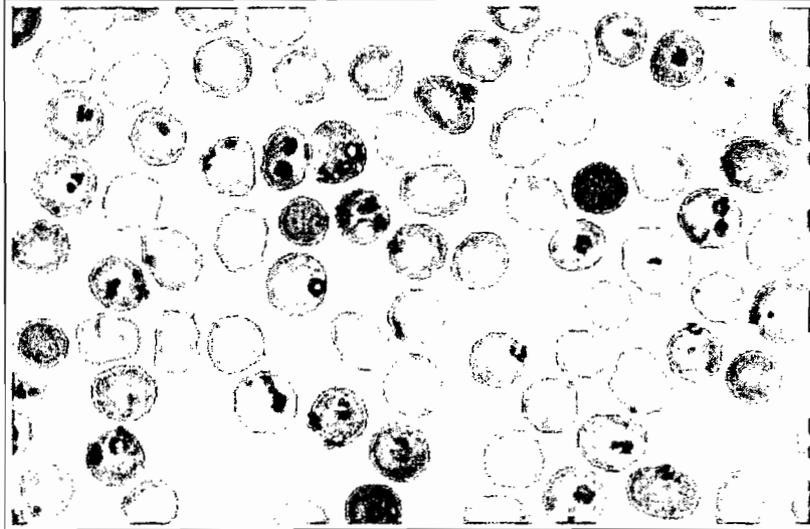


Figure 1.4: Photo of a Giemsa stained blood smear (Gkrania-Klotsas & Lever, 2007).

It is prudent to repeat the smear at least once if the first result is negative, particularly when chemoprophylaxis has been taken and clinical suspicion is high (Pasvol, 2005). The quality of the blood smear is very important for the diagnosis to be made and takes practice to be done well. In general, films taken daily for 3 days are an appropriate screen, though this may have to be prolonged when symptoms persist (Ashley *et al.*, 2006). Unfortunately, this diagnostic method is laborious, very time consuming and requires a relatively large quantity of culture reagents and parasites to conduct an accurate evaluation (Bloland, 2001). The quality of the reagents, microscope and the experience of the laboratory personnel are also factors contributing to the success of the diagnosis made (Bates *et al.*, 2004).

1.5.3 Antigen detection tests

A number of modern malaria diagnostic techniques are also available. Rapid diagnostic immunochromatographic test kits, commonly referred to as Rapid Diagnostic Tests (RDT's) or Malaria Rapid Diagnostic Devices (MRDD's), most often use a dipstick or cassette format and provide results in 2-15 minutes (Bell *et al.*, 2006). These tests detect antigens from malaria parasites in a finger-prick of blood, such as the histidine-rich protein 2 (HRP-2) from *P. falciparum* or the parasite-specific lactate dehydrogenase (LDH) from the parasite glycolytic pathway found in all species (Moody, 2001). Rapid diagnostic tests do not replace microscopy, but offer a useful alternative in situations where reliable microscopic diagnosis is not available (Gkrania-Klotsas & Lever, 2007). They are useful tests requiring minimal expertise, but are relatively expensive, not quantitative and can detect the presence of *P. falciparum* only (Pasvol, 2005).

1.5.4 Molecular tests

Molecular diagnosis detects parasite nucleic acids using a polymerase chain reaction (PCR) technique. This technique is more accurate than microscopy and has the ability to distinguish between the different *Plasmodium* species, identify mixed infections and detect low-level parasitemia. PCR- techniques are expensive, require a specialised laboratory and a great deal of proficiency, making it impractical in most developing countries (Bloland, 2001; Gkrania-Klotsas & Lever, 2007).

1.5.5 Serology

Serology detects antibodies against malaria parasites, using either indirect immunofluorescence assays (IFA) or enzyme-linked immunosorbent assays (ELISA). Serology does not detect current infection but rather measure past experience (Bloland, 2001; CDC, 2007).

Although reliable diagnosis cannot be made on the basis of signs and symptoms alone because of the non-specific nature of clinical malaria, clinical diagnosis of malaria is common in areas where the disease is prevalent. In much of the malaria-endemic areas, resources and trained health personnel are so scarce that presumptive clinical diagnosis is the only realistic option (Bloland, 2001). Three consecutive days of tests that do not indicate the presence of the parasite can rule out malaria (Tuteja, 2007).

The main reason for failure to diagnose malaria in non-endemic countries is failure to consider the disease (Kain *et al.*, 1998). Consideration of the possibility of malaria is an important step in diagnosis, particularly outside endemic areas and a travel history should be a routine part of any clinical consultation in febrile patients. Malaria must be considered in any febrile patient living in, or returning from an endemic country, regardless of whether they have been taking prophylactic antimalarial drugs (Gkrania-Klotsas & Lever, 2007). Malaria must also be considered in patients with fever after blood transfusion, organ transplantation or needle stick injury (Pasvol, 2005). Delay in diagnosis and treatment is a leading cause of death in malaria patients (Kain *et al.*, 1998).

Diagnosis of malaria can be difficult for a number of reasons. Where malaria is no longer endemic, e.g. The United States and countries of Western Europe, health care providers are not familiar with the disease which may delay diagnosis (CDC, 2007). Laboratory staff asked to make the diagnosis may be inexperienced in examining blood smears microscopically (Milne *et al.*, 1994). In highly malaria- endemic areas, many healthy individuals have parasitemia, thus

although a negative test rule out malaria, a positive test does not prove that malaria is the cause of illness (Greenwood *et al.*, 2005). In many malaria endemic countries, lack of resources is a major barrier to reliable and timely diagnosis. Health personnel are under trained, under equipped and underpaid. They often face excessive patient loads and must divide their attention between malaria and other equally severe infectious diseases such as pneumonia, diarrhea, tuberculosis and HIV (CDC, 2007). Antimalarial drugs taken for prophylaxis by travelers can delay the appearance of malaria symptoms by weeks or months, long after the traveler has left the malaria endemic area. This can happen particularly with *P. vivax* and *P. ovale* infections, both of which can produce dormant liver stage parasites, as the liver stages may reactivate and cause disease months after the infective mosquito bite (CDC, 2006). Patterns of drug resistance are changing rapidly and it is difficult for physicians to keep pace (Ashley *et al.*, 2006). The issue of most concern for patients with severe febrile illness is that treatable alternative diagnoses are being missed (Greenwood *et al.*, 2005). In tropical Africa, many patients treated for mild or severe malaria do not actually have the disease (Reyburn *et al.*, 2004), especially in adults diagnosed as having cerebral malaria (Makani *et al.*, 2003). Adults living in areas where HIV prevalence is high, much of the severe illness treated as malaria is probably HIV-related, although this assumption is not yet proven (Peters *et al.*, 2004).

1.6 Current status of malaria control

Prevention is the best way to fight disease, it eliminates the suffering of human illness, whereas treatment only decreases it. Public health programs implement disease prophylactic interventions suggested by epidemiologic studies. There are two principal methods for prophylaxis. One strategy is to limit human exposure to pathogenic microorganisms by decreasing or eliminating the pathogen's reservoir, or by interrupting disease transmission. Immunisation, which stimulates the body's natural immune defenses, offers another potential intervention in the fight against malaria (Ingraham & Ingraham, 2000).

1.6.1 Strategies and targets for malaria control

Beginning with the launch of the Roll Back Malaria (RBM) project in 1998, the United Nations Millennium Declaration in 2000, the Abuja Declaration by African Heads of State in 2000 (part of the African Summit on Roll Back Malaria), the World Health Assembly in 2005 and the RBM global strategic plan 2005–2015, significant contributions to the establishment of goals, indicators and targets for malaria control have been made. The global vision of these interventions is to reduce mortality by 50% by 2010 (WHO, 2008). To accelerate progress in

malaria control, the 2005 World Health Assembly (WHA) set targets of $\geq 80\%$ coverage for four key interventions: insecticide-treated nets (ITN's) for people at risk, appropriate antimalarial drugs for patients with probable or confirmed malaria, indoor residual spraying (IRS) of insecticide for households at risk and intermittent preventive treatment in pregnancy (IPTp) (WHO, 2008). The following interventions, as summarised in Figure 1.5 are often combined and are carried out for malaria control and will be discussed in the following sections.

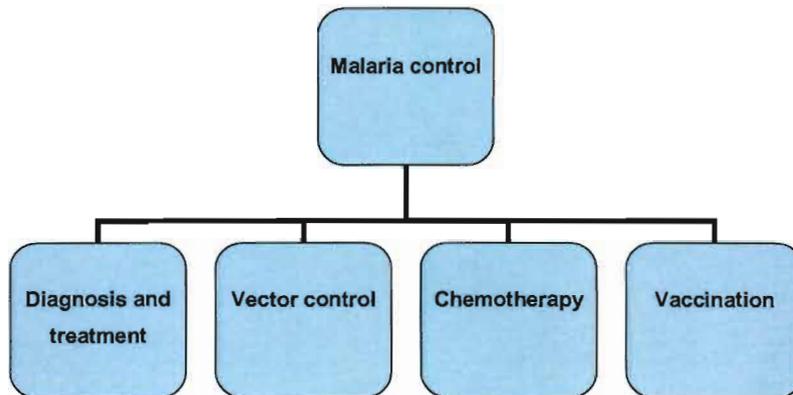


Figure 1.5: Strategies for malaria control (Modified from CDC, 2009).

1.6.1.1 Case management (diagnosis and treatment of patients suffering from malaria)

The objectives of an antimalarial treatment policy are to ensure rapid cure of the infection, reduce morbidity and mortality (including malaria-related anemia), prevent the progression of uncomplicated malaria into severe and potentially fatal disease, reduce the impact of malaria infection on the fetus during pregnancy, reduce the reservoir of infection, prevent the emergence and spread of drug resistance and prevent malaria in travelers (WHO, 2008).

The WHO recommends that anyone suspected of having malaria should receive diagnosis and treatment with an effective drug within 24 hours of the onset of symptoms. By June 2008, all except four countries and territories worldwide had adopted artemisinin combination therapy (ACT) as the first-line treatment for *P. falciparum*. Four ACT's are currently recommended: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine and artesunate-sulfadoxine-pyrimethamine (WHO, 2008).

1.6.1.2 Prevention of infection through vector control

Mosquito control has been at the center of past efforts to eradicate malaria, mainly through the use of the insecticide DDT for indoor residual house-spraying (CDC, 2008). Due to resistance and environmental concerns about residual insecticides, IRS programs were largely disbanded (Chen & Rogan, 2003; CDC, 2008). However, the recent success of IRS in reducing malaria cases in South Africa by more than 80% has revived interest in this malaria prevention tool (Mabaso *et al.*, 2004).

Insecticide-treated bed nets are a form of personal protection that has repeatedly been shown to reduce severe disease and mortality due to malaria in endemic regions. In community-wide trials in several African countries, ITN's have been shown to reduce mortality by about 20% (Lengeler, 2004; CDC, 2008). Regular re-treatment of nets with insecticides has proved difficult to sustain on a large scale, especially if users are required to pay for it (Greenwood *et al.*, 2005). However, several companies have developed long-lasting insecticide-treated nets (LLIN's) in which an insecticide is incorporated into the net fibers that retain lethal concentrations of insecticide for at least 3 years (Greenwood *et al.*, 2005; CDC, 2008). By the end of 2006, nearly all of the 45 countries in the African Region had adopted the policy of providing insecticidal nets free of charge to children and pregnant women (WHO, 2008). Other personal protection measures include the use of window screens, insect repellents and wearing light-colored clothes, long pants and long-sleeved shirts (CDC, 2008).

Other vector control measures include fogging or area spraying which is primarily reserved for emergency situations, halting epidemics or rapidly reducing adult mosquito populations when they have become severe pests. Source reduction or larval control through drainage of breeding sites is the method of choice for mosquito control when the mosquito species targeted are concentrated in a small number of discrete habitats. Sterile male release of *Anopheles* mosquitoes has been successfully applied in several small-scale areas, however, the need for large numbers of mosquitoes for release makes this approach impractical for most areas (CDC, 2008). Another potential option for reducing malaria is the development of genetically modified mosquitoes that are refractory to transmission of the pathogen, but this approach is still several years away from application in field settings (Christophides, 2005).

1.6.1.3 Disease prophylaxis with antimalarial drugs

Administration of antimalarial drugs to vulnerable population groups does not prevent infection, but drugs can prevent disease by eliminating the parasites that are in the blood. Pregnant

women are the vulnerable group most frequently targeted (CDC, 2009). Studies have shown that sulphadoxine-pyrimethamine (SP) given on two or three occasions during pregnancy was more effective at preventing infection of the placenta than chemoprophylaxis with chloroquine (Schultz *et al.*, 1994; Kayentao *et al.*, 2005). This approach, known as intermittent preventive treatment in pregnancy, protects against maternal anemia (Sulman *et al.*, 1999) and low birth weight (Beeson *et al.*, 2001). IPT has also been used to protect children, known as intermittent preventive treatment in infants (IPTi). Studies showed that the use of SP or amodiaquine at specific times during the first year of life lowered the incidence of malaria and severe anemia without any rebound in clinical malaria the following year (Shellenberg *et al.*, 2005). The systematic use of intermittent preventive treatment in pregnancy is restricted to the African Region, 33 of the 45 African countries had adopted IPT as a national policy by the end of 2006 (WHO, 2008).

1.6.1.4 Vaccination

Malaria vaccine research has progressed rapidly over the past few years. Vaccine development against *P. falciparum* and *P. vivax* is ongoing (Moorthy *et al.*, 2004). Most efforts so far have been directed at the development of pre-erythrocytic stage vaccines, designed to prevent invasion of hepatocytes by sporozoites or to destroy infected hepatocytes. The most current advanced pre-erythrocytic vaccine is RTS,S/AS02A, which is a hybrid molecule in which the circumsporozoite protein of *P. falciparum* is expressed with hepatitis B surface antigen (HBsAg) in yeast (Stoute *et al.*, 1997). However, it is likely to be at least a decade before an efficacious vaccine is available for widespread use in malaria-endemic countries.

Malaria control is however made difficult for a number of reasons. Drug resistant malaria parasites hinder case management by decreasing the efficacy of antimalarial drugs and by requiring the use of alternative drugs that are more costly, less safe and difficult to administer. Insecticide resistance decreases the efficacy of interventions that rely on insecticides such as insecticide treated bed nets and insecticide spraying. Due to inadequate health infrastructures in poor countries, the recommended interventions are not implemented. The people most exposed to malaria are often poor and lack education. They often do not know how to prevent or treat malaria. Even when they do know, they often do not have the financial means to purchase the necessary products, such as drugs or bed nets (CDC, 2009).

1.7 General principles regarding the treatment of malaria

In general, malaria is a curable disease if diagnosed and treated adequately and promptly. Once a definite diagnosis of malaria has been confirmed, treatment with specific antimalarial drugs and supportive measures must be initiated immediately. If the diagnosis of malaria is suspected but cannot be confirmed, or if the diagnosis of malaria is confirmed, but species identification is not possible, antimalarial treatment effective against *P. falciparum* must be initiated immediately (WHO, 2008). Treatment for malaria should not be initiated until the diagnosis has been confirmed by laboratory investigations (CDC, 2007). In endemic areas, the WHO recommends that treatment be started within 24 hours after the first symptoms appear. Treatment of patients with uncomplicated malaria can be conducted on an ambulatory basis with oral antimalarial drugs, but patients with severe malaria should be hospitalised if possible and treated aggressively with parenteral antimalarial therapy (WHO, 2006; CDC, 2007).

Treatment should be guided by the following factors (CDC, 2007):

- the infecting *Plasmodium* species;
- the area where the infection was acquired and its drug-resistance status;
- the clinical status of the patient;
- any underlying illness or condition of the patient;
- pregnancy;
- drug allergies or other medications taken by the patient.

There are only a limited number of drugs which can be used to treat or prevent malaria. Antimalarial drugs in common use can be divided into five major classes of compounds, namely quinine and related compounds, antifolate combination drugs, antibiotics, hydroxynapthaquinones and the artemisinin derivatives (Winstanley, 2000; Ashley *et al.*, 2006). These drugs will be discussed in detail in Chapter 2.

1.7.1 Antimalarial drug resistance and combination therapy

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today (Bloland, 2001). The high prevalence of infection, constant drug pressure, sexual reproduction and large parasite biomass contribute to the development of a resistant parasite (Daily, 2006). Resistance to antimalarial drugs has been documented for *P. falciparum* and *P. vivax* (White, 1999; WHO, 2006). *P. falciparum* has developed clinically significant resistance to nearly all antimalarial drugs in current use, with the possible exception of artemisinin and its

derivates (WHO, 2006). However, the geographical distribution of resistance to any single antimalarial drug varies greatly (Bloland, 2001). Resistance to several antimalarial drugs in selected areas is discussed in further detail in Chapter 2. The affordable and widely available antimalarial drug chloroquine that was the mainstay of malaria control is now ineffective in most *falciparum* malaria endemic areas and resistance to SP is increasing rapidly. The discovery and development of the artemisinin derivatives in China and their evaluation in South-East Asia and other regions have provided a new class of highly effective antimalarials. Artemisinin-based combination therapies (ACT's) are now generally considered as the best current treatment for uncomplicated *falciparum* malaria (WHO, 2006; WHO, 2008).

To counter the threat of resistance of *P. falciparum* to monotherapies and to improve treatment outcomes, combination therapy of antimalarials is now recommended by the WHO for the treatment of *falciparum* malaria. Antimalarial combination therapy is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and thus unrelated biochemical targets in the parasite (Barnes & White, 2005). By June 2008, all except four countries and territories worldwide had adopted ACT as the first-line treatment for *P. falciparum*. Four ACT's are currently recommended for use: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine and artesunate-sulfadoxine-pyrimethamine (WHO, 2008).

1.8 Current status of malaria in South Africa

Africa presents with the world's highest malaria incidence (Breman *et al.*, 2001). South Africa is at the southern extreme of malaria distribution in Africa. The population at risk for contracting malaria in South Africa is approximately 4.3 million. Malaria is endemic in the low-altitude areas of the northern and eastern parts of South Africa, along the border with Mozambique and Zimbabwe with transmission taking place mainly in the Limpopo, Mpumalanga and KwaZulu Natal provinces (Blumberg & Frean, 2007). Figure 1.6 is a map of South Africa indicating the malaria risk areas. Malaria transmission is distinctly seasonal, with transmission limited to the warm and rainy summer months of September to May, hence, malaria is unstable and epidemic-prone (Craig *et al.*, 1999).

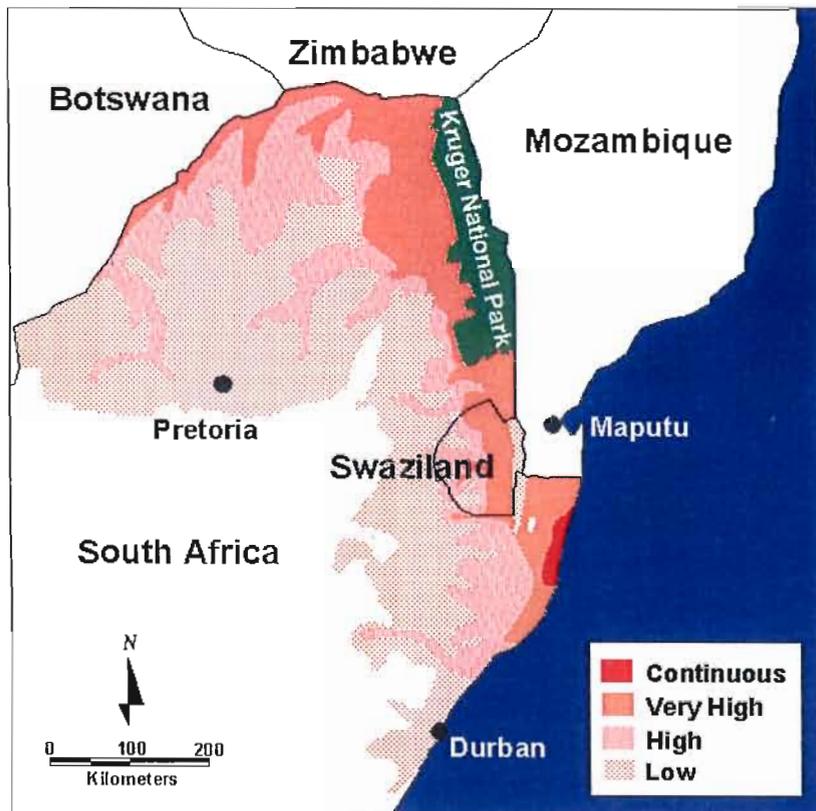


Figure 1.6: Malaria risk map of South Africa (MRP: available at www.malaria.org.za).

P. falciparum accounts for the majority of malaria cases in southern Africa and is the predominant species associated with severe and fatal disease. Almost all South Africans lack acquired immunity, including residents of seasonal malaria transmission areas and are therefore at risk for developing severe malaria (Blumberg & Frean, 2007). Malaria is a notifiable disease in South Africa (Gerritsen *et al.*, 2008).

The South African Malaria Control Programme has been active since 1945 in all three provinces considered as malaria risk areas as mentioned above and includes the following interventions (Blumberg & Frean, 2007, Gerritsen *et al.*, 2008):

- vector control through intensive IRS with DDT;
- case management through diagnosis with *P. falciparum*- specific HRP-2 rapid antigen detection tests;
- treatment of uncomplicated malaria with ACT, specifically artemether-lumefantrine;
- disease surveillance;
- epidemic preparedness and response;
- health promotion and education.

As a result of the above mentioned malaria control initiatives, as well as the Lebombo Spatial Initiative (a cross-border collaboration targeting malaria in eastern Swaziland, southern Mozambique and northern KwaZulu Natal), there has been a significant and sustained decrease in malaria case notifications, from 64 622 cases in the year 2000 to approximately 8000 in 2006 (DOH, 2003; Blumberg & Frean, 2007; Sharp *et al.*, 2007).

Despite successes in malaria control in South Africa, many challenges remain. These include importation of malaria cases, the potential for ongoing antimalarial drug resistance, vector insecticide resistance, a large HIV pandemic and a struggling health service (Blumberg & Frean, 2007).

1.9 Conclusion

It is clear that worldwide malaria cannot be controlled by the use of insecticides alone as it has become more expensive, less effective and harmful to the environment. Moreover, drug therapy today is significantly less effective than it was 30 years ago. Chloroquine, which replaced the centuries-old quinine cure, was extremely successful and inexpensive. It was effective both for treatment and prophylaxis until resistant strains of *P. falciparum* began to emerge. Drug resistance is a constantly evolving problem and so there is an ongoing search for new antimalarial drugs. Research is hampered by the fact that the market for these drugs is mainly in developing countries that cannot afford to support expensive research or buy expensive drugs. A vaccine could be the turning point in the global effort to control malaria, but vaccines are most effective against diseases for which natural immunity is strong and long-lasting. Unfortunately, natural immunity to malaria is weak, slow to develop and temporary. Still, active research is underway to develop innovative new vaccines (Ingraham & Ingraham, 2000). The three combined strategies of vector control, drug treatment and vaccination will ultimately be required to significantly reduce malaria transmission.

CHAPTER 2

CLASSIFICATION OF ANTIMALARIAL DRUGS

2.1 Introduction

The current approaches to curtail malaria include vector control, vaccination, immunotherapy and chemotherapy. Despite major efforts at vaccine development, chemotherapy remains the single most effective and efficient means to prevent and treat malaria (Tripathi *et al.*, 2005). However, the ability to treat and control *P. falciparum* infection through chemotherapy has been hampered by the advent and spread of resistance to antimalarial drugs (Valderramos & Fidock, 2006).

P. falciparum has developed clinically significant resistance to nearly all classes of antimalarial drugs, with the possible exception of the artemisinin derivatives (Barnes & White, 2005). However, the geographical distribution of resistance to any single antimalarial drug varies greatly (Bloland, 2001). This phenomenon has significantly increased the cost and complexity of treatment options for malaria (Wongsrichanalai *et al.*, 2002). Interventions aimed at preventing drug resistance generally focus on reducing overall drug pressure through more selective use of drugs, or using drug combinations which are inherently less likely to foster resistance or have properties that do not facilitate development or spread of resistant parasites (Bloland, 2001). The most currently recommended drug combinations for *falciparum* malaria are variants of artemisinin combination therapy where a rapidly acting artemisinin compound is combined with a longer half life drug of a different class, such as artesunate and mefloquine (Shanks, 2006). The rationale for most of the combinations in current use is to exploit synergy (White, 1999).

This chapter aims to emphasise the impact of antimalarial drug resistance, antimalarial combination therapy as a possible intervention against drug resistance and to describe the basic classification of the currently available antimalarial drugs.

2.2 Malaria and drug resistance

Antimalarial drug resistance is the ability of the malaria parasite to withstand attack by antimalarial compounds (WHO, 2006). The mechanism of drug resistance at the molecular level is attributed to spontaneously arising single or multiple point mutations in the parasite's genome that are drug insensitive (Peters, 1990; Hayton & Su, 2004). Multidrug resistance is defined as resistance to more than two currently used antimalarial compounds of different chemical classes (Wernsdorfer, 2004). Antimalarial drugs don't cause these genetic changes, but they favor or select the resistant strains once the genetic change has occurred (Ingraham & Ingraham, 2000). Reasons for the development and spread of drug resistance involve the interaction of drug-use patterns, characteristics of the drug itself, human host factors, parasite characteristics and vector and environmental factors (Bloland, 2001; Wongsrichanalai *et al.*, 2002).

For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required (Bloland, 2001). Antimalarial drug resistance is usually a result either of changes in drug accumulation or efflux, as seen in chloroquine, amodiaquine, quinine and mefloquine resistance, or reduced affinity of the drug target resulting from point mutations in the respective genes encoding the target, as in the case of pyrimethamine, proguanil, sulfonamide and atovaquone resistance (Ward *et al.*, 1995). Other mechanisms of drug resistance found in bacteria such as transferable resistance genes, the production of drug destroying enzymes, or the activation of accessory metabolic pathways do not appear to be involved in antimalarial resistance (White, 1999). Areas with reduced susceptibility of *P. falciparum* to some antimalarial drugs are shown in Figure 2.1:

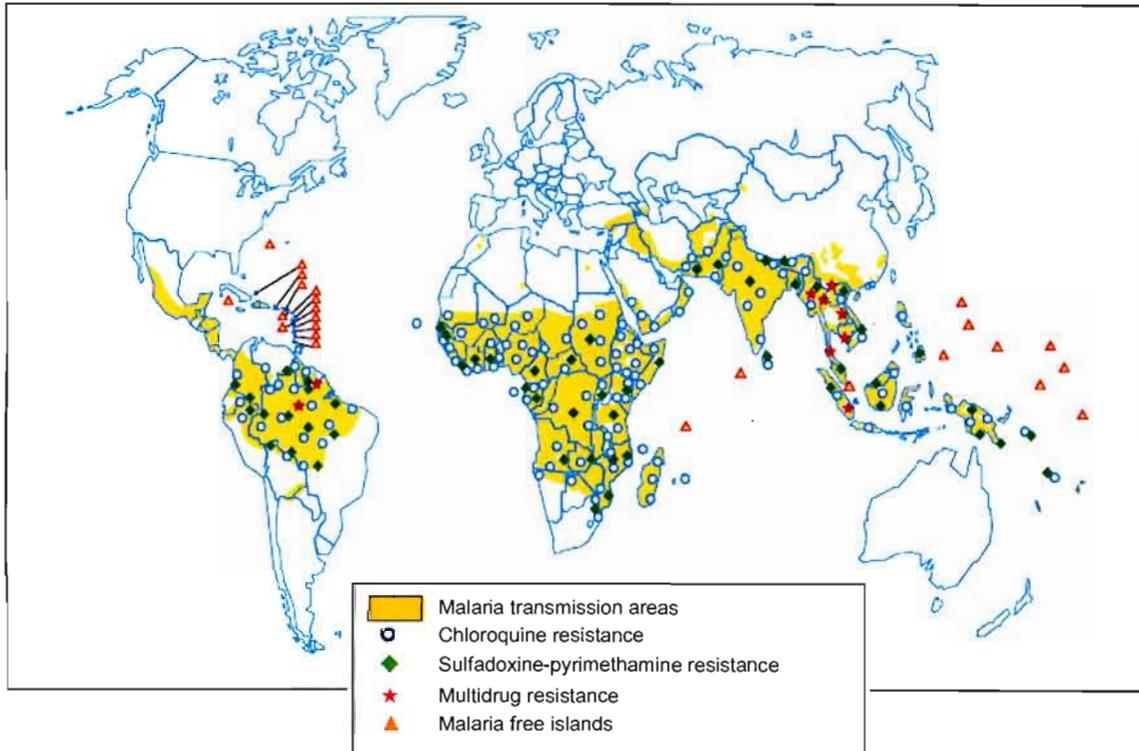


Figure 2.1: Areas with reduced susceptibility of *P. falciparum* (MAP: available at www.map.ox.ac.uk).

Drug resistance can cause treatment failure, however, not all treatment failure is due to drug resistance. Factors that can contribute to treatment failure include incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor or erratic absorption and misdiagnosis. In addition to causing treatment failure or apparent treatment failure in an individual, these factors may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to suboptimal drug levels (Bloland, 2001, Shanks, 2006).

2.3 The rationale of combination therapy

There is a growing consensus that drug resistance could be delayed or prevented by combining drugs with different biological targets in the parasite (White, 1999; Guerin *et al.*, 2002). Figure 2.2 shows the targets of the major classes of antimalarial drugs in the parasite.

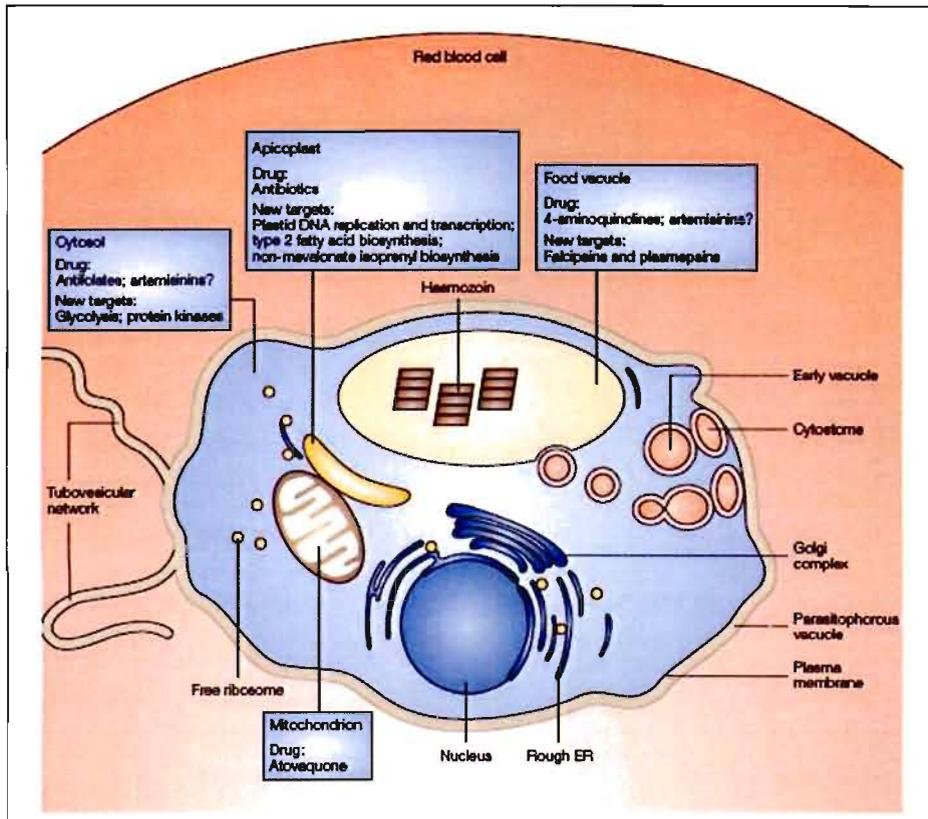


Figure 2.2: A schematic representation of an intra-erythrocytic *P. falciparum* trophozoite, illustrating the targets of some of the major classes of antimalarial drugs (Fidock *et al.*, 2004).

Combination therapy potentially offers a number of important advantages over monotherapies. First, they should provide improved efficacy. Appropriately chosen combination drugs must at least be additive in potency and provide synergistic activity. Second, drug combinations increase the likelihood that, in the setting of drug resistance, at least one agent will be clinically active. Third and probably the most important property is that drug combinations should reduce the selection of antimalarial drug resistance (Fidock *et al.*, 2004).

The class of drugs best suited to provide one of the partners in a combination regime is the artemisinin derivatives. They are highly potent and capable of reducing the parasite biomass by a function of 10^4 per asexual life cycle. This will leave a much smaller number of parasites for the partner drugs to kill, while its concentration in plasma remains high. Another advantage of artemisinin derivatives is their ability to kill gametocytes, thus interrupting malaria transmission. As a result, they are the drugs of choice during epidemics (Ashley *et al.*, 2006). However, artemisinins are natural products that are difficult to synthesise and cannot be sold at a cost of

less than US \$ 1-2 in curative combination regimes, a prohibitive price in most malaria-endemic regions (Fidock *et al.*, 2004). Current antimalarial combination therapy recommended by the WHO includes artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine and artesunate-sulfadoxine-pyrimethamine (WHO, 2008).

2.4 Classification of antimalarial drugs

The chemotherapy of malaria basically involves killing of the asexual parasites in the erythrocytes and providing supportive therapy to the host to boost its immune system (Winstanley, 2000; WHO, 2006). The sexual forms in the blood circulation are non-pathogenic, but are important in drug resistance development. The efficacy and specificity of anti-infective drugs depend on their ability to interfere with parasite metabolism that differs significantly from the human host (Tripathi *et al.*, 2005). During its life cycle in human erythrocytes, the *Plasmodium* parasite requires several metabolic adaptations and innovations which render it susceptible to chemotherapeutic attack (Ridley, 2002). The antimalarial drugs in common use can be classified as follows (Ashley *et al.*, 2006):

1. Quinolines and aryl-amino alcohols: quinine, quinidine, chloroquine, amodiaquine, mefloquine, primaquine, tafenoquine, halofantrine and lumefantrine.
2. Inhibitors of folate synthesis: pyrimethamine, proguanil, sulfadoxine and dapsone.
3. Antibiotics: tetracycline, doxycycline, clindamycin and azithromycin.
4. Hydroxynaphtaquinones: atovaquone
5. Artemisinin and derivatives: artemisinin, dihydroartemisinin, artemether and artesunate.

Antimalarial compounds should be evaluated in the context of life cycle stage effect, molecular target and half-life. Drug effect on each life cycle stage varies because of stage-specific biology and most antimalarials have no effect on the liver stage. However, as this stage is required to establish infection and initially generates a low burden of parasites, it may serve as an excellent potential target (Daily, 2006).

Primaquine, a tissue schizonticide, is the only agent available for elimination of hypnozoites (Rosenthal, 2004). Blood schizonticides eliminate the asexual stage erythrocytic forms and target only a small number of parasite biological functions, as shown in Figure 2.2 (Ingraham & Ingraham, 2000). Potential biological targets include the parasite's metabolism and detoxification of hemoglobin, where chloroquine, amodiaquine, quinine, mefloquine, halofantrine

and lumefantrine exert their possible effect (Daily, 2006). Inhibition of folate synthesis is mediated through sulfadoxine-pyrimethamine and dapsone (Tracy & Webster, 2001). The mitochondrion in the parasite's cytosol seems to be the target of atovaquone (Daily, 2006) and the sarco-endoplasmic reticulate Ca^{2+} -ATPase (SERCA) is thought to be the target of the artemisinin compounds (Eckstein-Ludwig *et al.*, 2003). Gametocytes are often resistant to standard antimalarials used to clear the asexual-stage parasites. This may be due to the unique biology of gametocytes, which shuts down a subset of metabolic pathways, rendering the antimalarials ineffective (Young *et al.*, 2005). The artemisinin compounds however are a notable exception and can clear gametocyte stages (Ashley *et al.*, 2006). Drugs that kill the sexual stages and thus prevent transmission to mosquitoes are called gametocytocidal (Rosenthal, 2004). Antimalarial compounds that eliminate the hepatic, asexual and gametocyte stages would thus be ideal (Daily, 2006).

Antimalarial drugs also vary by half-life. The benefit of a long half-life is decreased dosing and prolonged protection against new infections. The limitation of this strategy however is the presence of a long sub-therapeutic tail of the drug which will select drug resistant parasites (Watkins & Mosobo, 1993). Another consideration in the evaluation of antimalarial drugs is their cost. Aside from chloroquine and sulfadoxine-pyrimethamine, the newer antimalarials are expensive, which may affect their use in endemic areas where the local population often bears the cost of treatment (Wiseman *et al.*, 2005).

2.4.1 Quinolines and aryl-amino alcohols

Quinine is the only antimalarial drug, which over a period of time, has remained largely effective in treating malaria. A number of its derivatives are known to be good antimalarials (Skinner-Adams & Davis, 1999) and are discussed in detail below.

2.4.1.1 Quinine and quinidine

Quinine is an aryl-amino alcohol, an ancient drug derived from the bark of the South American *Cinchona* tree. The alkaloid was traditionally used as a remedy for intermitted fevers and was the first widely used antimalarial drug long before the causes of malaria were known (Tracy & Webster, 2001; Rosenthal, 2004). The chemical structure of quinine is shown in Figure 2.3:

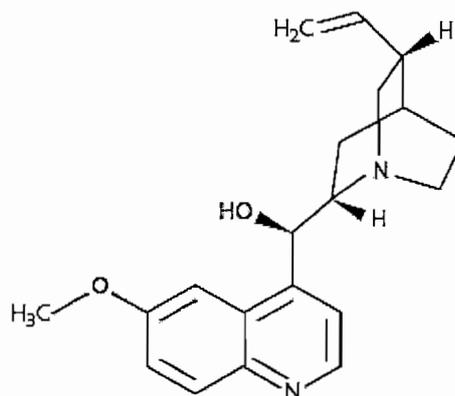


Figure 2.3: The chemical structure of quinine.

Quinidine, the stereoisomer of quinine, is more potent as an antimalarial, but more toxic (Krishna & White, 1996). Quinine is a rapidly acting, highly effective blood schizonticide against the four species of human malaria parasites (Rosenthal, 2004).

The mechanism of action of quinine is thought to involve inhibition of parasite heme-detoxification in the food vacuole, but is not well understood (Sullivan *et al.*, 1998). Resistance to quinine has been very slow to develop (Daily, 2006). However, quinine resistance is already common in Southeast Asia, especially border areas of Thailand and is also reported in both South America and Africa, where the drug may fail if used alone to treat *falciparum* malaria (Adagu *et al.*, 1995; Zalis *et al.*, 1998; Rosenthal, 2004).

Quinine hydrochloride or quinidine gluconate is the treatment of choice for severe *falciparum* malaria. Quinine sulphate is appropriate first-line therapy for uncomplicated *falciparum* malaria, except when the infection was transmitted in a chloroquine-sensitive area (Rosenthal, 2004). Quinine is commonly given concurrently with slower acting blood schizonticides such as a sulfonamide, or most often doxycycline to enhance the action of quinine, limit toxicity, as well as for the treatment of *falciparum* infections contracted in regions where sensitivity to quinine is reduced (Kremsner *et al.*, 1994). Quinine is less effective and more toxic than chloroquine against other human malaria and it is therefore not used to treat infections with these parasites (Tracy & Webster, 2001).

2.4.1.2 Chloroquine

Chloroquine is a synthetic 4-aminoquinoline derivative of quinine, formulated as the phosphate salt for oral use (Foley & Tilley, 1998). The chemical structure of chloroquine is shown in Figure 2.4:

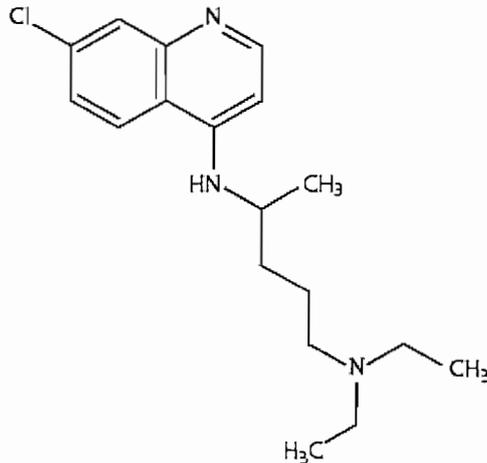


Figure 2.4: The chemical structure of chloroquine.

Chloroquine was synthesised in 1934 and has been the drug of choice for both treatment and chemoprophylaxis of malaria since the 1940's (Tracy & Webster, 2001). However, widespread resistance patterns have now rendered chloroquine virtually ineffective against *P. falciparum* infections in most parts of the world (Figure 2.1) (WHO, 2006). When not limited by resistance, chloroquine is a highly effective blood schizonticide against sensitive *P. falciparum* and other species of human malaria parasites (Rosenthal, 2004).

The mechanism of action of chloroquine remains controversial. Chloroquine probably interferes with parasite heme-detoxification by concentrating in parasite food vacuoles, preventing the polymerization of the hemoglobin breakdown product heme into hemozoin and thus eliciting parasite toxicity due to the buildup of free heme (Foley & Tilley, 1998).

Chloroquine is the preferred chemoprophylactic agent in regions with sensitive *falciparum* malaria. The compound is superior to quinine in that it is more potent and less toxic and it needs to be given only once weekly as a suppressive agent (Tracy & Webster, 2001). Chloroquine is the drug of choice for the treatment of non-*falciparum* and sensitive *falciparum* malaria (Tracy & Webster, 2001; Rosenthal, 2004). Chloroquine is still used to treat *falciparum* malaria in many areas with widespread resistance, in particular most of Africa owing to its safety

and low cost and the fact that many partially immune individuals respond to treatment even when infecting parasites are partially resistant to chloroquine. However, other agents are preferred to treat potentially resistant *falciparum* malaria, especially in non-immune individuals (Rosenthal, 2004).

2.4.1.3 Amodiaquine

Amodiaquine is a Mannich base 4-aminoquinoline closely related to chloroquine and it probably shares mechanisms of action and resistance patterns with chloroquine (Daily, 2006). The chemical structure of amodiaquine is shown in Figure 2.5:

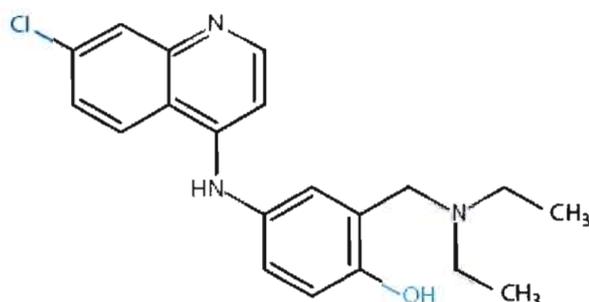


Figure 2.5: The chemical structure of amodiaquine.

Amodiaquine is rapidly converted in the liver to the active metabolite desethylamodiaquine, which contributes to nearly all of the antimalarial effect (Winstanley *et al.*, 1990). Prophylactic use of amodiaquine has been associated with an unacceptably high incidence of serious toxicity with developing agranulocytosis (Hatton *et al.*, 1986) and reports of significant hepatotoxicity (Sturchler *et al.*, 1987), limiting the use of the drug in recent years (Daily 2006). However, some authorities now advocate its use as a replacement for chloroquine, especially in combination regimes in areas with high rates of resistance (Olliaro *et al.*, 1996; Rosenthal, 2004). Amodiaquine in combination with sulfadoxine-pyrimethamine has been shown to improve treatment outcomes in highly drug-resistant areas (Sendagire *et al.*, 2005).

2.4.1.4 Mefloquine

Mefloquine is a synthetic 4-quinoline-methanol that is chemically related to quinine. The drug can only be given orally as the hydrochloride salt because severe local irritation occurs with parenteral use (Tracy & Webster, 2001). The chemical structure of mefloquine is shown in Figure 2.6:

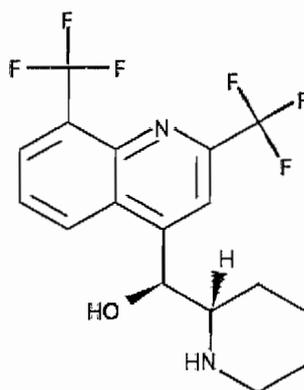


Figure 2.6: The chemical structure of mefloquine.

Mefloquine has strong blood schizontocidal activity against *P. falciparum* and *P. vivax* (Rosenthal, 2004). The exact mechanism of action of mefloquine is unknown. As a blood schizonticide, mefloquine behaves like quinine in many respects, but does not intercalate with DNA. Mefloquine may act by both inhibiting heme polymerisation and forming toxic complexes with free heme that damage membranes and interact with other plasmodial components (Sullivan *et al.*, 1998). Sporadic resistance to mefloquine has been reported from many areas. Regions of Southeast Asia, especially border areas of Thailand have high rates of multidrug resistance (Rojanawatsirivet *et al.*, 2004).

Mefloquine is recommended for oral use exclusively for the prophylaxis and chemotherapy of chloroquine-resistant or multidrug-resistant *falciparum* malaria. The drug is especially useful as a prophylactic agent for non-immune travelers who stay for only brief periods in areas where these infections are endemic (Schlagenhauf, 1999). Mefloquine's long half-life allows weekly dosing for prophylaxis, but unfortunately the occurrence of neuropsychiatric symptoms has reduced its acceptability by travelers (Schlagenhauf *et al.*, 2003). Mefloquine can be used for disease treatment, although the full treatment dose can result in gastrointestinal upset. Splitting the treatment dose may lessen side effects (ter Kuile *et al.*, 1995). This quinoline is most effective for treating uncomplicated drug resistant *falciparum* malaria when given 48 hours after the parasite burden has been substantially reduced by prior administration of an artemisinin antimalarial like artesunate (White, 1999). The drug is not appropriate for treating individuals with severe or complicated malaria, since quinine and quinidine are more rapidly active and drug resistance is less likely with these agents (Rosenthal, 2004).

2.4.1.5 Primaquine

Primaquine is a synthetic 8-aminoquinoline compound (Tracy & Webster, 2001). The chemical structure of primaquine is shown in Figure 2.7:

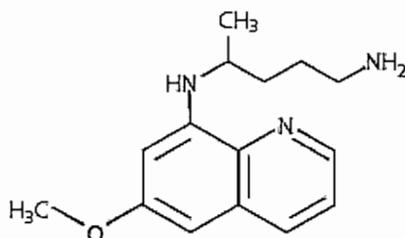


Figure 2.7: The chemical structure of primaquine.

This compound, in contrast with the other antimalarial drugs, is the only available agent which eradicates the dormant hypnozoite stages of *P. vivax* and *P. ovale* in the liver (Bunnag *et al.*, 1980; Tracy & Webster, 2001) and is thus reserved primarily for the terminal prophylaxis and radical cure of *vivax* and *ovale* relapsing malaria. Standard therapy for these infections includes chloroquine to eradicate erythrocytic forms (Rosenthal, 2004).

There are as yet no methods for cultivating *P. vivax in vitro*, so little is known about the antimalarial action of the 8-aminoquinolines, especially why they are far more active against tissue forms and gametes than asexual blood forms of plasmodia (Tracy & Webster, 2001). Primaquine may be converted to electrophiles that act as oxidation-reduction mediators (Bates *et al.*, 1990). Some strains of *P. vivax* in New Guinea, Southeast Asia and perhaps Central and South America are relatively resistant to primaquine, which makes it imperative that the drug not be misused (Smoak *et al.*, 1997; Rosenthal, 2004).

2.4.1.6 Tafenoquine

Tafenoquine is a promising newer 8-aminoquinoline which has a much longer half-life than primaquine, meaning shorter courses are effective to eradicate hypnozoites and prevent relapses (Walsh *et al.*, 2004; Ashley *et al.*, 2006).

2.4.1.7 Halofantrine

Halofantrine is a phenanthrene-methanol related to quinine, with blood schizontocidal properties similar to those of the quinoline antimalarials (Tracy & Webster, 2001; Rosenthal,

2004). This compound was originally developed and has been used as an alternative to quinine and mefloquine to treat acute malarial attacks caused by chloroquine- and multidrug resistant strains of *P. falciparum* (Tracy & Webster, 2001; Daily, 2006). The use of halofantrine with mefloquine is not recommended, because it may lead to erratic bioavailability, potentially lethal cardiotoxicity and extensive cross-resistance (Matson *et al.*, 1996; Tracy & Webster, 2001).

2.4.1.8. Lumefantrine

Lumefantrine is a racemic fluorine derivative related to halofantrine that belongs to the aryl-amino alcohol group of antimalarials (Daily, 2006). The chemical structure of lumefantrine is shown in Figure 2.8:

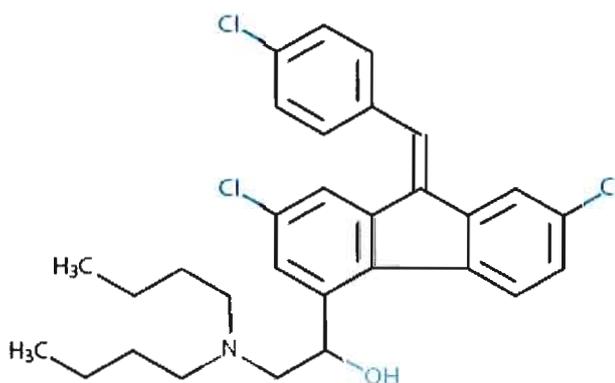


Figure 2.8: The chemical structure of lumefantrine.

The drug is only available in an oral preparation co-formulated with artemether, one of the artemisinin derivatives, as Coartem[®] (Olliaro, 1995; Ashley *et al.*, 2006). This ACT is highly effective against multidrug-resistant *P. falciparum* (WHO, 2006). Oral bioavailability of lumefantrine is variable and is highly dependant on administration with fatty foods (Ezzet *et al.*, 2000; WHO, 2006). Coartem[®] is highly effective for the treatment of *falciparum* malaria, but it is expensive and requires twice-daily dosing (Rosenthal, 2004). Coartem[®] does not appear to cause the cardiac toxicity seen with halofantrine (van Vugt *et al.*, 1999).

2.4.2 Inhibitors of folate synthesis

Inhibitors of enzymes involved in folate metabolism are generally used in combination regimes for the treatment and prevention of malaria (Ashley *et al.*, 2006, WHO, 2006). Commonly used drugs in this category include pyrimethamine, proguanil and the sulfonamide antibiotics.

Although these drugs have antimalarial activity when used alone, parasitological resistance can develop rapidly. When used in combination, they produce a synergistic effect in the parasite and can be effective even in the presence of resistance to the individual components (Bloland, 2001).

Pyrimethamine and proguanil act slowly against erythrocytic forms of susceptible strains of all four human malaria species. Sulfonamides and sulfones are weakly active against erythrocytic schizonts (Tracy & Webster, 2001, Rosenthal, 2004).

Pyrimethamine and proguanil selectively inhibit plasmodial dihydrofolate reductase, a key enzyme in the pathway for synthesis of folate, causing inhibition of DNA synthesis and depletion of folate co-factors (Rosenthal, 2004; Daily, 2006). Sulfonamides and sulfones are structural analogs of *para*-aminobenzoate that competitively inhibit another enzyme in the folate pathway, such as dihydropteroate synthase (Tracy & Webster, 2001). In many areas, resistance to folate antagonists and sulfonamides is common for *P. falciparum* and less common for *P. vivax* (Rosenthal, 2004).

Chemoprophylaxis with single folate antagonists is no longer recommended because of frequent resistance, but a number of agents are used in combination regimes (WHO, 2006). The combination of chloroquine (500mg weekly) and proguanil (200mg daily) is widely used as an alternative to mefloquine. This combination has lower efficacy, but is probably less toxic than mefloquine (Tracy & Webster, 2001; Rosenthal, 2004). The synergistic antifolate combination Fansidar[®], which consists of sulfadoxine (500mg per tablet) and pyrimethamine (25mg per tablet), has been used extensively for prophylaxis and suppression of human malaria, especially those caused by chloroquine-resistant strains of *P. falciparum*. Resistance to this formulation rapidly developed in Indonesia and is now widespread except in parts of Africa, where the drug combination is used primarily by indigenous populations to suppress attacks of chloroquine-resistant *falciparum* malaria (Tracy & Webster, 2001). Fansidar[®] is also used as presumptive therapy for travelers who develop fever while traveling in malaria-endemic regions and who are unable to obtain medical evaluation (Tracy & Webster, 2001; Rosenthal, 2004). This regimen should not be used for severe malaria as it is slower-acting than other better available agents. However, Fansidar[®] can also be used as an adjunct to quinine therapy to shorten the course of quinine and limit toxicity (Rosenthal, 2004).

The sulfone dapsone given with the biguanide chlorproguanil has also been effective for therapy of chloroquine-resistant *falciparum* malaria in Africa. Chlorproguanil-dapsone (LapDap[®]) is

highly effective in regions with fairly high levels of resistance to Fansidar® and its shorter half-life may prevent the selection of resistant parasites (Winstanley *et al.*, 1997).

2.4.3 Antibiotics

A number of antibiotics in addition to the folate antagonists and sulfonamides are modestly active antimalarials. Commonly used drugs in this class include the tetracyclines (doxycycline), clindamycin and azithromycin. The mechanisms of action of these drugs are unclear. They may inhibit protein synthesis or other functions in the mitochondrion and the apicoplast (Rosenthal, 2004). None of the antibiotics should be used as single agents for the treatment of malaria because their action is much slower than those of standard antimalarials (Ashley *et al.*, 2006).

Tetracycline and doxycycline are active against erythrocytic schizonts of all human malaria parasites (Tracy & Webster, 2001). Doxycycline is a synthetic tetracycline with a longer half-life, which makes dosing schedules shorter. Doxycycline may be preferred to tetracycline because of its longer half-life, more reliable absorption and better safety profile in patients with renal insufficiency where it may be used with caution (WHO, 2006). Doxycycline is commonly used in the treatment of *falciparum* malaria in conjunction with quinine or quinidine, allowing a better tolerated course of quinine (Rosenthal, 2004). The relative slowness of action of the tetracyclines makes concurrent treatment with quinine mandatory for rapid control of parasitemia (Tracy & Webster, 2001; Ashley *et al.*, 2006). Doxycycline has also become a standard chemoprophylactic drug for use in areas of Southeast Asia with high rates of resistance to other antimalarials, including mefloquine (Rosenthal, 2004).

Clindamycin is a lincosamide antibiotic which is slowly active against erythrocytic schizonts and can be used in conjunction with quinine or quinidine in persons for whom doxycycline is not recommended, such as children and pregnant women (Rosenthal, 2004; Ashley *et al.*, 2006). Clindamycin offers only limited advantages when compared to other available antimalarial drugs. Parasitological response to clindamycin is slow and recrudescence rates are high (Kremsner *et al.*, 1994). Azithromycin also has antimalarial activity and is now under investigation as an alternative chemoprophylactic drug (Puri & Singh, 2000).

2.4.4 Atovaquone

Based on the antiprotozoan activity of certain hydroxynaphthoquinones, atovaquone was developed as a promising synthetic derivative with potent activity against *Plasmodium* species.

Atovaquone is a highly lipophilic analog of ubiquinone and has potent activity against blood stages of all *Plasmodium* species (Hudson *et al.*, 1991). The use of atovaquone alone was found to result in a high prevalence of failure due to rapid development of resistance (Looareesuwan *et al.*, 1996). In contrast, excellent efficacy is obtained when atovaquone is used in combination with proguanil (Looareesuwan *et al.*, 1999). Malarone[®] is a fixed combination of atovaquone (250mg) and proguanil (100mg) and has been shown to be highly effective for both the treatment and chemoprophylaxis of *falciparum* malaria. This combination has an advantage over mefloquine and doxycycline in requiring shorter periods of treatment before and after the period at risk for malaria transmission, but it is more expensive than the other agents (Rosenthal, 2004)

2.4.5 Artemisinin and derivatives

Artemisinin, also known as qinghaosu in China, its country of origin, is a sesquiterpene lactone endoperoxide derived from the leaves of *Artemisia annua*, the sweet wormwood plant (Meshnick *et al.*, 1996). Artemisinin has been used as an antipyretic in China for over 2000 years (Klayman, 1985; Rosenthal, 2004). In 1979, the Chinese reported that artemisinin drugs were rapidly acting, effective and safe for the treatment of patients with *P. vivax* or *P. falciparum* infections (Tracy & Webster, 2001). The Chinese synthesised three derivatives with greater antimalarial potency and solubility than artemisinin (Figure 2.9 (a)) itself, namely dihydroartemisinin (Figure 2.9 (b)), a reduced product, artemether (Figure 2.9 (d)), an oil-soluble methyl- ester and artesunate (Figure 2.9 (c)), the water-soluble hemisuccinate salt of dihydroartemisinin. The latter two derivatives are rapidly metabolized *in vivo* to the active metabolite dihydroartemisinin. Important analogs currently under investigation are arteether and arteminic acid (Tracy & Webster, 2001; Rosenthal, 2004).

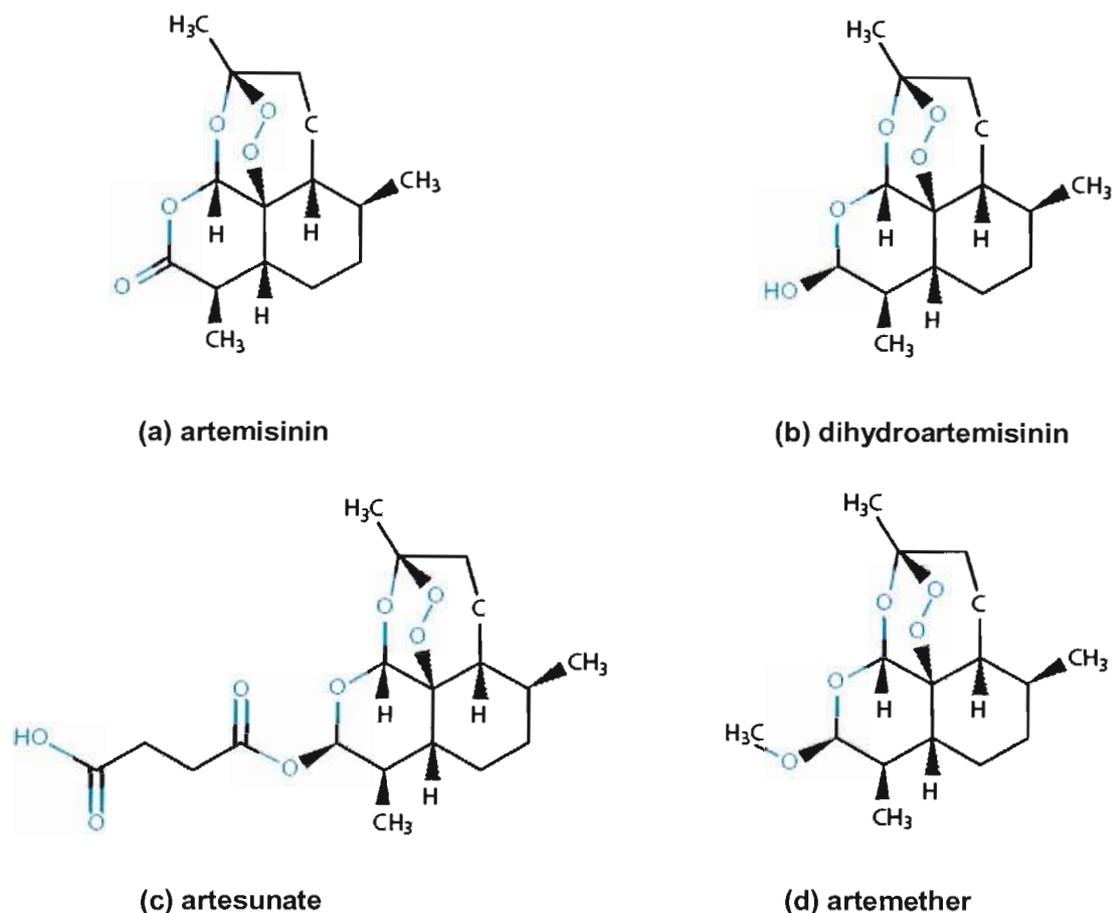


Figure 2.9: The chemical structures of (a) artemisinin, (b) dihydroartemisinin, (c) artesunate and (d) artemether.

These compounds have an unusually broad activity against asexual parasites of all *Plasmodium* species, killing all stages from young rings to schizonts (Tracy & Webster, 2001; WHO, 2006). In *P. falciparum* malaria, artemisinin also kills the gametocytes (Kumar & Zheng, 1990; Rosenthal, 2004), but do not affect either primary or latent liver stage parasites. Thus, artemisinin compounds are not useful either for chemoprophylaxis or for preventing relapses of *vivax* malaria (Tracy & Webster, 2001).

The current model of artemisinin action involves two steps. First, intraparasitic heme iron of infected erythrocytes catalyses cleavage of the endoperoxide bridge. This is followed by intramolecular rearrangement to produce carbon-centered radicals that covalently modify and damage specific malarial proteins (Meshnick *et al.*, 1996). Artemisinin compounds also show structural similarities to thapsigargin, a known inhibitor of sarco-endoplasmic reticulate Ca-ATPase (SERCA). This observation led investigators to demonstrate that artemisinins inhibit

*Pf*ATP6, a SERCA homologue in malaria (Eckstein-Ludwig *et al.*, 2003). How this mechanism results in parasite death is still under investigation (Daily, 2006). *In vitro* drug resistance to artemisinin derivatives has been reported (Dondorp & Nosten, 2009). Polymorphisms within the *Pf*ATP6 gene have been associated with the resistant phenotype in some studies (Jambou *et al.*, 2005). Thus, these drugs should be given as combination therapy to protect them from resistance (WHO, 2006).

Artemisinin compounds are the most rapidly acting, effective and safe drugs for the treatment of severe malaria, including infections due to chloroquine- and multidrug resistant strains of *P. falciparum*. Their potency is 10 to 100-fold greater *in vivo* than that of other antimalarial drugs (White, 1999; WHO, 2006). Artesunate and artemether in particular play a key role in the treatment of multidrug resistant *P. falciparum* malaria. They are the only drugs reliably effective against quinine-resistant strains (Rosenthal, 2004). Artemisinin drugs act more rapidly and produce less toxicity than the antimalarial alkaloids, moreover, they are just as effective against cerebral malaria (Tracy & Webster, 2001). The efficacy of the artemisinins is limited somewhat by their short plasma half-lives. Recrudescence rates are unacceptably high after a short-course or even seven days of therapy and these drugs are generally best used in conjunction with another agent (Kongpatanakul *et al.*, 2007). A brief course of these agents given in tandem with a longer-acting quinoline or antibiotic antimalarial like mefloquine or doxycycline usually prevents relapses and may delay the development of drug resistance (Adjuk *et al.*, 2004). Artemisinins are thus not useful in chemoprophylaxis due to their short half-lives (Rosenthal, 2004). In some areas of South-east Asia, combinations of artemisinin such as artesunate and mefloquine offer the only reliable treatment for even uncomplicated malaria, due to the development and prevalence of multidrug resistant *falciparum* malaria (White *et al.*, 1999).

Individual endoperoxide antimalarials differ in formulation and clinical utility. Dihydroartemisinin can be given only orally. The oil-soluble artemether can be given only orally or intramuscularly (Tracy & Webster, 2001). Artemether is available alone and as a fixed-dose combination with lumefantrine for combination therapy known as Coartem[®] in some countries (Rosenthal, 2004). Artemisinin is effective when given orally or as a rectal suppository (Tracy & Webster, 2001; WHO, 2006). Artesunate is perhaps the most versatile derivative, because it is effective when given orally, intramuscularly, intravenously or rectally (Rosenthal, 2004; WHO, 2006). The intravenous formulation is particularly suitable for treating cerebral malaria, whereas suppositories are especially advantageous for treating patients with severe malaria in isolated areas (Tracy & Webster, 2001).

Artemisinin and its derivatives are safe and appear to be better tolerated than most antimalarials (de Vries & Dien, 1996). The most commonly reported adverse effects have been mild gastrointestinal disturbances, dizziness, tinnitus and elevated liver enzyme values (Ashley *et al.*, 2006). The only potentially serious adverse effect reported with this class of drugs is type 1 hypersensitivity reactions in approximately 1 in 3000 patients (Leonardi *et al.*, 2001). Irreversible neurotoxicity has been depicted in animal studies, particularly with very high doses of intramuscular artemether, but this phenomenon has not been substantiated in humans (Hien *et al.*, 2004). While artemisinin derivatives are not recommended for use in the first trimester of pregnancy since they cause fetal resorption in animal studies, there has been no evidence of reproductive toxicity or teratogenicity from published data on their use of malaria treatment in hundreds of pregnant women (McGready *et al.*, 2001).

2.5 Antimalarial treatment regimes

In theory, recommended treatment regimes should be tailored specifically to a given region based on resistance patterns found in that area. Other considerations include cost-effectiveness, availability, ease of administration, capabilities of the health-care infrastructure, perceived efficacy and safety of the drug. In practice, currently recommended treatment regimes do not always reflect the current state of antimalarial drug resistance (Bloland, 2001).

When it comes to chemoprophylaxis in general, it is imperative to emphasise measures to prevent mosquito bites, since malaria parasites are increasingly resistant to multiple drugs and no chemoprophylactic regimen is fully protective (Gkrania-Klotsas & Lever, 2007). From the above discussion it can be concluded that recommendations for malaria chemoprophylaxis include the use of chloroquine in chloroquine-sensitive areas, mefloquine or atovaquone-proguanil for most other malarious areas and doxycycline for areas with very high prevalence of multidrug resistant *falciparum* malaria (WHO, 2006).

Chloroquine remains the drug of choice for the treatment of non-*falciparum* and *falciparum* infections contracted in areas without known resistance patterns. *P. vivax* and *P. ovale* infections should subsequently be treated with primaquine to eradicate liver forms of these parasites. *P. falciparum* malaria from most areas is best treated with oral quinine or intravenous quinidine, in either case plus doxycycline or, for children, clindamycin. Other agents that are generally effective against resistant *falciparum* malaria include mefloquine and the artemisinin derivatives artesunate and artemether, alone or in combination (WHO, 2006).

The future of antimalarial chemotherapy appears bleak unless prompt action is taken. It is imperative that the optimal indications and appropriate dosing regimens be followed for different parasite and human populations. Clinical evaluation of antimalarial chemotherapy must keep pace with the identification of promising new agents.

2.6 Conclusion

There is no clear single path to improve malaria control. Rather, a series of incremental steps involving better and more widespread use of methods that have already been shown to be effective, as well as the step-wise introduction of new treatments and partly effective control measures should be employed (Greenwood *et al.*, 2005). One of the cornerstones of the current approach to malaria control is the provision of effective, safe, affordable and practicable malaria treatment. However, as long as drugs are used, the chance of resistance developing to those drugs is present (Bloland, 2001). In order to delay the emergence of resistance and preserve effective drugs, combination therapy is recommended. Artemisinin-based combination therapies currently offer the best chance to reduce global morbidity and mortality of malaria infection.

Combination therapy costs more than individual single drug treatments, but this approach should make considerable savings over the longer term by delaying the onset of resistance. Resistance itself has a price too high to pay through increased morbidity and mortality (White, 1999). A thorough understanding of the complex parasite biology, transmission and drug resistance will provide new options for effective therapy and reduce transmission of this often deadly disease.

CHAPTER 3

ENHANCEMENT OF ANTIMALARIAL DRUG EFFICACY WITH PHEROID™ TECHNOLOGY

3.1 Introduction

Malaria still remains one of the most imperative parasitic diseases worldwide. The rapidly escalating prevalence of drug resistant strains of *P. falciparum*, the high cost of conventional antimalarial medicine and the small number of widely available chemoprophylactic and -therapeutic agents have contributed to the increasing burden of malaria globally. Although a considerable progress has been made in comprehending the cell biology, pharmacogenomics, etiology and pathophysiology of malaria in the last decade, the scenario in the area of therapeutics is disappointing (Renslo & McKerrow, 2006). To date, despite major research efforts, there exists no effective vaccine against malaria (Tuteja, 2007). Most of the currently existing antimalarial agents were introduced over 50 years ago. Although these agents are effective, most of them are no way close to the modern concept of “drug” in terms of tolerability, therapeutic regimen, duration of treatment, specificity and patient compliance (Renslo & McKerrow, 2006). The poor rate of discovery in the antimalarial segment seen in the last few decades has necessitated effective management of existing drugs by modulating their delivery (Date *et al.*, 2007).

The most feasible and practical strategy that could be implemented to tackle the aforementioned crisis associated with malaria is to develop novel drug delivery systems in order to improve the efficacy, specificity, tolerability and therapeutic index of existing antimalarial agents (Date *et al.*, 2007). For the purpose of this study, the technology identified to optimise the delivery and thus efficacy of antimalarial drugs is the Pheroid™ drug delivery system. This pliable, but stable lipid-based submicron emulsion type delivery system can be formulated with various active pharmaceutical ingredients for novel and effective dosage forms (Grobler *et al.*, 2006).

This chapter focuses on the biopharmaceutical and biological issues to be considered in the design of a drug delivery strategy for treating parasitic infections such as malaria. Biopharmaceutical limitations associated with some antimalarial drugs, with emphasis on artesunate and mefloquine are highlighted. The role of colloidal carriers in addressing these limitations is explained. Emphasis is placed on the Pheroid™ drug delivery system in which its characteristics and clinical pharmaceutical applications are discussed.

3.2 Biopharmaceutical limitations associated with antimalarial drugs

Drugs used to treat malaria are frequently limited in efficacy, plagued by severe side effects and thus poor patient compliance or are hamstrung by drug resistance (Renslo & McKerrow, 2006). For proof of concept, a few examples will be discussed in terms of this scenario.

The use of quinine is limited by its narrow therapeutic index, which is characterised by cardiotoxicity and the development of cinchonism, as well as hypoglycemia and hypersensitivity reactions (Bonington *et al.*, 1990; White, 2007). Thus, the poor tolerability and long duration of treatment (≥ 7 day treatment course) has led to low patient adherence with quinine (Fungladda *et al.*, 1998). Primaquine is also characterised by dose-limiting side-effects of which acute hemolytic anemia in patients with glucose-6-phosphate deficiency is the most important (Baird & Rieckman, 2003). Limited oral bioavailability due to pre-systemic metabolism and excretion has also been reported as another limitation regarding the administration of primaquine (Singh & Vingkar, 2008).

Due to incomplete drug absorption, the resulting plasma concentration of halofantrine hydrochloride is highly variable and often low following an oral tablet administration (Khoo *et al.*, 1998). Although absorption of halofantrine can be substantially increased when co-administered with a fatty meal (Humberstone *et al.*, 1996), this practice is now contraindicated due to the uncontrolled increase in halofantrine plasma levels, which can result in prolongation of the QTC-interval of the electrocardiogram (ECG) of the heart (Karbwang & Na Bangchang, 1994). The oral bioavailability of artemether is low (~40%) due to its poor aqueous solubility and degradation in stomach acids (Karbwang *et al.*, 1997), whereas the current oily intramuscular injection suffers from disadvantages such as pain on injection and slow and erratic absorption (Hien *et al.*, 2004). Although the three day treatment course of artemether-lumefantrine shows high efficacy in most clinical trials, it has to be taken as a six-dose regimen and must be co-administered with at least 1.2g of fat, both of which might compromise patient adherence and thus its effectiveness in the normal context of use (Ashley *et al.*, 2007a; Ashley

et al., 2007b). With regard to the purpose and scope of this study, emphasis is placed on the biopharmaceutical problems associated with artesunate and mefloquine.

As an artemisinin derivative, the efficacy of artesunate is impaired by its short plasma half-life of approximately 10 minutes (Krishna *et al.*, 2004), its low solubility in water or oil, the requirement of a high dosage with oral administration and a high rate of recrudescence when used alone as monotherapy (Kongpatanakul *et al.*, 2007). Not surprisingly, *in vitro* drug resistance to artemisinin and its derivatives has been reported (Dondorp & Nosten, 2009). As artemisinin and its derivatives currently are the most valuable and important antimalarial drugs available, great effort needs to be made to protect these drugs against drug resistance, which is done mainly through the strategy of combination therapy (WHO, 2008). Malaria treatment is therefore far from optimal and this can lead to relapse infections and increased drug resistance.

Mefloquine hydrochloride can only be taken orally because severe local irritation occurs with parenteral use. Mefloquine is eliminated slowly with a terminal elimination half-life of approximately 20 days. Neuropsychiatric toxicities associated with mefloquine have received a great deal of publicity. However, tolerability is improved by splitting administration of the drug into two doses separated by six to eight hours (Rosenthal, 2004), or by administering it after artesunate (Smithuis *et al.*, 2004). Moreover, resistance to mefloquine was reported as early as five years after its introduction as a prophylactic agent in parts of Thailand (Wongsrichanalai *et al.*, 2002). There is thus a growing concern about toxicity and especially resistance emerging towards the drug.

3.3 Drug delivery

It is widely recognised that inadequate drug delivery is the single most important factor delaying optimised application of new classes of molecular therapeutics. Considerable research efforts is therefore currently focusing on the continued development of improved drug delivery systems, which besides enhancing efficacy, may also improve safety (Grobler *et al.*, 2006). Although several drug delivery technologies are available, most of them are hampered to some extent by stability problems, high cost to market, limited field application, low solubility and bioavailability (Muller & Keck, 2004). Over the past ten years, a more holistic approach to the concept of a drug delivery system has been embraced. This includes not only the drug or prodrug and its formulation matrix, but also the dynamic interactions among the drug, its formulation matrix, its container and the physiologic milieu of the patient (Shargel *et al.*, 2004).

The primary goal of modern parasitic chemotherapy is to target the drug specifically to the parasite in order to minimise the adverse effects arising during treatment. Thus, the sole objective behind the design of a drug delivery system for this purpose should be to modulate physiochemical- and pharmacokinetic properties of the antiparasitic agent in order to improve biospecificity rather than bioavailability, with concomitant minimisation of the adverse effects associated with it (Edwards & Krishna, 2004). Additionally, the following factors must also be considered when designing novel drugs or drug delivery systems with respect to antiparasitic agents (Date *et al.*, 2007):

- parasite-host cell interaction;
- biological barriers to overcome in order to reach the target organ, tissue or cells;
- complete information about the receptors present on the cells infected by the parasite;
- changes occurring in the infected cell after the invasion of the parasite or with the progression of disease;
- antigens or receptors present on the surface of the parasite;
- pathophysiology of the disease.

Biopharmaceutical aspects like physiochemical- and pharmacokinetic properties such as solubility, absorption, distribution, metabolism and elimination of a drug strongly influence the route of administration, therapeutic response, adverse effects and in turn the design of a drug delivery system (Date *et al.*, 2007). Other biopharmaceutical aspects that govern the design of drug delivery systems include permeability of drug through cell membranes, mechanism of cellular uptake (especially in case of intracellular parasitic infections like malaria), stability, activity and kinetics of the drug in the target cell environment (Kayser & Kiderlen, 2003).

Considering the nature of parasitic infections such as malaria, the ideal drug delivery system for antimalarial drugs should exhibit the following properties (Date *et al.*, 2007):

- it should allow oral administration of the antiparasitic drug;
- with respect to intracellular targeting, it should allow high drug payloads, low toxicity, low immunogenicity and protect the drug from extracellular degradation;
- it should shorten the duration of the treatment by improving pharmacokinetic or pharmacodynamic profile of the antimalarial agent;
- it should be versatile enough to allow delivery of combination of antimalarial agents, which is a normal practice in the current situation;
- it should prove to be affordable when cost to benefit ratio is considered;

- it should have the ability to selectively transport the antimalarial agent to the parasite to render maximum efficacy and minimum adverse effects.

In view of this, colloidal drug delivery systems have been studied intensively due to their versatile nature and attractive advantages (Porter *et al.*, 2008), which will be discussed in the following section.

3.4 Pheroid™ technology: a novel drug delivery system

Pheroid™ technology, based on what was previously called Emzaloid™ technology, is able to enhance the absorption and/or efficacy of various categories of pharmaceutical active ingredients (Grobler *et al.*, 2006). This technology has been shown to result in major improvements in the control of size, charge and the hydrophilic-lipophilic characteristics of therapies when compared to other drug delivery systems (Saunders *et al.*, 1999; Tzaneva *et al.*, 2003).

The intellectual property on which Pheroid™ technology is based was purchased by the North-West University of South Africa in December 2003 from MeyerZall (Pty) Ltd., whose founder, Piet Meyer, had initially developed the technology for the treatment of his own psoriasis. The novelty of Pheroid™ technology is underlined by patents registered in Europe, the United States, South Africa and China. These patents describe the use of Pheroid™ technology as a delivery system to promote the absorption and increase the efficacy of dermatological, biological and oral medicines in various pharmaceutical groups (Grobler *et al.*, 2006). The effectiveness of Pheroid™ technology has been illustrated by several national and international clinical trials with products based on this technology (Saunders *et al.*, 1999; Goodfield *et al.*, 2003).

3.4.1 Classification of Pheroid™ technology as a lipid-based colloidal drug delivery system

The vast majority of drug delivery systems can structurally be classified as colloids (Grobler *et al.*, 2006). The intention in using colloidal systems as carriers of drugs is to enhance the efficacy of the administered compounds while reducing the unwanted side effects (Vonarborough *et al.*, 2005). The Pheroid™ drug delivery system is a colloidal system that contains unique and

stable lipid-based submicron- and micron-sized structures called Pheroid™(s), uniformly distributed in a dispersion medium (Grobler *et al.*, 2006). Colloidal delivery systems consist of two distinctive phases, namely the dispersed phase in a continuous phase (Attwood, 2003). Commonly used colloidal dosage forms include liposomes (Cimato *et al.*, 2004), emulsions and micro-emulsions (Tadros *et al.*, 2004), polymeric microspheres (Kawaguchi, 2000) and macromolecular microspheres (Vasir *et al.*, 2003). In the design of the Pheroid™, one or more features of each of these dosage forms have been incorporated and it is therefore important to discuss some of these features (Grobler *et al.*, 2006).

3.4.1.1 Liposomes

Liposomes are the most extensively investigated amongst various colloidal carriers (Date *et al.*, 2007). They are spherical phospholipid vesicles consisting of single or multiple lipid bilayer membranes that form spontaneously in an aqueous medium (Cimato *et al.*, 2004), as illustrated in Figure 3.1. The molecules in the lipid bilayer consist of a hydrophilic head and a hydrophobic tail (Honeywell-Nguyen & Bouwstra, 2007). The spherical structures can have diameters ranging from 80nm to 100µm (Sharma & Sharma, 1997).

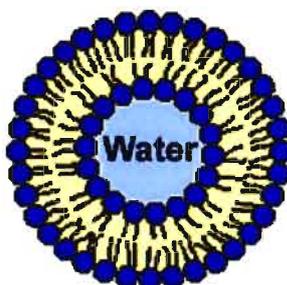


Figure 3.1: Schematic illustration of a liposome: a lipid bilayer enclosing an aqueous core (Daniels, 2001).

Liposomes can encapsulate water-soluble ingredients in their inner aqueous space and oil-soluble ingredients in the phospholipid membranes without the use of surfactants or emulsifiers (Hager *et al.*, 1993). Various antimalarial drugs have been encapsulated in liposomes. Gabriëls and Plaize-Vercammen (2003) have formulated artesunate liposomes to reduce its dosing frequency and they found 30% release in 24 hours in the *in vitro* release test using the dialysis bag technique. The encapsulation of β -artemether in liposomes for the treatment of recrudescence malaria has been reported by Chimanuka and co-workers (2002). The egg phosphatidylcholine cholesterol liposomes prepared by the group with 100% entrapment efficiency was demonstrated to successfully circumvent the recrudescence parasitemia in

Plasmodium chabaudi-infected OF1 mice. Chloroquine-encapsulated liposomes, with or without surface modification have been studied by different groups (Date *et al.*, 2007). Owais *et al* (1995) have tagged antibody against infected erythrocytes on the chloroquine liposomes and tested their efficacy in chloroquine-resistant *Plasmodium berghei* infection in mice. The chloroquine delivered in these liposomes intravenously at a dosage of 5mg/kg of body weight per day on days four and six post-infection, completely cured the animals (75% to 90%) of chloroquine-resistant *P. berghei* infections. Liposomes have also been used as carriers to deliver malaria vaccines (Green *et al.*, 1995).

3.4.1.2 Emulsions and micro- or nano-emulsions

An emulsion is a system consisting of two immiscible liquid phases, one of which is dispersed through the other (El-Aasser & Sudol, 2004). The dispersed phase is small droplets in the disperse medium, usually stabilised by an emulsifying agent (Attwood, 2003). Since most drugs are hydrophobic and water-insoluble, oil-water emulsions are used as vehicles to deliver such drugs (El-Aasser & Sudol, 2004). Micro-emulsions, also referred to as nano-emulsions, are oil in water emulsions where a single lipid layer encloses a fluid lipid center, in contrast to a liposome's bilayer-enclosed hydrophilic core, as seen in Figure 3.2.

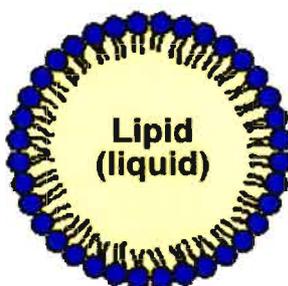


Figure 3.2: Schematic illustration of a particle of nano-emulsions: a lipid monolayer enclosing a liquid core (Daniels, 2001).

Nano-emulsions might thus be more suitable for the delivery of lipophilic drugs. Droplet diameter ranges between 50 and 1000nm (Daniels, 2001). The main application of nano-emulsions is as formulations for controlled drug delivery and targeting (Solans *et al.*, 2005).

3.4.1.3 Polymeric microspheres and/or nanoparticles

Polymeric microspheres are solid colloidal particles, ranging in size from 1-1000 nm, consisting of various biocompatible polymeric matrices in which the therapeutic moiety can be adsorbed, entrapped or covalently attached (Lockman *et al.*, 2002), as illustrated in Figure 3.3.

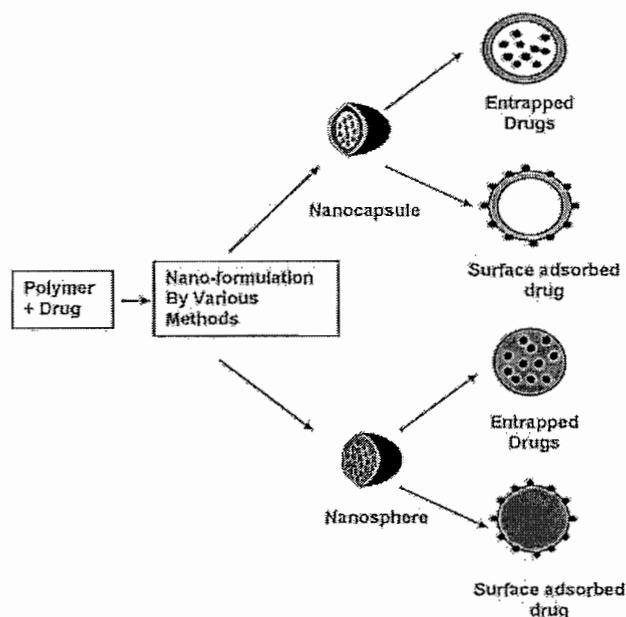


Figure 3.3: Schematic illustration of a nanoparticle. The drug molecules are either entrapped inside or adsorbed on the surface (Modified from Tiyaboonchai, 2003).

Biodegradable and –compatible synthetic polymers like poly(_{D,L}-lactide-co-glycolide) (PLGA) and polyalkylcyanoacrylates (PACA) are preferred for obtaining nanoparticles (Covreur & Vauthier, 2006). Nanoparticles of primaquine were prepared with the aim of liver targeting (Labhasetwar & Dorle, 1990; Mbela *et al.*, 1992). Labhasetwar & Dorle (1990) prepared gelatin, albumin, gluteraldehyde and polyacrylamide nanoparticles and demonstrated their sustained release in *in vitro* experiments. Mosqueira *et al* (2004) synthesised halofantrine loaded polyethylene glycol (PEG)-coated polylactic acid (PLA) nanocapsules. The efficacy and pharmacokinetics of halofantrine nanocapsules on intravenous administration were compared to that of halofantrine solution. In the four-day test, the halofantrine-loaded nanocapsules showed activity that was similar to or better than that of the solution given as a single dose in severely infected mice. Nanocapsules increased the area under the curve for halofantrine in plasma more than six-fold, compared to the solution throughout the experimental period of 70 hours.

Furthermore, nanocapsules induced a significantly faster control on parasite development than the solution in the first 48 hours of treatment.

3.4.1.4 Macromolecular microspheres

In macromolecular microspheres, the drug can either be entrapped within a macromolecular membrane or be bound covalently to the macromolecules, which are soluble in body fluids. Proteins such as albumin, gelatin and polysaccharides such as dextran and cellulose derivatives are the common macromolecules used in these drug delivery systems (Vasir *et al.*, 2003).

3.4.1.5 Pheroids™

As is the case with liposomes, Pheroids™ generally contain a lipid bilayer, but it contains no phospholipids or cholesterol. In contrast to liposomes, Pheroids™ are formed by a self-assembly process similar to that of low-energy emulsions and micro-emulsions. As in emulsions, Pheroids™ are dispersed within a dispersion medium, but it contains not only two liquid phases, but also a dispersed gas phase which is associated with the fatty acid dispersed phase. The specific ratio of pegylated to ethylated fatty acids used in the assembling of the Pheroids™ adds some of the reservoir characteristics of the polymeric microspheres, while the formulation of natural depots is reminiscent of the structure of macromolecular microspheres (Grobler *et al.*, 2006). A schematic representation of a Pheroid™ is shown in Figure 3.4:

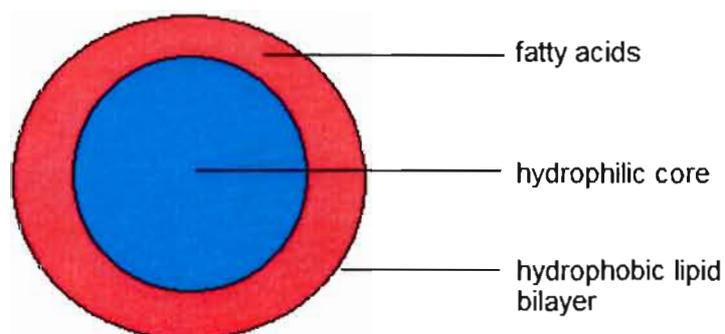


Figure 3.4: A Pheroid™ vesicle schematically illustrated.

3.5 The composition and various types of Pheroids™

The main components of Pheroid™ particles are the ethyl esters of the essential fatty acids linoleic acid and linolenic acid, as well as the *cis*-form of oleic acid and water. The formulation is saturated with nitrous oxide (du Plessis *et al.*, 2009). The essential fatty acids used in the Pheroid™ formulation are important for normal cellular functions, but cannot be manufactured by the human body. Their functions include homeostasis of energy, modulating the immune system, maintaining the integrity of cell membranes and regulating some components of programmed cell death. The Pheroid™ delivery system thus has inherent therapeutic qualities in contributing to the normal functioning of cells as well (Grobler *et al.*, 2006).

Nitrous oxide (N₂O) is a volatile anaesthetic gas that is both lipid and water soluble (Eger, 2005) and has at least three known functions in the formulation, namely helping with the self-assembly process of Pheroid™ vesicles (Uys, 2006) and the miscibility of the fatty acids in the dispersed medium, as well as playing a role in the stability of Pheroid™ vesicles or microsponges that are formed (Grobler *et al.*, 2006). Molecular modeling indicates that there is some interaction between the fatty acids and the nitrous oxide, resulting in stable vesicular Pheroid™ structures. It was noted in controlled experiments on various formulations that if either the nitrous oxide or the essential fatty acids were absent from the formulation, the efficacy and stability of the formulation was decreased dramatically (Grobler *et al.*, 2006).

As a result of the different target sites in the body for pharmaceutical applications, versatility is one of the requirements of an acceptable carrier system (Date *et al.*, 2007). The design of the Pheroid™ allows for manipulation of both its structural and functional features, which can be performed through the following changes (Grobler *et al.*, 2006):

- changing the fatty acid composition or concentrations;
- the addition of non-fatty acids or phospholipids such as cholesterol;
- the addition of cryo-protectants;
- the addition of charge-inducing agents;
- changing the hydration medium (ionic strength, pH);
- changing the method of preparation;
- changing the character and the concentration of the active compound.

The manipulation is thus done in terms of size, function and structure of the vesicles (Grobler *et al.*, 2006). Three types of Pheroid™ particles can be classified, as summarised in Table 3.1 and illustrated in Figure 3.5 (Grobler *et al.*, 2006):

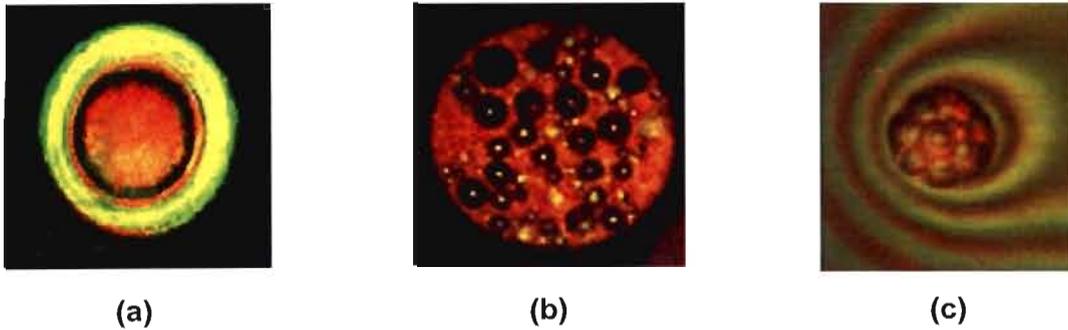


Figure 3.5: Confocal laser scanning micrographs of a Pheroid™ (a) vesicle, (b) microsponge and (c) pro-Pheroid (Grobler, 2004).

Table 3.1: Classification of Pheroid particles (Grobler et al., 2006)

Type of Pheroid™	Description
Vesicles	Basic pheroid vesicles with a diameter of 200-400nm. The vesicle consists of a highly elastic double layer membrane with the lipids packed loosely (Figure 3.5 (a)).
Microsponges	Very small, sponge-like vesicles with a diameter of less than 30 nm (Figure 3.5 (b)). These nanosponges can entrap hydrophobic molecules in their membrane, while small hydrophilic molecules can be entrapped within the aqueous central area.
Pro-pheroids	Pro-pheroid spheres, or reservoirs, ranging from 5 to 100µm in diameter, serving as a depot (Figure 3.5 (c)). They have a hydrophobic centre, where the pheroid lipid phase is entrapped, surrounded by a hydrophilic area. The hydrophilic area serves as a vesicle forming and release zone. The vesicles are gradually released to attain continuous release.

The Pheroid™ formulation can thus be specifically manipulated to yield different types of vesicles, ensuring a fast transport rate, high encapsulation efficiency, rapid delivery and stability of the delivery system for a specific drug (Grobler, 2004).

3.6 Characteristic features that make Pheroid™ technology an interesting carrier

Research has confirmed the following key characteristics of the Pheroid™ drug delivery system as depicted in Figure 3.6, which will be discussed in detail below.



Figure 3.6: Key advantages of the Pheroid™ drug delivery system (Modified from Grobler, 2004).

3.6.1 Toxicity profile

Pheroids™ consist primarily of essential fatty acids. Essential fatty acids are necessary for various cell functions in the human body, but cannot be manufactured by human cells, it therefore has to be ingested. Since these fatty acids are part of the natural biochemical

pathways, Pheroids™ cause no cytotoxicity (Grobler, 2004). During an extensive *in vivo* study on Sprague Dawley rats, no signs of toxicity were observed with the administration of an oral pro-Pheroid™ formulation at a concentration of 50mg/kg (Elgar, 2008). Cytokine studies demonstrated that Pheroids™ elicit no immune responses in man. Some drugs such as proteins may induce an immunologic response, but masking the proteins using Pheroids™ may reduce recognition by the immune system (Grobler, 2004).

3.6.2 Stability

Pheroids™ are sterically stabilised without the disadvantages of increased size or decreased elasticity. The Pheroid's™ interior volume is stabilised due to its composition through hydrogen bonding and waterbridge interactions and not by cholesterol, as is the case in most lipid-based delivery systems. Due to the presence of a gas as well as the pliable pegylated tails added to the fatty acids, extremely elastic structures are formed (Grobler, 2004). Pegylation serves to sterically stabilise the Pheroids™ and maintain their interior spaces. Furthermore, polyethylene glycol (PEG) has been shown to contribute to increased bioavailability, drug stability, lower toxicity and enhanced drug solubility (Torchilin, 2001). Pheroids™ are not shattered under moderate pressure or extravasation, e.g. to deform in order to cross densely packed cohesive capillary walls without fractioning (Grobler *et al.*, 2006).

3.6.3 Drug entrapment efficiency

The Pheroid™ is polyphilic and drugs that have different solubilities as well as insoluble drugs can be entrapped. Entrapment efficiency in all compounds tested is high (between 85% and a 100%). The entrapment efficiency of Pheroid™-based formulations is generally determined by confocal laser scanning microscopy (CLSM) and both the Pheroid™ and the active compound are visualised through fluorescence labeling (Figure 3.7) (Grobler, 2004).

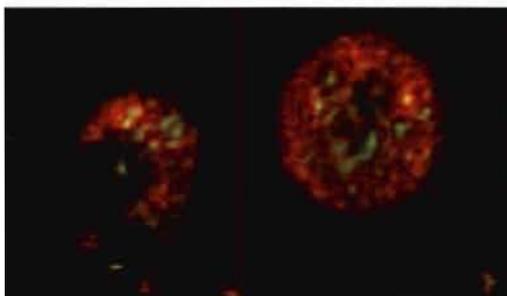


Figure 3.7: Pheroids™ containing auto fluorescent active molecules (with permission from Anne Grobler, NWU, Potchefstroom).

3.6.4 Types of Pheroids™

A variety of Pheroid™ types can be formulated, as seen in Figure 3.5, depending on the composition and the method of manufacturing. Pheroids™ can be manipulated in terms of size, charge, lipid composition and membrane packaging. The desired types can repeatedly be obtained. The Pheroid™ system can therefore be optimised for the active compound and indication of the drug. The type of Pheroid™ formulated for a specific compound determines the loading capacity of that Pheroid™. Microsponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other in the sponge-like spaces. Geographical separation of active compounds into different interior spaces minimises interaction between compounds or drug interactions (Grobler, 2004).

3.6.5 Pheroid™-cell membrane interaction

Since Pheroids™ are comprised of fatty acids, affinity exists between the Pheroid™ and cell membranes. This affinity is showed in the CLSM micrograph of Figure 3.8. It interacts with the cell membrane and penetrates the cell through the endosome sorting mechanism, resulting in effective and fast delivery (Grobler, 2004). Cell membranes are dynamic and are constantly changing, with not only movement of proteins and lipids laterally on the membrane, but also with molecules moving in and out of the membrane (Mastrangelo *et al.*, 1978). The fluidity of the Pheroid™ membrane should increase the movement of hydrophobic or hydrophilic molecules or compounds laterally in the membrane to connecting cells (Grobler *et al.*, 2006). The Pheroid™ penetrates keratinised tissue, skin, intestinal lining, vascular system, fungi, bacteria and parasites. The rate of binding to and uptake of Pheroids™ by cells are high and fast, comparable to the rate observed for active transport across cell membranes (Grobler, 2004).

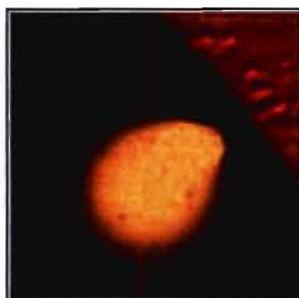


Figure 3.8: A CLSM illustrating affinity between a primary fibroblast and a Pheroid™ vesicle (with permission from Anne Grobler, NWU, Potchefstroom).

3.6.6 Drug protection

Many drugs have reduced therapeutic effects because of the partial degradation of the drug before it reaches the specific target (Vogelson, 2001). The Pheroid™ protects entrapped drugs from metabolism and inactivation in the plasma or other body fluids. No leakage of the drug from the Pheroid™ before it reaches the target site has been observed (Grobler, 2004).

3.6.7 Absorption and bioavailability profile

Pheroids™ enhance the absorption and bioavailability of oral, topical, parenteral and nasal administration of active compounds in all products tested so far and will be as discussed in detail in Section 3.7. An increase in the bioavailability leads to a reduction in the minimal inhibitory concentration with increased therapeutic efficacy (Grobler, 2004).

3.6.8 Pharmacokinetics

Entrapment in Pheroids™ changes the pharmacokinetics of active compounds, resulting in a decrease in the time needed to achieve maximum concentration levels (T_{max}). As opposed to small molecule drug compounds that have a large volume of distribution on administration, entrapment of active compounds in Pheroids™ reduces the volume of distribution and consequently the concentration at the target site is increased. The narrow therapeutic index is thus enhanced with less toxicity (Grobler, 2004).

3.6.9 Drug targeting

The use of different combinations of fatty acids and/or other added molecules is used to target Pheroids™ at sub-cellular level to some extent. Pheroids™ entrapped with small peptides and antibodies have been shown to interact with specific micro-domains on cells in culture (Grobler, 2004).

3.6.10 Drug resistance

Drug resistance in infectious diseases was reduced or eliminated in all *in vitro* studies done. The possible mechanism for this property may be due to the intracellular release of drugs beyond the membrane and thus beyond the drug efflux pumps found in drug resistant organisms. This is probably the case in the decrease in resistance observed for chloroquine-resistant malaria parasites. The effect of commercially available chloroquine phosphate in water versus a Pheroid™-entrapped chloroquine formulation was tested against a *P. falciparum* resistant reference strain (W2). The strain is known to have a 50% inhibition concentration (IC₅₀) value of between 200 - 300 nm for chloroquine. The determined IC₅₀ value for the Pheroid™-formulated chloroquine was reduced to 25 - 30 nm (Grobler, 2004).

From the above discussion it can be concluded that the Pheroid™ drug delivery system combines advantages of traditional colloidal drug carriers, but at the same time avoids or minimises the drawbacks associated with them.

3.7 Examples of possible clinical applications of Pheroids™

The effect of a drug delivery system or carrier should ideally be measured by its contribution to therapeutic efficacy (Date *et al.*, 2007). Pheroid™™ technology has been successfully employed for efficient delivery of numerous therapeutic agents by various delivery routes.

3.7.1 Transdermal therapy

All Pheroid™-based products currently on the market are topical products, supported by results from various clinical trials (Hager *et al.*, 1993; Cimato *et al.*, 2004). Acyclovir, an antiviral agent was formulated in a Pheroid™ micro-formulation and compared *in vitro* with a reference sample

in a phosphate buffer solution (PBS). The Pheroid™-based acyclovir had a better drug delivery across the skin (van der Walt, 2007). The *in vitro* transdermal delivery of anti-tuberculosis drugs, namely isoniazid and rifampisin in a Pheroid™ micro-formulation, showed an increase of drug delivery. The drug quantities as determined over 12 hours showed levels above the minimum inhibitory concentration (Botes, 2007).

3.7.2 Treatment of infectious diseases

The Pheroid™ platform has shown applicability to infectious disease treatment. For proof of concept, an oral pro-Pheroid™ formulation of anti-tuberculosis drugs was utilised in an *in vivo* study, measuring the efficacy of the Pheroid™ delivery system in which rifampisin, isoniazid, pyrazinimide and ethambutol were entrapped, against the same drugs in solution with water. Rifampisin in the pro-Pheroid™ showed a 305% increase in the plasma concentration, where isoniazid and pyrazinimide showed a 20% and 19% increase respectively. This pro-Pheroid™ formulation thus showed better absorption and thus bioavailability (Mathee, 2007).

Regarding the treatment of malaria, *in vitro* drug efficacy studies showed that chloroquine, mefloquine, artesunate and artemether had better efficacy in a Pheroid™ formulation than in water. Chloroquine showed a fifteen-fold efficacy enhancement against a chloroquine resistant *P. falciparum* strain. Mefloquine, artesunate and artemether had an efficacy enhancement of 314%, 238% and 254% respectively (Langley, 2007). An *in vivo* study using the *Plasmodium berghei* mouse model showed better efficacy of chloroquine in Pheroids™ during the first eleven days after infection, compared to chloroquine in water (Langley, 2007).

The *in vitro* efficacy study of human immunodeficiency virus (HIV) drugs on M7-Luc cells showed enhanced viability (van der Merwe *et al.*, 2008).

3.7.3 Vaccination

Vaccination is a vital part of disease control and preventative strategies. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor. There is thus an urgent need for effective vaccine adjuvants to enhance the immunogenicity and –stimulatory properties of vaccines. The Pheroid™ carrier is *per se* an adjuvant as it is based on a micro-colloidal carrier system that conferred marked superiority in drug delivery over competitive products (Grobler, 2004).

The Pheroid™ delivery system was investigated as a vehicle for the delivery of antigens in an effort to enhance the efficacy of existing vaccines. Indeed, a Pheroid™-based rabies vaccine showed a nine-fold increase in antibody response in comparison to the commercial vaccine. The South African State Vaccine Institute in collaboration with the Department of Immunology of the University of Cape Town executed an investigation of the efficiency of a peptide-based hepatitis vaccine. The use of Pheroid™ technology led to more than a ten-fold increase in the efficacy of the peptide based hepatitis B vaccine. The Pheroid™ system therefore has a dual role in vaccination. Firstly, as a delivery system for disease specific antigens and secondly as an immunostimulatory adjuvant (Grobler, 2004).

3.7.4 Peptide drug delivery

Peptide or protein drugs are poorly absorbed after oral administration (Johnson & Tracy, 1999). The possible enhancement of the absorption of various peptide drugs with Pheroid™ technology has been investigated.

The nasal and intestinal absorption of calcitonin in combination with Pheroid™ vesicles and micro-sponges led to an increase in absorption *in vivo* (du Plessis *et al.*, 2009). The *in vivo* effect of the nasal administration of insulin was determined using Sprague Dawley rats. Insulin formulated in either Pheroid™ vesicles or micro-sponges showed a decrease in glucose levels after nasal administration (de Bruyn, 2006).

In a transdermal preparation, Pheroids™ increased the transdermal flux of human growth hormone, and in an *in vivo* study on Sprague Dawley rats, an increase in plasma concentration after nasal administration was observed (Steyn, 2006). In a transdermal preparation, Pheroids™ increased the flux of vasopressin compared to the control sample (Coetzee, 2007). Pheroid™ technology thus shows great potential in delivering peptide drugs via various administration routes.

3.8 Conclusion

Ineffective drug regimes cannot curb increasing infection rates, high mortality rates or the high financial means associated with malaria. In such scenario, drug delivery systems have a crucial role to play in effective management of the current and emerging antimalarial agents. In view of this, colloidal carriers, especially the Pheroid™ drug delivery system have shown great potential

in improving efficacy and tolerability of a number of active pharmaceutical ingredients, including antimalarial drugs. Its application in the pharmaceutical industry is potentially limitless and includes areas such as malaria, tuberculosis, HIV and vaccination.

CHAPTER 4

IN VITRO EVALUATION OF ARTESUNATE AND MEFLOQUINE IN COMBINATION WITH PHEROID™ TECHNOLOGY

4.1 Introduction

In vitro studies are a vital component in the antimalarial drug discovery process. *In vitro* drug sensitivity assays are primarily used to determine parasite drug susceptibility, or the measure to which parasite growth is inhibited and is based on the cultivation of *P. falciparum in vitro* in human erythrocytes (Noedl *et al.*, 2003; Fidock *et al.*, 2004).

The principles of *in vitro* drug sensitivity assays were initially based on a sub-optimal, short-term culture method reported in 1912, applied for the WHO macro-test system in the 1960's and used until the 1980's. These *in vitro* principles underwent a major modification in 1976 when a new method for continuous culture of *P. falciparum* was reported by Trager and Jensen (1976). This technical improvement led to the elaboration of several *in vitro* drug sensitivity assays in the late 1970's and 1980's, namely the WHO micro-test, the 48-hour test and the radioisotope micro-test. However, two novel *in vitro* drug sensitivity assays that have been introduced over the past few years are an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies directed against either plasmodial lactate dehydrogenase (LDH) or histidine-rich protein 2 (HRP-2) and a fluorometric assay with deoxyribonucleic acid (DNA) -binding fluorescent dyes. These two assays are non-morphological and non-radioactive and are also based on Trager and Jensen's (1976) culture method (Basco, 2007).

This chapter describes the importance of *in vitro* studies as part of the antimalarial drug discovery process, as well as the major types of *in vitro* drug sensitivity assays for malaria and how *in vitro* methods were implemented in this study to determine if the efficacy of existing antimalarial drugs could be enhanced in combination with Pheroid™ technology.

4.2 Classification of *in vitro* antimalarial drug sensitivity assays

In vitro drug sensitivity assays are vital in research application regarding malaria for the following reasons (Basco, 2007):

- drug development;
- monitoring drug resistance;
- validating molecular markers of drug resistance;
- indirect measurement of plasma drug concentrations in bioassays.

The currently available antimalarial assays can be classified according to the methods used to quantify parasite growth in relation to drug concentrations, namely direct, visual counting of parasites using light microscopy, incorporation of radioisotope precursors into the parasite and non-radioactive methods. The last technique includes fluorescence-activated cell sorter (FACS) or flow cytometry assays, fluorometric assays, ELISA-based methods and non-ELISA-based colorimetric techniques. The above mentioned *in vitro* drug sensitivity assays involve direct exposure of human malaria parasites to antimalarial drugs in culture plates (Basco, 2007). Table 4.1 presents a summary of the different types of antimalarial *in vitro* drug sensitivity assays. The different types of assays do not necessarily yield directly comparable results, as the assays have different end-points and indirect indicators of parasite growth that reflect different aspects of parasite metabolism and maturation (Basco, 2007). For the purpose and scope of this study, only flow cytometry is discussed in detail in Section 4.6.3.

Table 4.1: Major types of *in vitro* drug sensitivity assays for malaria (Modified from Basco, 2007).

Morphological assays	Radioisotope assay	Non-radioactive assays
<ul style="list-style-type: none"> • Macro-test • Micro-test • 48-hour test 	<ul style="list-style-type: none"> • [³H]hypoxanthine 	<ul style="list-style-type: none"> • Colorimetry • Flow cytometry • Fluorometric assay • ELISA

4.3 Experimental design

The purpose of this study was to determine if the efficacy of the selected antimalarial drugs, artesunate and mefloquine could be enhanced in combination with Pheroid™ technology. The chloroquine sensitive 3D7 strain of *P. falciparum* was cultivated according to standard procedures as described in Section 4.4. *In vitro* drug sensitivity assays were carried out with both artesunate and mefloquine at various concentrations and under different experimental conditions (Table 4.2). Parasitic growth was quantified with a flow cytometric method and expressed as a percentage (% parasitemia). Each experiment was either performed in duplicate or triplicate. The drug concentration at which parasitemia (%) was inhibited by 50% (IC₅₀ value) was calculated with non-linear regression analysis. Table 4.2 presents a summary of each experiment performed and this will be elaborated on in the results and discussion section of each experiment.

Table 4.2: Summary of *in vitro* drug sensitivity assays performed.

Variable parameters	Experiment		
	1	2	3
Entrapment	Manufactured in oil phase	Manufactured in oil phase	Entrapped for 24 h after manufacturing
Incubation period	48 hours	72 hours	72 hours
DNA stain	YOYO®-1	To-Pro®-1	YOYO®-1

4.4 Cultivation of *P. falciparum*

The continuous cultivation of *P. falciparum* was first described by Trager and Jensen (1976) as the candle jar method (Schuster, 2002; Basco, 2007). *In vitro* growth of *Plasmodium* requires the imitating of the conditions during the erythrocytic cycle of the parasite in the human host (Schuster, 2002).

4.4.1 Materials

P. falciparum isolates were supplied by The University of Pretoria (South Africa). Roswell Park Memorial Institute (RPMI) -1640 powder, D-(+)-glucose powder, HEPES (N-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)), hypoxanthine, gentamycin solution, sodium

bicarbonate, sodium chloride (NaCl) and sorbitol were purchased from Sigma- Aldrich (Pty) Ltd. (St. Louis, MO, USA). Albumax® II serum replacement was obtained from Gibco (New Zealand). Sterile water for injection was purchased from Medirex (South Africa). Vacutainers used for blood collection was purchased from BD Biosciences® (South Africa). A special gas mixture containing 5% oxygen (O₂), 5% carbon dioxide (CO₂) and 90% nitrogen gas (N₂) was purchased from Afrox (Germiston, South Africa). Liquid nitrogen was obtained from Afrox (Potchefstroom, South Africa). Culture flasks and other consumables were purchased from Scientific Group (South Africa).

4.4.2 Cultivation method

P. falciparum was cultivated according to a modified method of Trager and Jensen (1976) and De Ridder (2007). All the processes described below were performed under sterile conditions using a 4ft class II laminar flow hood (Laboratory and Air Purification Systems, Midrand, South Africa).

A 3D7 chloroquine-sensitive strain of *P. falciparum* was used during this study. The strain was cryopreserved in liquid nitrogen. The parasites were thawed in a water bath (Sonorex Digital 10P Bandelin, Germany) at 37°C. The content was transferred to a 15 ml centrifuge tube. A 12% and 1.6% NaCl solution was added very slowly respectively and mixed thoroughly. Preheated culture medium (10 ml) and fresh erythrocytes (\pm 0.5 ml) were added and mixed well. The content was transferred to 75 cm³ culture flasks and gassed for a minimum of 30 seconds with a special gas mixture that consisted of 5% O₂, 5% CO₂ and 90% N₂. The culture flasks were sealed airtight and incubated at 37°C.

The complete culture medium consisted of the following reagents (per 100 ml):

- | | |
|-------------------------|-------------------------|
| • RPMI 1640 medium | 1.04 g |
| • D-(+)-glucose | 0.4 g |
| • HEPES | 0.6 g |
| • Hypoxanthine | 0.0044 g |
| • Gentamycin (40 mg/ml) | 0.12 ml |
| • Sodium bicarbonate | 4.2 ml of a 5% solution |
| • Albumax® II | 0.5 g |

The reagents were dissolved in sterile water for injection and filtered through a 0.22 µm filter under a vacuum. Wash medium, used to wash human whole blood for the removal of leukocytes, contains the same components without the serum replacement Albumax® II.

Fresh erythrocytes were obtained from O⁺ human whole blood, collected in vacutainers with anticoagulant. The whole blood was washed with the wash medium to remove leukocytes, as leukocytes are detrimental to parasite growth. The washing process consisted of repeatable steps of centrifuging (Centrifuge PLC Series, Gemmy Industrial Corp., Taiwan) of the blood (2000 revolutions per minute (r.p.m.) for eight minutes) with wash medium and the removal of the buffy coat or supernatant by aspiration. This process was repeated three times in an attempt to remove all the leukocytes. The erythrocytes were resuspended in wash medium and stored at 4°C. Ethical approval for this project was obtained from the Ethics Committee of the North-West University with approval number NWU – 0008 – 08 – S5 (Annexure A contains the approval report).

Routine cultivation of the isolates consisted of the replacement of culture medium at least three times a week. Fresh erythrocytes were added on a weekly basis to maintain a haematocrit and parasitemia of approximately 5%. The cultures were maintained at an atmosphere of 5% O₂, 5% CO₂ and 90% N₂ at 37°C.

For the purpose of this study, synchronised cultures were used. Cultures are synchronised to ensure that all the parasites in culture are in the same phase of morphology (Lambros and Vanderberg, 1979; De Ridder, 2007). Cultures are normally synchronised in the ring phase or early trophozoite phase (refer to Figure 1.3, Chapter 1). Synchronisation was achieved by transferring the culture from the culture flask to a 15 ml centrifuge tube for centrifugation at 2000 r.p.m. for eight minutes. The supernatant was removed via aspiration. Four milliliters of a sterile 15% (w/v) sorbitol solution was added very slowly to the packed infected erythrocytes and mixed well. The content was left to incubate for five minutes at 37°C. After the incubation time, 8 ml of a sterile 0.1% (w/v) glucose solution was added and incubated at 37°C for another five minutes. The content was centrifuged once again at 2000 r.p.m. for eight minutes. The supernatant was removed and the infected erythrocytes were resuspended in 10 ml preheated culture medium and cultivated as described above.

4.5 Preparation and characterisation of Pheroid™ vesicles

Novel drug delivery systems are a new approach for enhanced drug efficacy in the treatment of malaria. Pheroid™ technology, a proven drug delivery system is a sub-micron emulsion type formulation that consists primarily of modified essential fatty acids (Grobler, 2004).

4.5.1 Materials

Vitamin F ethyl ester was obtained from Kurt Richter Pharma (Germany), Cremophor® EL from BASF (Germany) and D- α -tocopherol from Chempure (South Africa). Medicinal nitrous oxide was purchased from Afrox (Klerksdorp, South Africa) and Nile Red® from Molecular Probes™ Inc. (Oregon, USA).

4.5.2 Basic method for preparation of Pheroid™ vesicles

The manufacturing of Pheroid™ vesicles were performed in the formulation facility at the North West University, Unit for Drug Research and Development (Potchefstroom, South Africa). The formulation facility is an organised laboratory specifically established for the manufacturing of Pheroids™ and is performed according to good manufacturing practices (GMP). The following formulation listed in Table 4.3 was used to prepare 100 ml of Pheroid™ vesicles.

Table 4.3: The basic Pheroid™ formulation (du Plessis *et al*, 2009).

Raw materials	Quantity (w/v)
Vitamin F ethyl ester	2.8 g
Cremophor® EL	1.0 g
D- α -tocopherol	0.2 g
N ₂ O.H ₂ O	96.0 g

The non-ionic surfactant Cremophor® EL and vitamin F ethyl ester were weighed (Schimaszu UW6200H, Japan) and heated on a hot plate to 55°C. When the mixture reached the desired temperature, the anti-oxidant D- α -tocopherol was added and mixed well. This mixture (4% w/v) represents the oil phase of the Pheroid™ formulation. The water phase (96%) consists of double distilled water that was gassed under pressure with nitrous oxide for four days. The nitrous oxide water (N₂O.H₂O) was weighed in an Erlenmeyer flask and heated to 70°C on a hot

plate. When the water reached the desired temperature, the oil phase was added to the water phase and homogenised with a Heidolph DiAx 600 homogeniser at 13 500 r.p.m. until the mixture cooled down to a temperature of $\pm 40^{\circ}\text{C}$. The emulsion was transferred to a 100 ml amber glass bottle to protect it from light and sealed airtight. The product was placed on a GFL shaker (GFL Gesellschaft für Labortechnik, Germany) until it reached room temperature (25°C) where after it was stored in a refrigerator at 6°C for 24 hours before *in vitro* efficacy studies for malaria were performed.

4.5.3 Characterisation of Pheroid™ structures

Particles in a colloidal suspension or emulsion are seldom the same size and often vary in shape (Uys, 2006). Particle size and particle size distribution analysis was performed by laser diffraction using a Malvern Mastersizer Micro (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Refer to Annexure B for the particle size analysis reports.

The micro-structure of the Pheroid™ vesicles was visualised under a confocal laser scanning microscope (CLSM) (Nikon PCM 2000 with digital camera DMX 1200, The Netherlands). This microscope has the ability to view fluorescent stained particles at varying depths. One hundred microliters of a Pheroid™ sample was stained with the fluorescent marker Nile Red® for 5-10 minutes. Twenty microliters of the stained Pheroid™ sample was placed on a microscope slide and covered with a cover-slip for visualisation on the CLSM. Refer to Annexure C (Figure C.1 and Figure C.2) for the CLSM- micrographs.

4.5.4 Entrapment of antimalarial drugs in Pheroid™ vesicles

There are two approaches for entrapment of compounds in Pheroid™ vesicles. Active ingredients can either be dissolved in the oil phase of the Pheroid™ formulation during the manufacturing process, or added separately to the final manufactured Pheroid™ product and stored in the refrigerator for 24 hours for entrapment to take place. Refer to Annexure C for the CLSM- micrographs that show the entrapment (green fluorescence) of artesunate (Figure C.3) and mefloquine (Figure C.4) in Pheroid™ vesicles (red fluorescence).

4.6 In vitro growth inhibition assays

Flow cytometry, based on the detection and count of *P. falciparum* in infected erythrocytes in the presence of a DNA-binding fluorescent dye was used as analytical method in assessing the efficacy of the test and reference formulations of this study.

4.6.1 Materials

Giemsa® stain, sodium phosphate and potassium phosphate were purchased from Sigma-Aldrich (Pty) Ltd. (St. Louis, MO, USA). Methanol was purchased from Merck Chemicals (Pty) Ltd. (South Africa). First grade frosted 1.2 mm microscope slides were purchased from Lasec (South Africa). The Pheroid™ formulations were manufactured by the North West University, Unit for Drug Research and Development (Potchefstroom, South Africa). Phosphate buffered saline (PBS) was purchased from Scientific Group (South Africa). Artesunate and mefloquine were purchased from Iffect Chemphar Co. Ltd. (Hongkong, China). Annexure D contains the certificates of analysis for these drugs.

4.6.2 The experimental method

P. falciparum was cultivated as described in Section 4.3 to maintain a parasitemia and haematocrit of approximately 5%. The initial parasitemia per culture flask was determined with light microscopy of three Giemsa® stained blood smears. Thin blood smears of the culture were made by placing a drop of parasite culture on a microscope slide. The sample was smeared across the length of the slide using another slide and was left to air dry. The dried smears were fixed with 96% methanol and stained with Giemsa® solution for ± five minutes. The Giemsa® solution consisted of 300 µl phosphate buffer and 300 µl Giemsa® stain per slide. The blood smear slides were rinsed under running water to remove unbound stain and left to air dry. The blood smears were viewed under a light microscope (Nikon Y5100, X 100 magnification). Ten fields of approximately 100 cells of infected versus uninfected erythrocytes were counted on each slide. The parasitemia for each slide was determined with the following formula:

$$\% \text{ Parasitemia} = \frac{\text{Number of infected erythrocytes}}{\text{Number of erythrocytes}} \times 100$$

The percentage parasitemia and haematocrit was adjusted to approximately 1% and 2% respectively for each experiment before incubation with the antimalarial drugs.

Stock solutions of artesunate and mefloquine were prepared by dissolving each compound in 200 µl 96% methanol and made up to 100 ml with PBS. Serial dilutions of the stock solutions were made by continuously diluting the stock solutions with the Pheroid™ formulation (1:250 dilution) for the test samples and control medium for the reference samples to obtain the desired concentration range of each compound. The control medium was either PBS, low hypoxanthine medium or N₂O.H₂O. The amount of methanol in the final concentration range is thus minimal and will have no toxic effects on the parasites. The concentration range was prepared in either duplicate or triplicate for each of the test and reference samples.

Incubation of the samples with *P. falciparum* was carried out in 96-well microtitre plates. A total volume of 200 µl per well was used that consisted of 20 µl of the serially diluted drug solution and 180 µl infected erythrocytes that was adjusted to the desired % parasitemia and haematocrit for each experiment. A typical 96-well plate layout after compilation of the aforementioned processes is illustrated in Figure 4.1.

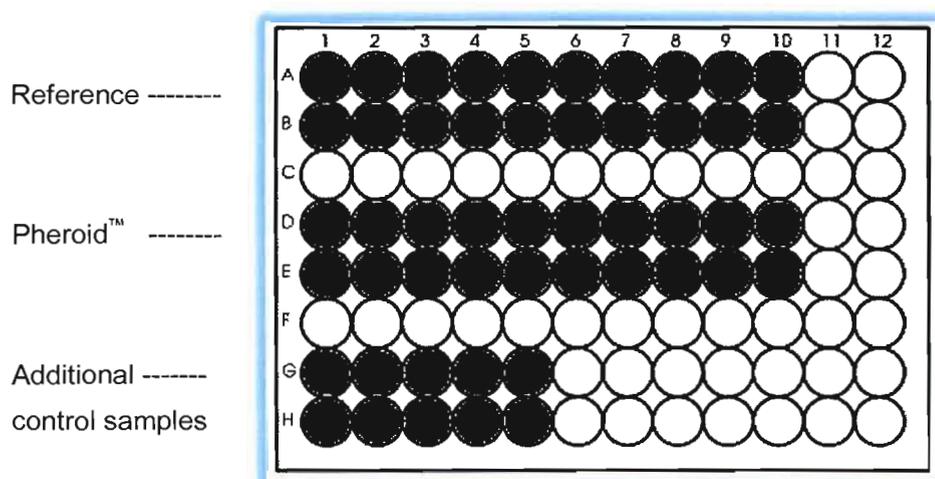


Figure 4.1: A 96-well plate layout ready for incubation.

The 96-well plate was placed in an incubation container and gassed with the aforementioned special gas mixture for ± five minutes. The container was incubated at 37°C in a CO₂ free incubator for 48 or 72 hours, depending on the experiment done and gassed every 24 hours.

4.6.3 Flow cytometry

Flow cytometry is a laser-based technology that measures multi-parametric physical characteristics of biological particles or cells. As the name implies, flow cytometry is the

measurement of cells in a flow system. Cells are prepared as single-cell suspensions for flow cytometric analysis, as this allows the cells to flow single file in a liquid stream past an excitation light source, namely a laser beam (Radcliff & Jaroszeski, 1998). The cells in a sample is usually fixed and tagged or labeled with a fluorescent probe for cell surface marker analysis, cell counts, measurement of DNA content and cell cycle analysis (Basco, 2007). Fluorescent probes are typically monoclonal antibodies that have been conjugated to fluorochromes. These probes can also be fluorescent stains or dyes that are not conjugated to antibodies (Radcliff & Jaroszeski, 1998).

The modern flow cytometer is a highly sophisticated instrument that combines modern techniques in fluidics, laser optics, electronics and computerised data processing (Riley & Idowu, 2008). The basic principle of flow cytometry is illustrated in Figure 4.2 and described in detail.

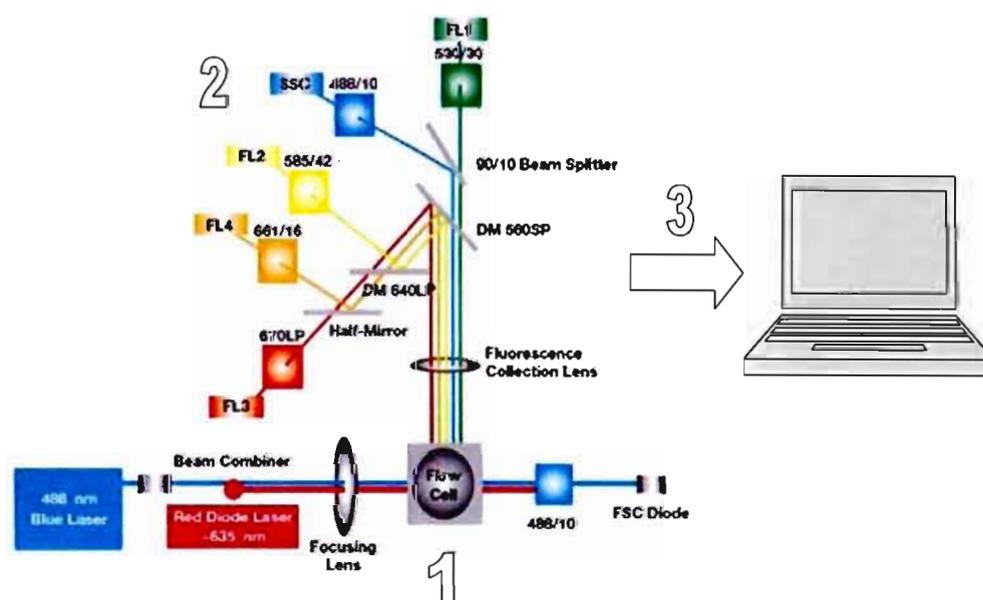


Figure 4.2: The basic principle of flow cytometry (Modified from CNBC: available at www.cnbc.pt/services/flow_cytometry/).

A fluidic system (1) transports particles or cells from a prepared suspension past a focused laser beam that is generated by an illumination system. Particle interrogation takes place, one cell at a time, in a flow chamber. The resulting scattered light and fluorescence is gathered by an optical and electronics system (2) that translates the light signals into information that is saved by the data storage and computer control system (3). After data from a sample has been

stored, retrospective graphical data analysis can be performed with the aid of software (Radcliff & Jaroszeski, 1998).

As the laser beam strikes the individually prepared cells, two physical events occur that yield information about the cells, namely light scattering and fluorescence. Light that is scattered in the forward direction (in the same direction as the laser beam) is analysed as one parameter and light scattered at 90° relative to the incident beam is collected as a second parameter. Forward-scattered (FSC) light is a result of diffracted light and this provides basic morphological information such as relative cell size. Larger particles scatter more light in the forward direction than smaller particles. Light scattered at 90° to the incident beam (side-scattered (SSC) light) is the result of refracted light and this parameter is an indicator of granularity or complexity within the cytoplasm of cells. Fluorescence results when fluorochrome-labeled cells are illuminated by the laser beam and emit light with a specific spectral composition. Fluorescence is detected using a network of mirrors, optics and beam splitters that direct the emitted fluorescent light within the range of wavelengths associated with each of the three fluorescent channels. Fluorescence generated from a green fluorochrome is typically detected in a band of wavelengths that is designated as the FL1 parameter. Refer to Figure 4.2 to see its position in the system. Similarly, fluorochromes emitting orange-red light are typically detected in another range of wavelengths that is designated as the FL2 parameter. Red fluorescence is detected in a third wavelength range designated as FL3 (Radcliff & Jaroszeski, 1998, Basco, 2007).

Several flow cytometric methods have been developed to measure *Plasmodium spp.* parasitemia *in vitro* and *in vivo* (Janse & Van Vianen, 1994). These methods take advantage of the lack of a nucleus in mammalian erythrocytes, in contrast to *Plasmodium* infected erythrocytes that contain nucleic acids from the different stages of *Plasmodium*. The former characteristic can be measured easily with flow cytometry, using DNA-specific fluorochromes or dyes (Jiménez-Díaz *et al.*, 2005). Amongst these, YOYO®-1 and To-Pro®-1 are nucleic acid stains used in this study.

4.6.3.1 Materials

YOYO®-1 and To-Pro®-1 were purchased from Molecular Probes™ Inc. (Oregon, USA), FACS reagents and consumables were purchased from Scientific Group (South Africa). Gluteraldehyde and Triton® X-100 were purchased from Sigma-Aldrich (Pty) Ltd. (St. Louis, MO, USA).

4.6.3.2 Method of analysis

All flow cytometric analyses were carried out with a FACSCalibur™ (BD Biosciences). Cell counts of 10 000 in each sample were analysed. Staining of parasitised erythrocytes was performed by using two DNA-stains namely YOYO®-1 and To-Pro®-1. A modification of the original method for YOYO-1-staining described by Barkan *et al* (2000) was performed.

During the first and third experiment, the content of the 96-well plate was transferred to individual 1 ml Eppendorf® tubes and centrifuged at 2000 r.p.m. for five minutes. After aspiration of the supernatant, 500 µl of PBS containing 0.25% glutaraldehyde was added and incubated at room temperature for 15 minutes. Glutaraldehyde is a fixation agent. After the incubation period, the samples were once again centrifuged at 2000 r.p.m. for five minutes and the supernatant removed. The samples were resuspended in 500 µl PBS with a final concentration of 0.01% Triton®-X 100 and incubated at room temperature for ten minutes. Triton®-X 100 is a membrane permeating agent. The samples were centrifuged again at 2000 r.p.m. with the supernatant removed and finally resuspended in 500 µl PBS. A 10 µM stock solution (10 ml) YOYO®-1 was prepared of which 25 µl was added to each sample to yield a final YOYO®-1 concentration of 0.5 µM in each sample. The samples were vortexed (Stuart Scientific Autovortex SA6, UK) and incubated in the dark at room temperature for one hour. After the incubation period, each sample was vortexed again and read onto the FACSCalibur™.

During the second experiment, 100 µl of each sample from the 96-well plate was transferred to FACS tubes, to which 400 µl PBS was added. To-Pro®-1 (4 µl) was added to each sample to yield a final To-Pro®-1 concentration of 1 µM in each sample. The samples were vortexed and incubated in the dark at room temperature for one hour and vortexed again before reading onto the FACSCalibur™. Data was collected on a MAC-OC computer and analysed with FCS Express™ version 3, (DeNovo Software, L.A, CA). The data obtained was collected in a two parameter correlated dot plot or cytogram. In a dot plot, each cell recorded is shown as a single dot. This is the form in which data are shown during data acquisition. The parasitemia (%) was determined on a SSC/FL1 dot plot as shown in Figure 4.3.

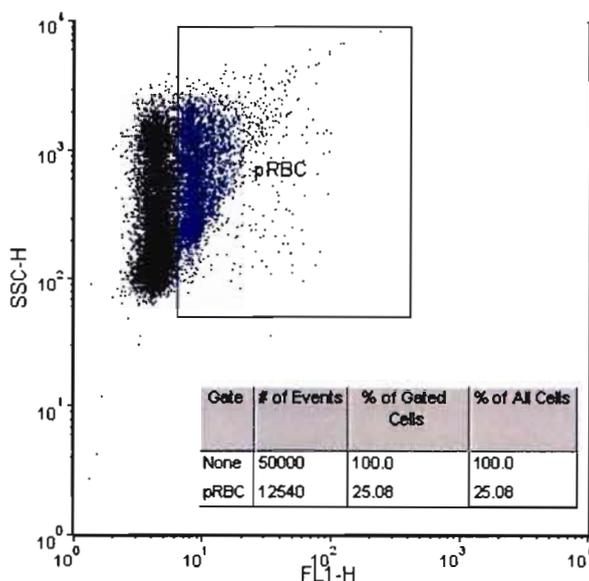


Figure 4.3: SSC/FL1 dot plot of infected and uninfected erythrocytes. The infected erythrocytes (pRBC) are gated.

The SSC/FL1 dot plot is a presentation of two properties of the cells measured. As explained in Section 4.6.3, cell complexity or granularity increases along the Y-axis (SSC) and fluorescence increases to the right on the X-axis (FL1). In *P. falciparum* infected erythrocytes, cell complexity and size increase and the presence of nuclei cause increase in fluorescence. The cells in the gated area of Figure 4.3 are the infected erythrocytes and thus an indication of the percentage parasitemia.

4.6.4 Statistical evaluation

The preliminary data was converted to percent inhibition with respect to drug free control samples. This was accomplished by calculating the mean of the replicate percentage parasitemia values obtained from drug free control samples, which was set as a nominal value representing a percentage parasitemia of 100%. The mean of all the other replicate parasitemia values were then set against the representative 100% value and a corresponding percentage value was calculated for the mean of each replicate set. The data was processed using Microsoft Excel™ 2007 and Graphpad Prism™ version 5 (GraphPad Software Inc., San Diego, CA, USA). The data are presented as mean ± standard error of mean (SEM). D'Agostino & Pearson omnibus normality test confirmed that the data obtained represented normal (Gaussian) distribution. Statistical significance of the difference between the reference and the Pheroid™ vesicle formulation was determined with a two-way repeated measures

analysis of variance (ANOVA) with Bonferroni post-tests. The Bonferroni post-tests narrow down exactly where the differences are statistically significant, namely the difference between the mean responses of the reference group versus the Pheroid™ vesicle group at a particular drug concentration. Differences were considered extremely significant (***) when the probability (p) value was less than 0.0001, very significant (**) when $p < 0.001$, significant (*) when $p < 0.05$ and not significant (ns) when $p > 0.05$.

The IC_{50} values were calculated with non-linear regression analysis using Graphpad Prism™ version 5 (GraphPad Software Inc., San Diego, CA, USA). Drug concentrations were transformed to log values and plotted against the normalised response, namely the decrease in parasitemia (%). The plot was fitted with a sigmoidal curve with the following equation:

$$Y = 100/[1 + 10^{(X - LogIC_{50})}]$$

This model assumes that the dose response curve has a standard slope, equal to a Hill slope (or slope factor) of -1.0. Statistical significant differences between IC_{50} values of the reference and Pheroid™ vesicles were calculated using the extra sum of squares F test and differences were considered significant (*) when $p < 0.05$.

4.7 Results and Discussion

The aim of this study was to test the *in vitro* efficacy of artesunate and mefloquine, alone and in combination with Pheroid™ vesicles against a 3D7 chloroquine-sensitive strain of *P. falciparum* via flow cytometry, using a standard 48 hour *in vitro* test. It was also important to evaluate the effect of two different formulation methods with Pheroid™ vesicles, namely co-formulation of artesunate and mefloquine in the oil phase of Pheroid™ vesicles during manufacturing, as well as entrapment of the drugs in Pheroid™ vesicles for 24 hours after manufacturing, utilising *in vitro* drug sensitivity assays. A third aim was to evaluate whether the drugs co-formulated with Pheroid™ vesicles or entrapped within the Pheroid™ vesicles for 24 hours have an influence on the stage specificity of the malaria parasite by using the *in vitro* efficacy assay with an extended incubation period of 72 hours. In this study, three different *in vitro* drug sensitivity experiments were performed with artesunate and mefloquine according to varied experimental conditions as summarised in Table 4.2. In the following sections, each of the experiments will be described in detail in terms of methods used and results obtained.

4.7.1 Artesunate Experiment 1

The drug efficacy of artesunate was tested against a 3D7 chloroquine-sensitive strain of *P. falciparum*. Artesunate was manufactured in the oil phase of the Pheroid™ vesicles. The control medium used was PBS and the incubation period was 48 hours. The DNA-binding fluorescent dye used for quantification of the parasites was YOYO®-1. The experiment was performed in triplicate and the results are presented as mean \pm SEM (n = 3). The parasitemia expressed as a % relative to drug free controls are shown in Table E.1 (Annexure E) and graphically depicted in Figure 4.4.

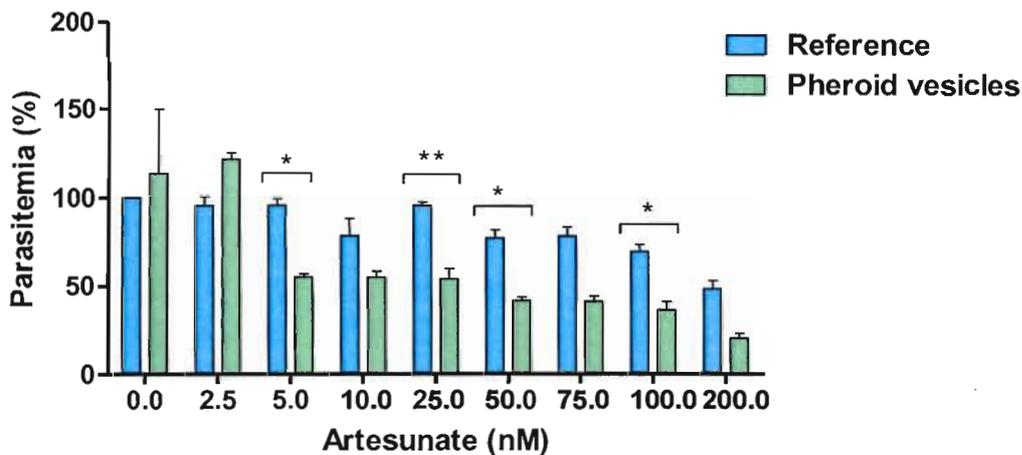


Figure 4.4: *In vitro* activity of artesunate against a 3D7 chloroquine-sensitive strain, Experiment 1 (** indicates $p < 0.001$ at 25.0 nM and * $p < 0.05$ at 5.0, 50.0 and 100.0 nM).

Parasite growth inhibition of the 3D7 chloroquine-sensitive strain was consistent according to ascending drug concentration. The Pheroid™ vesicle formulation had a better inhibitory effect than the reference group from 5.0 nM to 200.0 nM. Statistical significant differences were calculated at 5.0, 25.0, 50.0 and 100.0 nM ($p < 0.05$). According to the data obtained, the Pheroid™ vesicle formulation had a better inhibitory effect than the reference group, as there was a greater decrease in percentage parasitemia (from 0nM to 200nM) from 113.78% to 20.76% (93.02%) than 100.00% to 48.54% (51.46%). Figure 4.5 illustrates the IC_{50} values of the reference and Pheroid™ vesicles groups, calculated with non-linear regression analysis. The IC_{50} value decreased from 81.54 ± 0.14 nM for the reference to 8.10 ± 0.10 nM for the Pheroid™ vesicles. This decrease of ~90 % was statistically significant ($p < 0.05$).

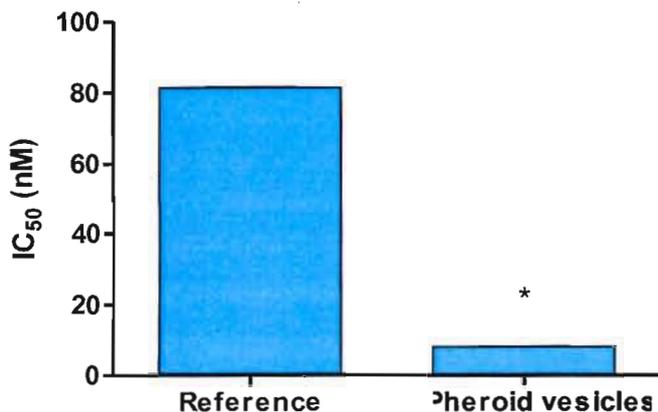


Figure 4.5: The IC₅₀ values of the reference and Pheroid™ vesicles presented as the mean ± SEM (n=3) of artesunate, Experiment 1 (* indicates a statistical significant difference at $p < 0.05$).

The IC₅₀ is defined as the concentration of inhibitor (in this case the drug) that provokes a response halfway between the baseline (bottom) and maximum (top) response. The fact that the data was normalised, the IC₅₀ may not be the same as the concentration that provokes a response of $Y = 50$. For instance, the data may be normalised to percentage of maximum response, without subtracting a baseline. The baseline of the reference is about 48% and the maximum is about 100%, so the IC₅₀ is the concentration of artesunate that evokes a response of about 78% (at about 80 nM, halfway between 40% and 100%). The concentration that provokes a response of $Y = 50$ in this experiment is thus not the IC₅₀ value (Figure 4.4).

4.7.2 Artesunate Experiment 2

Artesunate was dissolved in the oil phase of the Pheroid™ vesicles during the manufacturing process and tested against a 3D7 chloroquine-sensitive strain in triplicate. The control medium consisted of a low hypoxanthine medium. The incubation period of 48 hours was extended to 72 hours. The reason for this intervention can be explained as follows: *P. falciparum* completes a morphological cycle every 48 hours as mentioned in Section 1.3.2, Chapter 1. Synchronised ring phase cultures were used during this study as discussed in Section 4.4.2. Thus, when ring phase parasites are incubated for 48 hours for drug sensitivity testing, the parasites should be in the ring phase again after the 48 hour incubation period. When extending the incubation period to 72 hours, the parasites should be in the schizont phase. The incubation period for *in vitro* drug sensitivity assays varies from 24 hours to 96 hours, but the standard assay is based on 48 hours in which *P. falciparum* completes the erythrocytic cycle. A prolonged incubation

time of 72 hours have the advantage of determining whether Pheroid™ vesicles have delayed inhibitory effects on parasites after the first completed cycle.

The quantification of *P. falciparum* with flow cytometry relies on staining of the DNA content of the parasite in erythrocytes as explained in Section 4.6.3. Much more parasite DNA is however present in the schizont phase of *P. falciparum* than in the ring phase. Refer to Figure 1.3 in Chapter 1 to see the morphological difference between these two phases. Thus, extending the incubation period to 72 hours might enhance the sensitivity of the flow cytometric analysis of *P. falciparum* infected erythrocytes. The DNA stain YOYO®-1 was replaced by To-Pro®-1. YOYO®-1 is classified as a cell impermeable DNA stain and To-Pro®-1 is classified as semi cell permeable to cell impermeable (Haugland, 2002). The results are presented as mean ± SEM (n = 3) in Table E.2 (Annexure E) and graphically depicted in Figure 4.6.

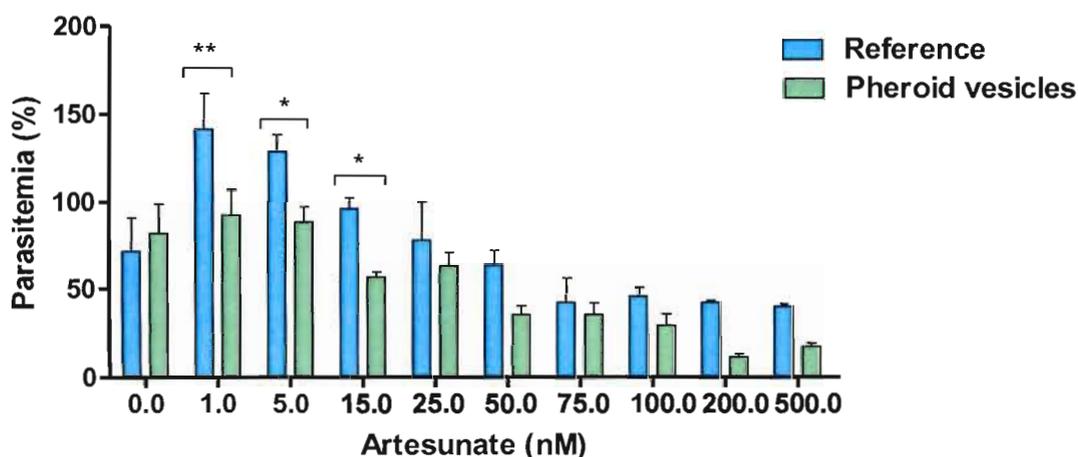


Figure 4.6: *In vitro* activity of artesunate against a 3D7 chloroquine-sensitive strain, Experiment 2 (** indicates $p < 0.001$ at 1.0 nM and * $p < 0.05$ at 5.0 and 15.0 nM).

The column graph shows once again an overall tendency of parasite growth inhibition according to ascending drug concentration from 1.0 nM onward. The Pheroid™ vesicle formulation seems to have had an enhanced efficacy in parasite decrease in relation to the reference group, with statistical significant differences at 1.0, 5.0 and 15.0 nM. According to the data obtained, the Pheroid™ vesicle formulation had a better inhibitory effect than the reference group, as there was a greater decrease in percentage parasitemia (from 0nM to 200nM) from 82.15% to 17.45% (64.70%) than 71.78% to 40.15% (31.63%). It should be stated that changing the fluorescent dye to To-Pro®-1 did not markedly improve the sensitivity of the assay (results not shown). Changing the control medium to culture medium with a low hypoxanthine concentration also had little influence on the results. Figure 4.7 shows the IC₅₀ of the reference

and Pheroid™ vesicle groups evaluated over the extended incubation period of 72 hours. The IC₅₀ value for Experiment 2 decreased from 111.30 ± 0.14 nM for the reference to 30.90 ± 0.07 nM for Pheroid™ vesicles. This ~72% decrease was statistically significant (p = 0.028).

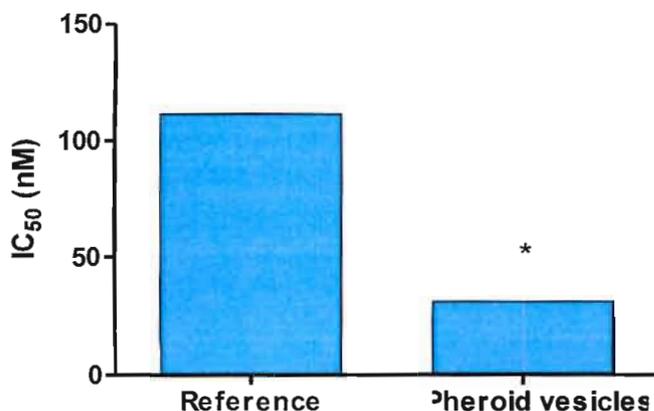


Figure 4.7: The IC₅₀ values of the reference and Pheroid™ vesicles presented as the mean ± SEM (n=3) of artesunate, Experiment 2 (* indicates a statistical significant difference at p < 0.05).

The IC₅₀ concentration of this experiment was somewhat higher (111.30 ± 0.14 nM and 30.90 ± 0.07 nM) after 72 hours incubation when compared to the reference and Pheroid™ vesicles (81.54 ± 0.14 nM and 8.10 ± 0.10 nM) of Experiment 1 (48 hours incubation). This is consistent with other studies that indicated that differences in parasite phases of development can lead to up to two-fold shifts in the IC₅₀ values 24 hours, 48 hours and 72 hours post-infection (Fidock *et al.*, 2004). The decrease in IC₅₀ between the reference and Pheroid™ vesicles after the 72 hour incubation period was less pronounced (~72 %) compared to the decrease in IC₅₀ after the 48 hour incubation period (~90%), although this was not statistically significant. It can therefore be concluded that artesunate co-formulated with Pheroid™ vesicles did not have any significant delayed inhibitory effects.

4.7.3 Artesunate Experiment 3

Artesunate was not manufactured in the oil phase of the Pheroid™ vesicles, but added afterwards and left for 24 hours for entrapment to take place. The control medium used was PBS and the incubation period was 72 hours. The DNA-binding fluorescent dye used for quantification of the parasites was YOYO®-1, as the question arose whether To-Pro®-1 does

permeate cells effectively. The experiment was performed in duplicate and the results are presented as mean ± SEM (n = 2). The parasitemia expressed as a % relative to drug free controls are shown in Table E.3 (Annexure E) and graphically depicted in Figure 4.8.

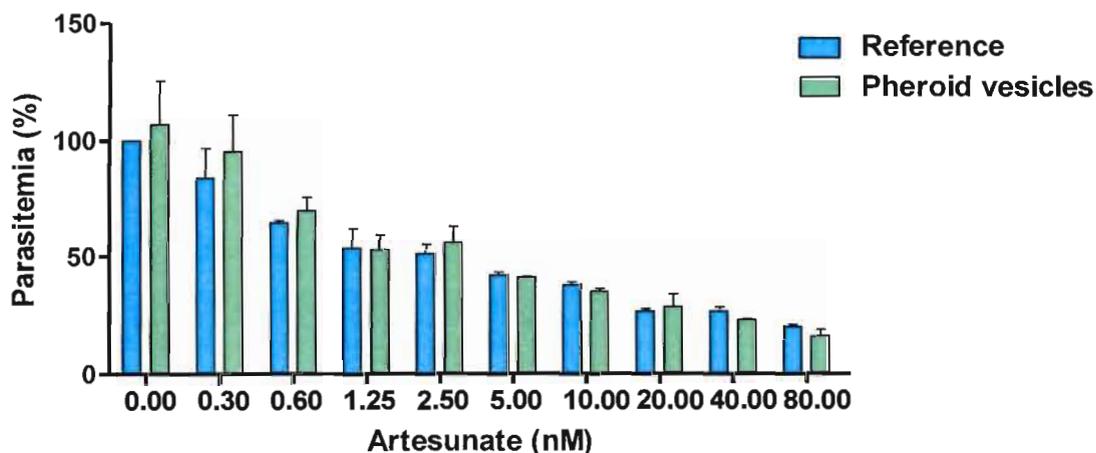


Figure 4.8: *In vitro* activity of artesunate against a 3D7 chloroquine-sensitive strain, Experiment 3.

Figure 4.8 indicates a consistent decrease in percentage parasitemia according to ascending drug concentrations. There were only slight differences between the reference and Pheroid™ vesicles, with the Pheroid™ vesicles resulting mostly in higher parasitemia levels. No statistically significant differences were obtained between the two groups. Figure 4.9 shows the IC₅₀ values of the experiment.

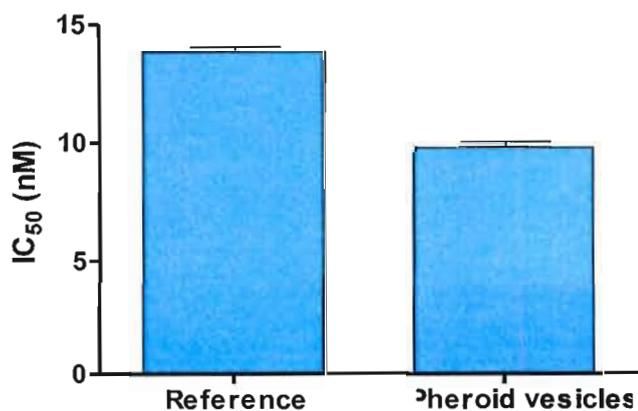


Figure 4.9: The IC₅₀ values of the reference and Pheroid™ vesicles presented as the mean ± SEM (n=2) of artesunate, Experiment 3.

There was a slight difference between the IC₅₀ concentrations of the reference (13.86 ± 0.2 nM) and Pheroid vesicles (9.79 ± 0.23 nM). This difference (29%) was not statistically significant (p = 0.24). The IC₅₀ concentration of this experiment was also very low compared to Experiment 1 and 2. The results indicated that entrapment of artesunate in Pheroid™ vesicles after manufacturing for a period of 24 hours did not increase the antimalarial efficacy of artesunate. Further research is needed to determine whether increasing the entrapment period might have an influence.

There were large variations in the IC₅₀ concentrations obtained between the different experiments. It is important to note that there is no universally accepted, standardised protocol for malaria *in vitro* drug sensitivity assays. Different experimental conditions can profoundly influence the level of drug response (Basco, 2007). This was also seen in this study. It is therefore recommended to compare results to experimental specific conditions. However, in this study more emphasis was placed on the difference between the efficacy of the reference and Pheroid™ vesicles than between different experimental conditions.

4.7.4 Mefloquine Experiment 1

The drug efficacy of mefloquine was tested against a 3D7 chloroquine-sensitive strain of *P. falciparum*. Mefloquine was manufactured in the oil phase of the Pheroid™ vesicles. The control medium used was PBS and the incubation period was 48 hours. The DNA-binding fluorescent dye used for quantification of the parasites was YOYO®-1. The experiment was performed in triplicate and the results are presented as mean ± SEM (n = 3). The parasitemia expressed as a % relative to drug free controls are shown in Table E.4 (Annexure E) and graphically depicted in Figure 4.10.

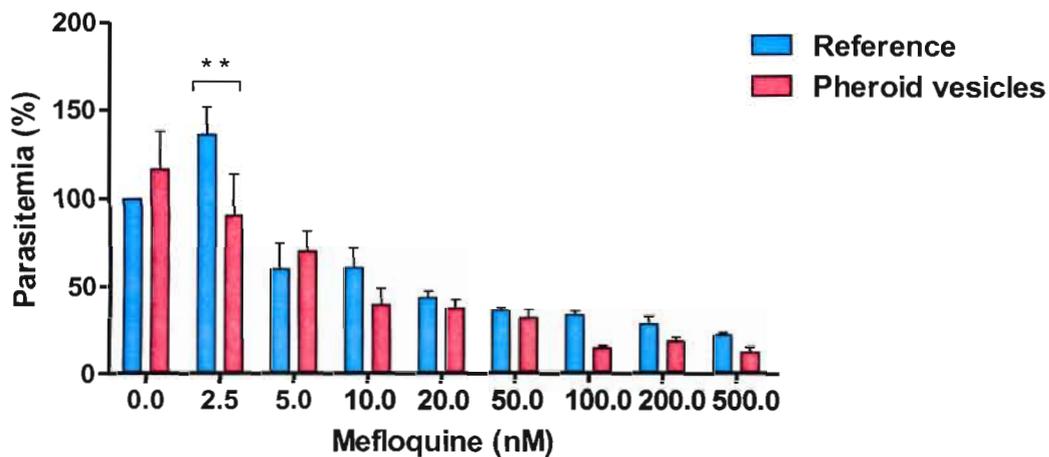


Figure 4.10: *In vitro* activity of mefloquine against a 3D7 chloroquine-sensitive strain, Experiment 1 (** indicates $p < 0.001$ at 2.5 nM).

Parasite growth inhibition decreased relatively from 5.0 nM onward. The Pheroid™ vesicle formulation indicated better parasite growth inhibition than the reference group at 2.5 nM and again from 10.0 nM onward. A statistical significant difference was calculated at 2.5 nM. According to the data obtained, the Pheroid™ vesicle formulation had a better inhibitory effect than the reference group, as there was a greater decrease in percentage parasitemia from 116.50% to 12.29% (104.21%) than 100.00% to 22.33% (77.67%). Figure 4.11 shows the IC_{50} values of the reference and Pheroid™ vesicles in the standard 48 hour *in vitro* efficacy assay.

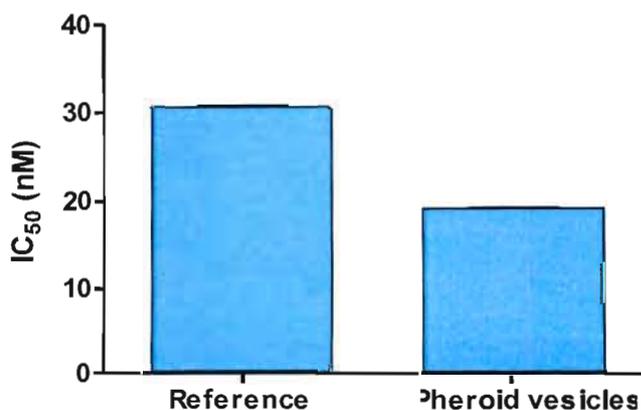


Figure 4.11: The IC_{50} values of the reference and Pheroid™ vesicles presented as the mean \pm SEM (n=3) of mefloquine, Experiment 1.

The IC₅₀ concentration decreased from 30.60 ± 0.16 nM for the reference to 19.05 ± 0.13 nM for the Pheroid™ vesicles. This ~36% decrease was not statistically significant (p = 0.45).

4.7.5 Mefloquine Experiment 2

Mefloquine was dissolved in the oil phase of the Pheroid™ vesicles during the manufacturing process and tested against a 3D7 sensitive strain in triplicate. The control medium consisted of a low hypoxanthine medium. The incubation period was extended to 72 hours as explained in Section 4.7.2 and the DNA-binding fluorescent dye YOYO®-1 was replaced by To-Pro®-1 as explained in Section 4.7.2. The results are presented as mean ± SEM (n = 3) in Table E.5 (Annexure E) and graphically depicted in Figure 4.12.

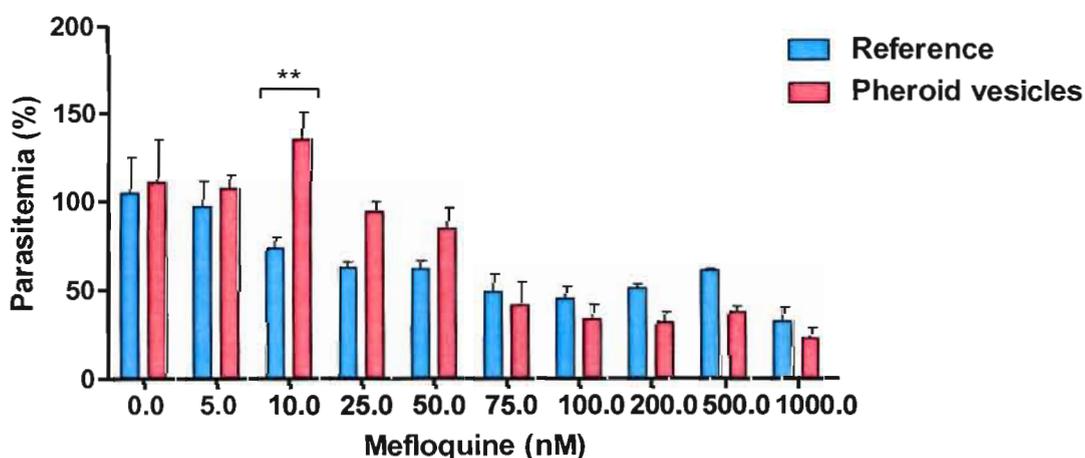


Figure 4.12: *In vitro* activity of mefloquine against a 3D7 chloroquine-sensitive strain, Experiment 2 (** indicates p < 0.01 at 10.0 nM).

The column graph shows once again an overall tendency of parasite growth inhibition according to ascending drug concentration, especially for the reference group. The Pheroid™ vesicle formulation had a better inhibitory effect only from 75.0 nM onward in relation to the reference group. A statistical significant difference was obtained at 10.0 nM where the reference group had a better inhibitory effect than the Pheroid™ vesicle group. According to the data obtained, the Pheroid™ vesicle formulation had a better inhibitory effect than the reference group, as there was a more pronounced decrease in percentage parasitemia from 110.74% to 23.14% (87.57%) than 104.62% to 32.40% (72.22%). Again it is important to note that changing the type of DNA-binding fluorescent dye and its concentration, as well as changing the control

medium did not seem to affect the results. The following IC₅₀ values (Figure 4.13) were calculated:

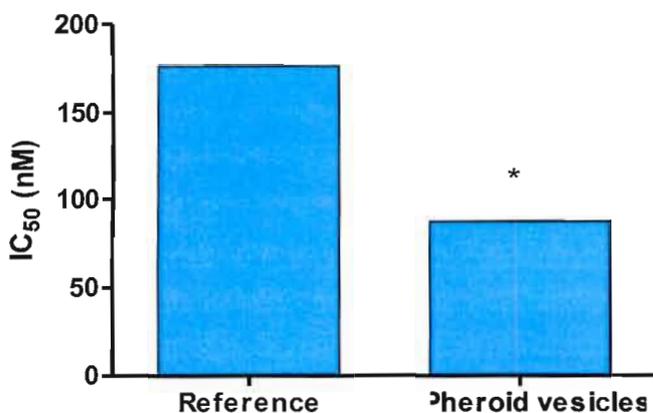


Figure 4.13: The IC₅₀ values of the reference and Pheroid™ vesicles presented as the mean ± SEM (n=3) of mefloquine, Experiment 2 (* indicates a statistical significant difference at p < 0.05).

The IC₅₀ concentration decreased from 176.40 ± 0.22 nM for the reference to 87.13 ± 0.13 nM for Pheroid™ vesicles. This ~51% decrease was statistically significant (p = 0.0027). The IC₅₀ concentration obtained from this experiment was much higher than the 48 hour experiment. This was a similar trend to that observed with artesunate. However, the IC₅₀ concentration decreased by a larger percentage (51%) compared to Experiment 1 (36%). These results indicated that the extended incubation period markedly improved the efficacy of mefloquine in the reference as well as Pheroid™ vesicles.

4.7.6 Mefloquine Experiment 3

Mefloquine was not manufactured in the oil phase of the Pheroid™ vesicles, but added afterwards and left for 24 hours for entrapment to take place. The control medium used was PBS and the incubation period was 72 hours. The DNA-binding fluorescent dye used for quantification of the parasites was YOYO®-1, as the question arose whether To-Pro®-1 does permeate cells effectively. The experiment was performed in duplicate and the results are presented as mean ± SEM. The parasitemia expressed as a % relative to drug free controls are shown in Table E.6 (Annexure E) and graphically depicted in Figure 4.14.

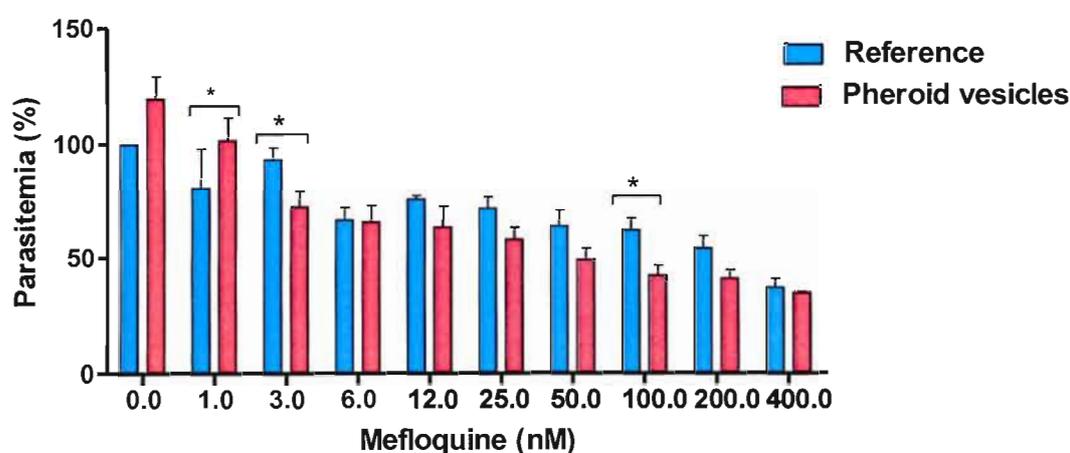


Figure 4.14: *In vitro* activity of mefloquine against a 3D7 chloroquine-sensitive strain, Experiment 3.

Figure 4.14 indicates a consistent decrease in parasite levels according to ascending drug concentration. The Pheroid™ vesicle formulation had a better inhibitory effect than the reference group from 3.0 nM onward. Statistical significant differences were calculated at 1.0, 3.0 and 100.0 nM ($p < 0.05$). According to the data obtained, the Pheroid™ vesicle formulation had a better inhibitory effect than the reference group, as there was a greater decrease in % parasitemia (from 0nM to 200nM) from 119.28% to 34.83% (84.45%) than 100.00% to 37.07% (62.93%). Figure 4.15 shows the IC_{50} values of the experiment.

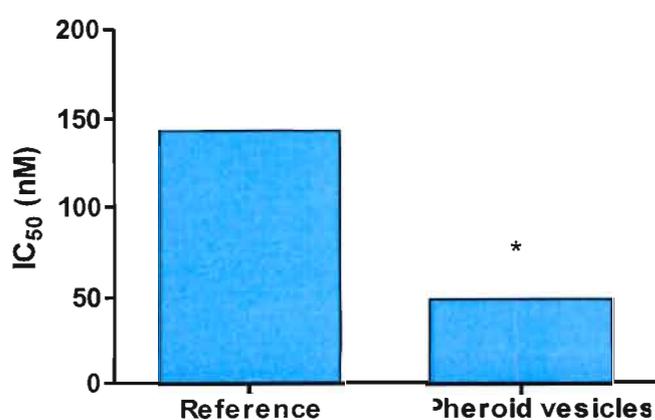


Figure 4.15: The IC_{50} values of the reference and Pheroid™ vesicles presented as the mean \pm SEM ($n=3$) of mefloquine, Experiment 3 (* indicates a statistical significant difference at $p < 0.05$).

The IC₅₀ concentration decreased from 141.3 ± 0.11 nM for the reference to 48.67 ± 0.12 nM for Pheroid™ vesicles. This difference (66%) was statistically significant (p = 0.03). These results indicated that entrapment of mefloquine in Pheroid™ vesicles for 24 hours increased the efficacy of mefloquine significantly. In contrast to the results obtained with artesunate, the type of drug formulation together with the Pheroid™ does not seem to influence the efficacy of mefloquine on 3D7 parasites.

4.8 Conclusion

Novel drug delivery systems that enhance the efficacy of existing antimalarial drugs are of potential clinical value. The Pheroid™ platform has shown some diligence in efficacy enhancement of existing antimalarial drugs. In a study conducted by Langley (2007), the efficacy of existing antimalarial drugs in combination with Pheroids™ was investigated *in vitro* against a RB-1 chloroquine resistant strain of *P. falciparum* by means of light microscopy. The efficacy of artesunate and mefloquine was enhanced by 218.0% and 195.0% in a Pheroid™ vesicle formulation respectively. Slabbert (2008) evaluated the efficacy of mefloquine *in vitro* against a RSA11 chloroquine-resistant strain of *P. falciparum* in combination with different Pheroid™ microsponge formulations with flow cytometry. The results revealed that the most effective Pheroid™ formulation was when mefloquine was incorporated into the Pheroid™ during the manufacturing process. The results revealed a 46.0% reduction in parasite growth at the highest drug concentration.

In continuation of previous research done regarding antimalarial drugs in combination with Pheroid™ technology, this study aimed to expand on the knowledge obtained with the use of a highly sensitive flow cytometric method, evaluating a chloroquine sensitive strain of *P. falciparum*.

The first experiment aimed to identify whether artesunate and mefloquine, co-formulated with Pheroid™ vesicles had enhanced efficacy when compared to a reference, evaluated according to the standard 48 hour *in vitro* assay. In the second experiment the same formulations were used, but the incubation period was extended to 72 hours. During the third experiment, artesunate and mefloquine were entrapped for 24 hours in Pheroid™ vesicles and also evaluated over a period of 72 hours.

The calculated IC_{50} concentrations indicated that artesunate co-formulated with Pheroid™ vesicles and evaluated after 48 hours seems to be most efficacious. This result was expected as artesunate is rapid-acting with a short half-life. Artesunate is also a lipophilic drug and the drug dissolved and thus solubilised in the oil phase of the Pheroid™ vesicles during manufacturing may have enhanced entrapment of the drug. The IC_{50} concentrations of mefloquine co-formulated with Pheroid™ vesicles, evaluated over a period of 48 hours was the least effective. The extended incubation period of 72 hours seemed to have a marked influence on the activity of mefloquine in both the reference and Pheroid™ vesicles. This result is also expected as mefloquine has a long half-life and is long-acting. In contrast to the results obtained with artesunate, there seems to be little difference between co-formulating mefloquine during manufacturing or entrapment of the drug after manufacturing of Pheroid™ vesicles. This result is somewhat unexpected and should be investigated further.

CHAPTER 5

BIOAVAILABILITY EVALUATION OF ARTESUNATE AND MEFLOQUINE IN COMBINATION WITH PHEROID™ TECHNOLOGY

5.1 Introduction

Considering the small number of new and innovative antimalarial medicine approved since 1990, the search for more efficient and less toxic antimalarials, the development of a successful vaccine and the design of nanotechnology-based delivery systems applied to antimalarial drugs are likely to be the main strategies in combating this disease (Santos-Magalhães & Mosqueira, 2009). Nanocarriers have been proposed for malaria diagnosis, treatment and vaccine formulation (Sharma *et al.*, 2008; Date *et al.*, 2007; Peek *et al.*, 2008). Nanocarriers are useful tools to improve the pharmacokinetic profile of effective antimalarial drugs that, due to poor water solubility, low bioavailability and high toxicity have been limited in application in chemotherapy (Forrest & Kwon, 2008). For the purpose of this study, the nanotechnology identified to optimise the delivery and thus efficacy of selected antimalarial drugs is the Pheroid™ drug delivery system. This pliable, but stable lipid-based submicron emulsion type drug delivery system can be formulated with various active pharmaceutical ingredients for novel and effective dosage forms (Grobler *et al.*, 2006).

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites in guinea pigs are critical for the successful conduct of preclinical and/or biopharmaceutical and clinical pharmacological studies (FDA, 2001). Artesunate and mefloquine were evaluated for their pharmacokinetic properties in a mouse model. The drugs were tested in both a reference and a Pheroid™ vesicle formulation. A sensitive and selective liquid chromatography- tandem mass spectrometric (LC-MS/MS) method was developed and partially validated for the analysis.

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This chapter begins with a description of bioavailability in general. The development and validation of a LC-MS/MS assay for application to the pharmacokinetic study of artesunate, its active metabolite dihydroartemisinin (DHA), as well as for mefloquine are outlined.

5.2 Methods for assessing bioavailability

Bioassays are used to test the absorption efficiency of a compound in an animal host as well as the effects of a compound's metabolites. In bioassays, a drug is administered to an animal host at various doses by the oral or parenteral route. After a drug has been absorbed, metabolised and distributed in the blood circulation, venous blood samples are obtained at various time intervals (Basco, 2007). Estimates of an effective clinical dosage are based on a combination of estimates of oral absorption, bioavailability, clearance and volume of distribution (Pelkonen, 2001).

Bioavailability indicates a measurement of the rate and amount of therapeutically active drug that reaches the systemic circulation and which is available at the site of action. Bioavailability studies are used to define the effect of changes in the physiochemical properties of the drug substance and the effect of the drug product on the pharmacokinetics of the drug. Bioavailability can thus be considered as a performance measure of a drug product *in vivo* (Shargel *et al.*, 2005).

Measurement of drug concentration in blood, plasma or serum after drug administration is the most direct and objective way to determine systemic drug bioavailability. Appropriate blood sampling can give an accurate description of the plasma drug concentration-time profile of the therapeutically active compound, as illustrated in Figure 5.1.

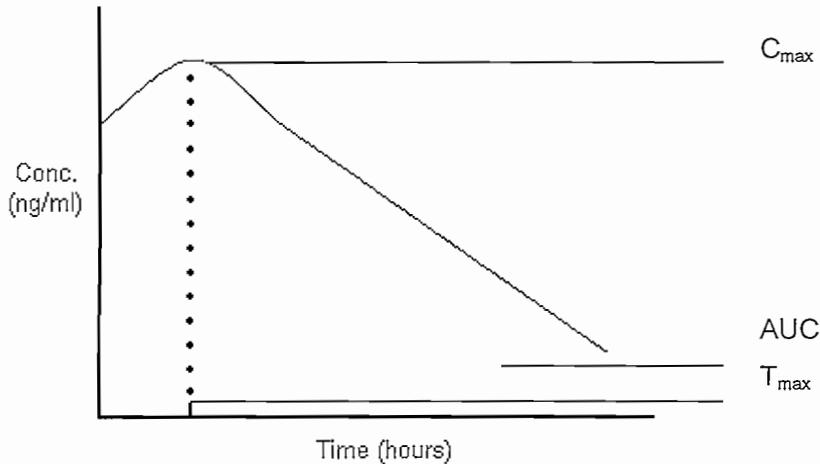


Figure 5.1: A typical drug concentration-time profile.

The following pharmacokinetic parameters, as shown in Figure 5.1, are explained:

- Time of peak plasma concentration (T_{max}) corresponds to the time required to reach maximum drug concentration after drug administration. At T_{max} peak drug absorption occurs and the rate of drug absorption equals the rate of drug elimination. Drug absorption still continues after T_{max} is reached, but at a slower rate.
- Peak plasma drug concentration (C_{max}) represents the maximum plasma drug concentration obtained after oral administration of a drug. C_{max} provides an indication that the drug is sufficiently systemically absorbed to provide a therapeutic response.
- Area under the drug plasma level-time curve (AUC) is a measurement of the extent of drug bioavailability. The AUC reflects the total amount of active drug that reaches the systemic circulation. The AUC is from $t = 0$ to $t = \infty$ and is equal to the amount of unchanged drug reaching the general circulation divided by the clearance:

$$[AUC]_0^{\infty} = \frac{FD_0}{\text{clearance}} = \frac{FD_0}{kV_D}$$

where F = fraction of dose absorbed, D_0 = dose, k = elimination rate constant and V_D = volume of distribution (Shargel *et al.*, 2005).

5.3 Method development

Artemisinin and its semi-synthetic derivatives are rapidly metabolised to their major metabolite DHA *in vivo*. DHA possesses greater antimalarial activity than the parent drug (Grace *et al.*, 1998). Therefore, it is important to monitor DHA in addition to the parent artemisinin drug such as artesunate for pharmacokinetic studies (Xing *et al.*, 2007).

Various methods have been developed for the quantification of DHA and its analogues in biological samples, such as high-performance liquid chromatography (HPLC) with electrochemical detection (Lai *et al.*, 2007). However, some limitations of these methods include long run time, poor reproducibility and inadequate sensitivity. Currently, liquid chromatography coupled with mass spectrometry techniques have been widely employed for the analysis of drug compounds in biological fluids because of their excellent specificity, high speed and sensitivity (Liu *et al.*, 2009).

Artesunate, DHA and mefloquine were evaluated for their pharmacokinetic properties in a mouse model. The drugs were tested in both a reference and a Pheroid™ vesicle formulation. The drugs were manufactured in the oil phase of the Pheroid™ vesicles. A sensitive and accurate LC-MS/MS method was developed for quantification of the study samples. The samples were pre-treated by a simple liquid-liquid extraction procedure with ethyl acetate. Concentration versus time graphs and pharmacokinetic models were constructed for the reference and Pheroid™ groups. The study was conducted in accordance with Dr. L. Wiesner at the University of Cape Town (UCT).

Typical method development and establishment for a bioanalytical method include determination of selectivity, accuracy and precision, calibration curve linearity and stability of an analyte in spiked samples (FDA, 2001).

5.3.1 Preparation of calibration standards

A calibration standard is a biological matrix to which a known amount of analyte has been added or spiked. Calibration standards are used to construct calibration curves from which the concentration of analytes in quality control samples and in unknown study samples are

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determined. A calibration (standard) curve is thus an indication of the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample (FDA, 2001).

Stock solutions of artesunate and DHA were prepared in ethanol at 1 mg/ml. Blank mouse plasma was spiked with the stock solutions to obtain standard (STD) 1 at a concentration of 8 µg/ml. A stock solution of mefloquine was prepared in water at a concentration of 1 mg/ml. Blank mouse whole blood (1240 µl) was spiked with the stock solution (10 µl) to obtain STD 1 at a concentration of 8 µg/ml. Serial dilution with blank mouse plasma and blank mouse whole blood resulted in STD 2 (4 µg/ml), STD 3 (2 µg/ml), STD 4 (1 µg/ml), STD 5 (0.5 µg/ml), STD 6 (0.25 µg/ml), STD 7 (0.125 µg/ml), STD 8 (0.0625 µg/ml), STD 9 (0.0313 µg/ml) and STD 10 (0.0156 µg/ml). Standards 4 to 10 were used for constructing calibration curves. The calibration standards were briefly vortexed, aliquotted into labelled polypropylene Eppendorf® tubes and stored at - 20°C.

5.3.2 Sample preparation: liquid-liquid extraction procedures

An optimised (buffer and organic solvent) liquid-liquid extraction method was developed for accurate and precise extraction of the test compounds and their internal standards (ISTD) from mouse plasma for artesunate and DHA and from mouse whole blood for mefloquine.

The analytes were extracted by a simple single-step liquid-liquid extraction with ethyl acetate. Artesunate and DHA were separated by isocratic elution with methanol and ammonium acetate (10 mM) with 0.1% acetic acid (65:35, v/v). Mefloquine was separated by gradient elution with acetonitrile and 0.1% formic acid (75:25, v/v).

5.3.2.1 Artesunate and DHA

The extraction procedure was performed on ice and in polypropylene test tubes. The plasma samples were thawed on ice and briefly vortexed. Twenty five microliters of a sodium carbonate buffer (0.1 M, pH 10) was aliquotted into clean Eppendorf® tubes and 15 µl of the plasma sample was added. The ISTD (artemisinin) was spiked at an appropriate concentration into the sodium carbonate buffer and another 25 µl of the buffer was added to the extraction tubes. Ethyl acetate (250 µl) was added as the organic solvent, the samples were vortexed for 1 minute and centrifuged for 5 minutes at high speed. The organic phase

(200 µl) was transferred to clean polypropylene tubes and the organic layer evaporated under vacuum in a rotor evaporation system at 30°C for 45 minutes. Mobile phase (50 µl) which consisted of methanol and ammonium acetate (10 mM) with 0.1% acetic acid (65:35, v/v) was added to the dry samples. The samples were vortexed for 30 seconds and transferred to 96-well polypropylene plates. Twenty microliters was injected into the HPLC column.

5.3.2.2 Mefloquine

Fifty microliters of a sodium carbonate buffer (0.1 M, pH 10.8) was aliquotted into clean polypropylene tubes and 10 µl of the whole blood sample was added. The ISTD (amodiaquine) was spiked at an appropriate concentration into water and 50 µl was added to the extraction tube. Ethyl acetate (500 µl) was added as the organic solvent. The samples were vortexed for 1.5 minutes and centrifuged for 5 minutes at high speed. The organic phase (400 µl) was transferred to clean polypropylene tubes and the organic layer evaporated under vacuum in a rotor evaporation system at 30°C for 45 minutes. The samples were reconstituted with 100 µl acetonitrile : 0.1% formic acid (75:25, v/v). The samples were vortexed for 30 seconds, placed into autosampler vials and 4 µl was injected into the HPLC column.

5.3.3 Selectivity: chromatography and mass spectrometry

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in a sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, whole blood) should be tested for potential interfering substances in the matrix. Each analyte to be quantified should be tested to ensure that there is no interference (FDA, 2001).

5.3.3.1 High-performance liquid chromatography (HPLC)

Chromatographic separation was performed on a Phenomenex Luna PFP (2) (5µ, C18, 110A, 50x2 mm) analytical column (Phenomemex, Torrance, CA, USA) using an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of methanol and ammonium acetate (10 mM with 0.1% acetic acid) for artesunate and DHA and acetonitrile with 0.1% formic acid (75:25 v/v) for mefloquine. The mobile

phase was delivered at a constant flow rate of 0.5 ml/min using a gradient. The organic phase was increased from 55 to 90% over 2 minutes and was kept at 90% for another 2.5 minutes.

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The organic phase was then decreased back to the original 55% in 0.1 minute and was kept at 55% for another 1.4 minutes for column equilibration. The flow-rate was increased to 0.7 ml/min during the equilibration step. The column was kept in a column compartment at 35°C. An autosampler injected 20 µl of the artesunate and DHA extraction samples and 4 µl of the mefloquine extraction sample into the HPLC column. The injection needle was rinsed with mobile phase before each injection for 10 seconds using the flush port wash station. The samples were stored at 5°C while awaiting injection. The first 1.3 and last 1.5 minutes were diverted to waste to keep the source clean. Representative chromatograms are presented in Figures 5.2 - 5.7.

5.3.3.2 Mass spectrometry (MS)

Analyte quantification was performed by electro-spray ionisation (ESI) - triple quadrupole mass spectrometry by multi- reaction monitoring (MRM) detection in the positive mode, using an AB Sciex API 3200 mass spectrometer (AB Sciex Instruments, USA). The settings of the apparatus are summarised in Tables 5.1 and 5.2.

Table 5.1: ESI settings:

	Artesunate, DHA	Mefloquine
Curtain gas	20	20
Collision gas	5	5
Ionspray voltage (V)	5000	4500
Source temperature (°C)	300	500
Gas 1 (psi)	50	50
Gas 2 (psi)	60	60

Table 5.2: MS/MS settings for the selected antimalarial drugs and ISTD's:

	*AS	DHA	ISTD (artemisinin)	*MQ	ISTD (amodiaquine)
Q1 mass [M+H]⁺	402.2	302.2	300.2	379.2	356.1
Q3 mass	267.1	163.1	209.2	321.1	283.1
Dwell time (ms)	150	150	150	150	150
Declustering potential (V)	16	16	21	56	36
Entrance potential (V)	4.5	3.5	3	10	5
Collision cell entrance potential (V)	18	18	20	18	18
Collision energy (V)	17	21	15	45	29
Collision cell exit potential (V)	6	4	4	6	6
Scan type	MRM	MRM	MRM	MRM	MRM
Polarity	positive	positive	positive	positive	positive
Pause time (ms)	5	5	5	5	5

* Artesunate, mefloquine.

5.3.4 Results: method validation

A sensitive and specific LC-MS/MS method was developed and validated for application to pharmacokinetic determination of artesunate, DHA and mefloquine in a mouse model.

5.3.4.1 Selectivity: chromatograms and MS spectra

Typical chromatograms obtained from the calibration standards spiked with artesunate, DHA and mefloquine, together with their ISTD's, as well as plasma and whole blood samples from treated mice and blank plasma and whole blood samples are shown in Figures 5.2 - 5.7.

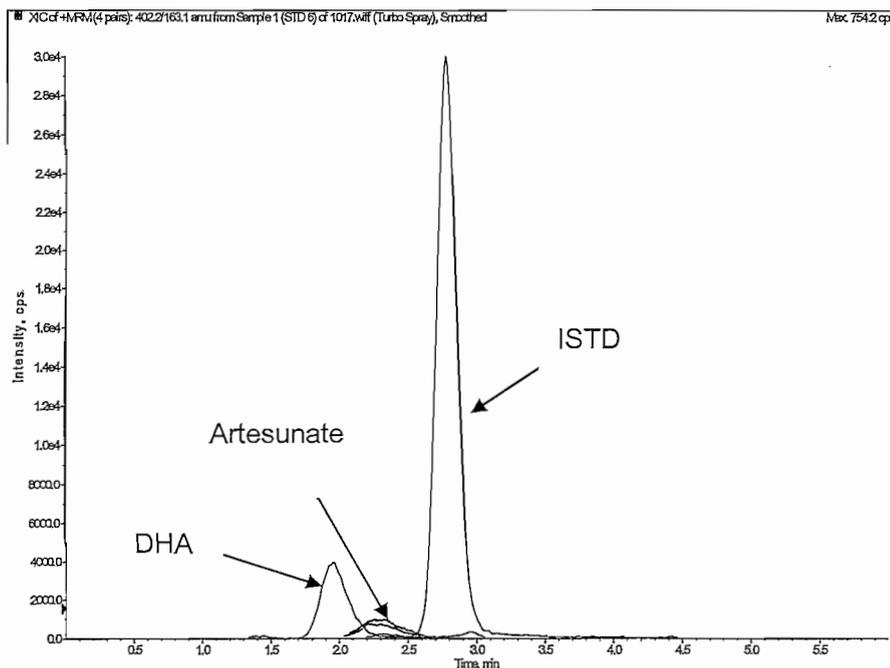


Figure 5.2: Representative chromatogram of mouse plasma spiked with 0.25 µg/ml artesunate, DHA and ISTD (artemisinin) (STD 6).

Artesunate, DHA and the ISTD were detected at retention times of approximately 2.35 min., 1.99 min. and 2.78 min. respectively.

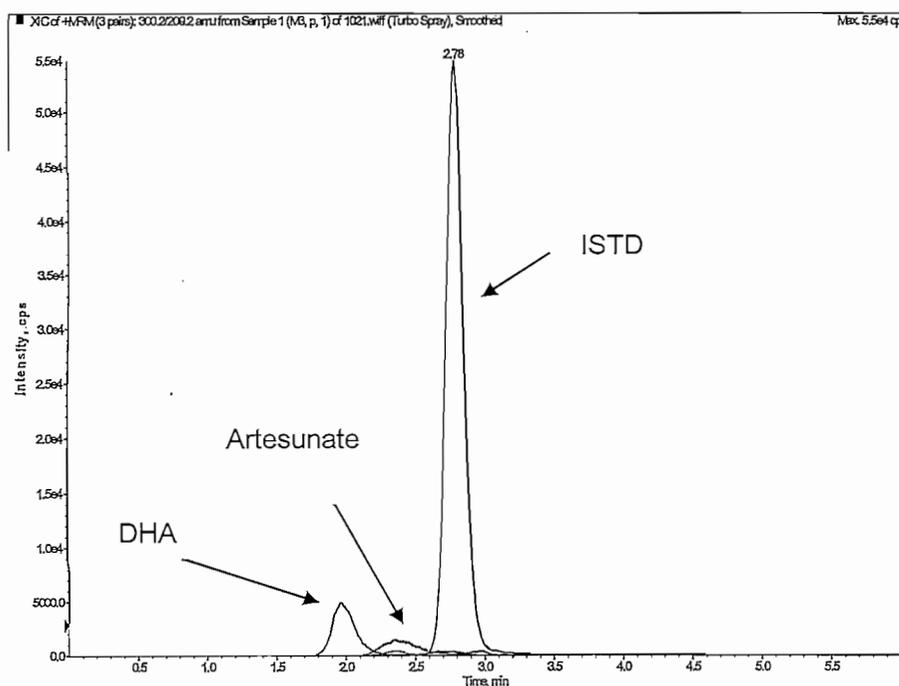


Figure 5.3: Representative chromatogram of Mouse 3 treated with 20 mg/kg artesunate in combination with Pheroid™ vesicles. Mouse plasma was obtained 0.25 h after treatment.

The same approximate retention times for artesunate, DHA and the ISTD were obtained for this treated sample in combination with Pheroid™ vesicles, indicating that the Pheroid™ matrix had no significant effect on the method.

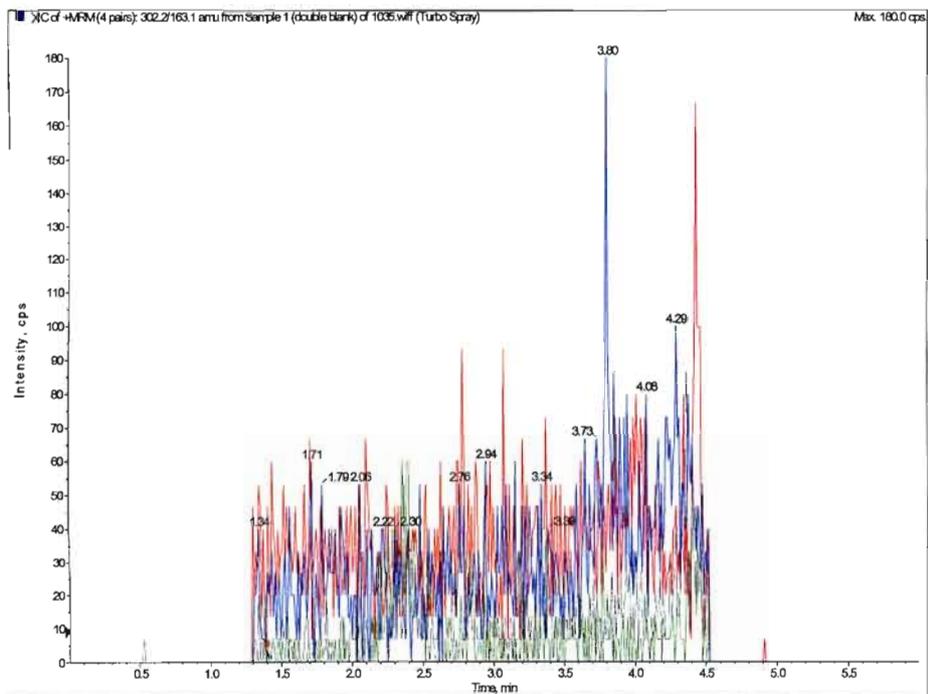


Figure 5.4: Representative chromatogram of a double blank sample.

Blank mouse plasma showed no significant interfering peaks at the retention times of artesunate (2.35 min.), DHA (1.99 min.) and the ISTD (2.78 min.).

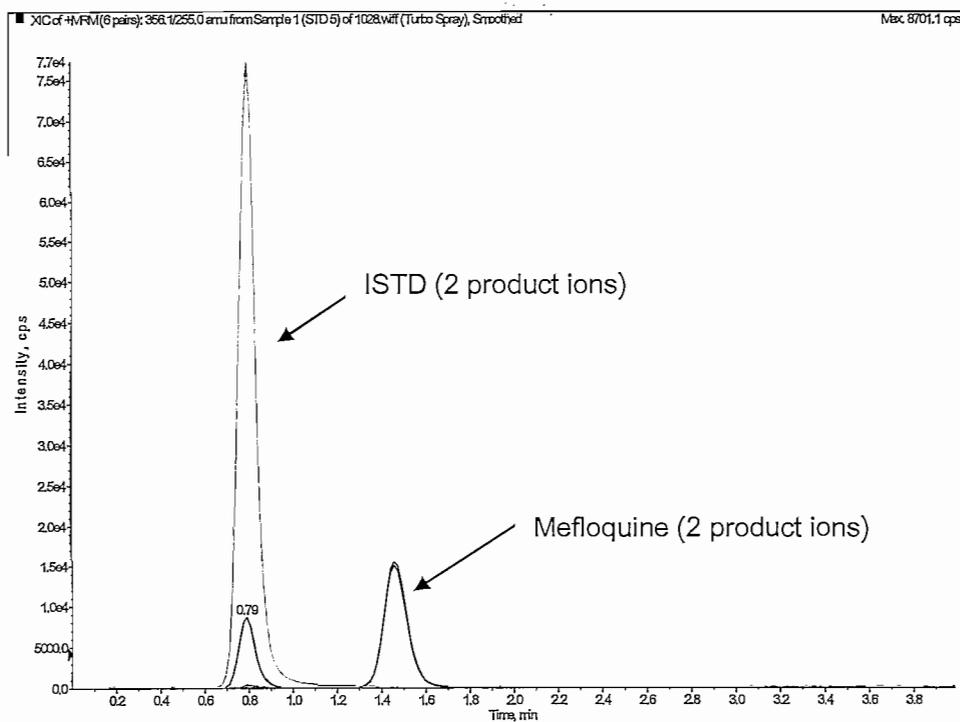


Figure 5.5: Representative chromatogram of mouse plasma spiked with 0.5 µg/ml mefloquine and ISTD (amodiaquine) (STD 5).

Mefloquine and the ISTD were detected at retention times of approximately 1.45 min. and 0.79 min. respectively.

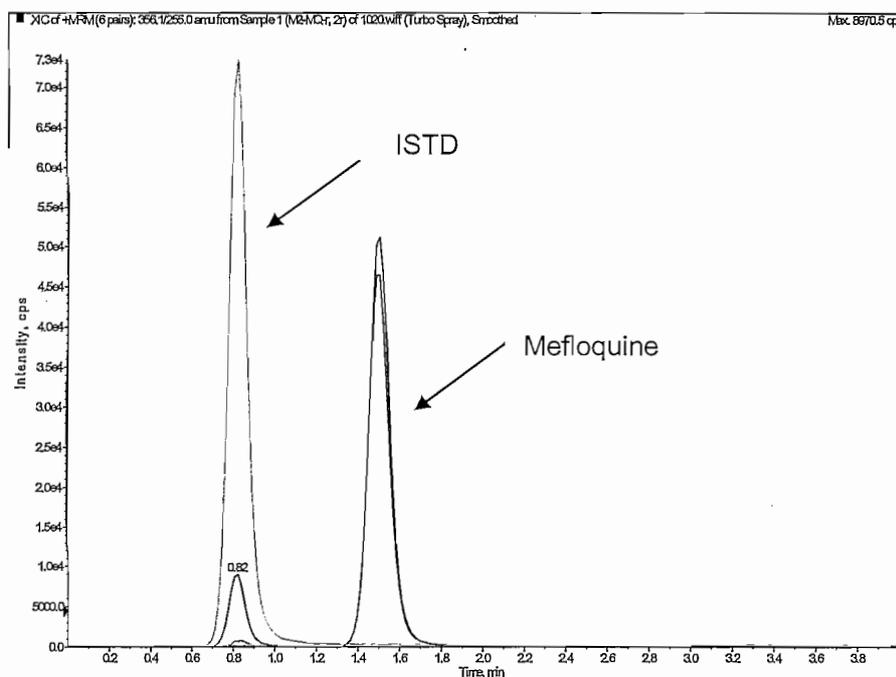


Figure 5.6: Representative chromatogram of Mouse 2 treated with 20 mg/kg mefloquine in combination with Pheroid™ vesicles. Mouse plasma was obtained 2.0 h after treatment.

The same approximate retention times for mefloquine and the ISTD were obtained for this treated sample in combination with Pheroid™ vesicles, indicating that the Pheroid™ matrix had no significant effect on the method.

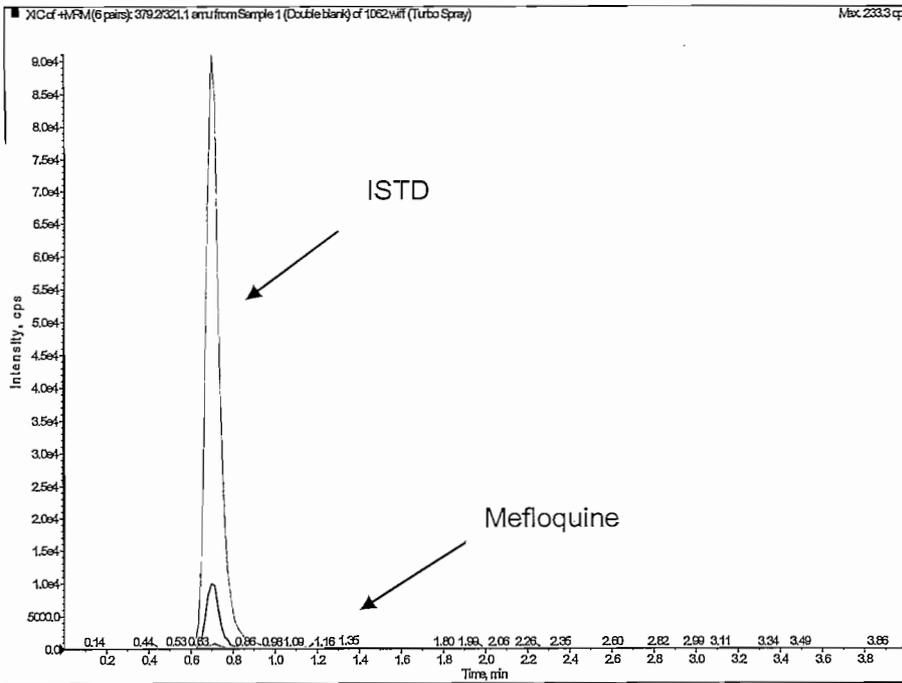


Figure 5.7: Representative chromatogram of a double blank sample.

Blank mouse plasma showed no significant interfering peaks at the retention times of mefloquine (1.45 min.) and the ISTD (0.79 min.).

The product ion mass spectra obtained for artesunate, DHA and mefloquine, together with their ISTD's are shown in Figures 5.8 - 5.12.

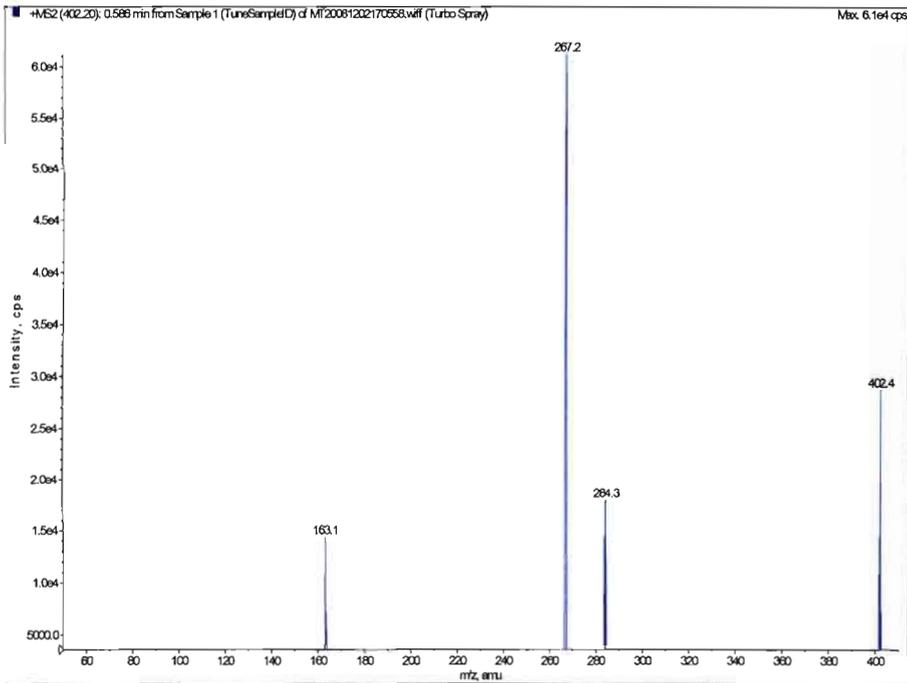


Figure 5.8: Typical MS/MS spectrum of artesunate, indicating the analyte (m/z) at 267.2.

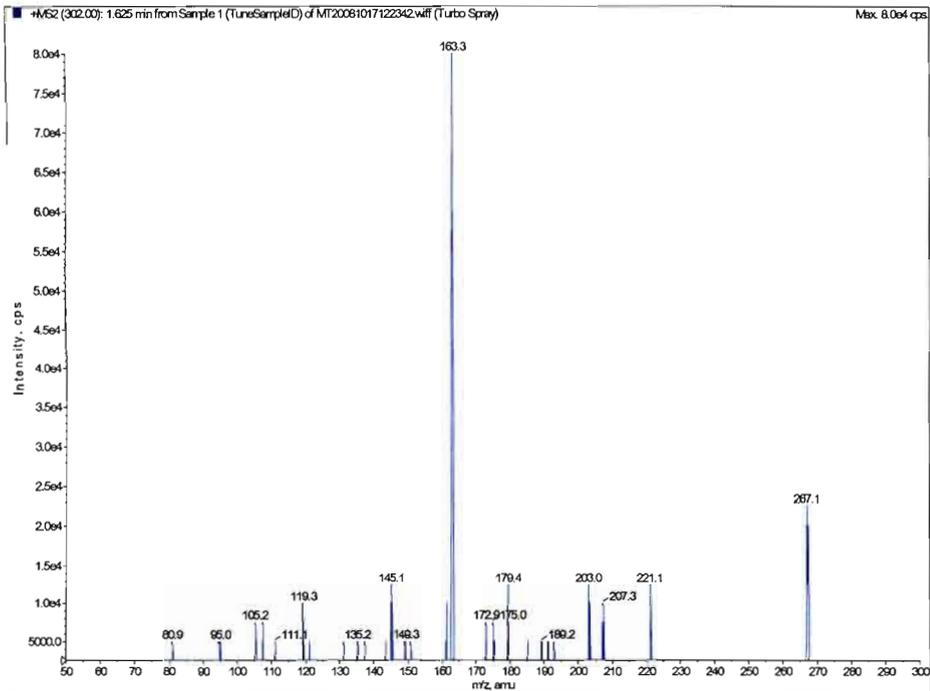


Figure 5.9: Typical MS/MS spectrum of DHA, indicating the analyte (m/z) at 163.3.

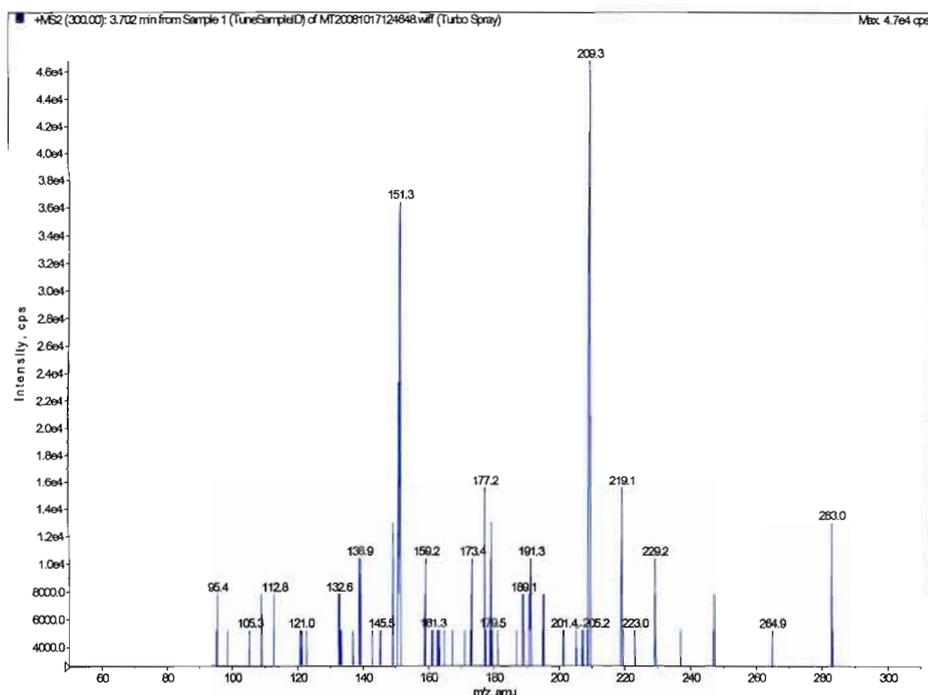


Figure 5.10: Typical MS/MS spectrum of artemisinin (ISTD), indicating the analyte (m/z) at 209.3.

The mass spectra of artesunate, DHA and artemisinin (ISTD) were characterised by intense fragment ions at m/z 267.2, m/z 163.3 and m/z 209.3 respectively, which correlates well with the m/z obtained by Liu *et al* (2009), which were 267.1 for artesunate, 163.2 for DHA and 209.1 for the internal standard artemisinin.

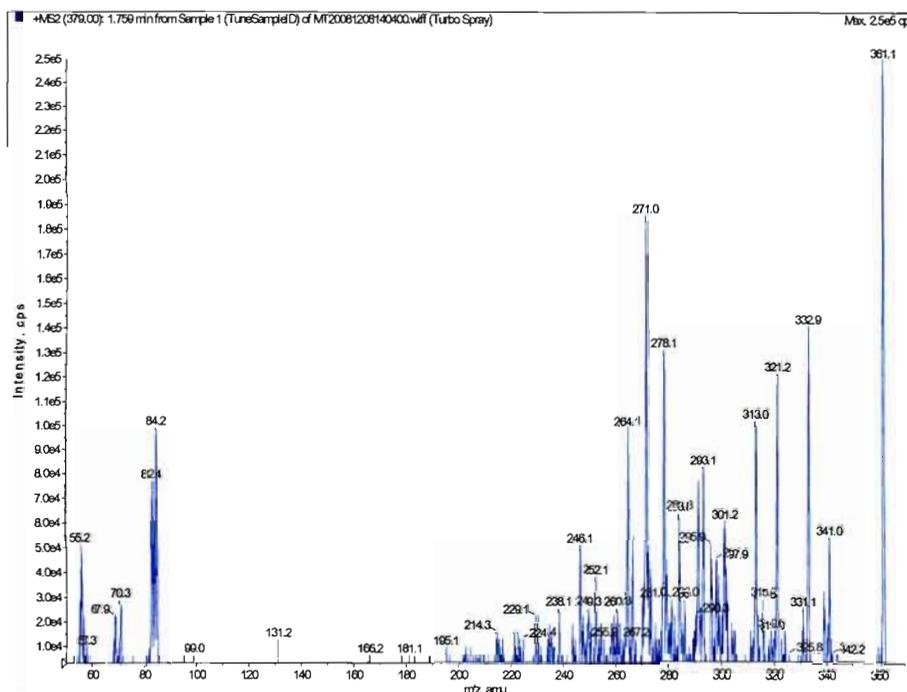


Figure 5.11: Typical MS/MS spectrum of mefloquine, indicating the analyte (m/z) at 361.1.

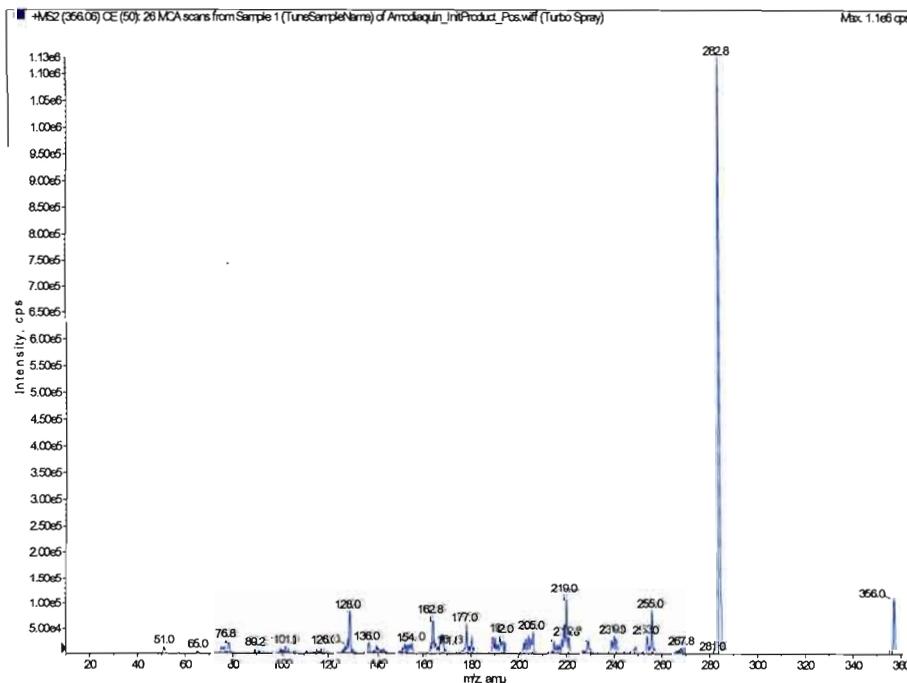


Figure 5.12: Typical MS/MS spectrum of amodiaquine (ISTD), indicating the analyte (m/z) at 282.8.

The mass spectra of mefloquine and its ISTD were characterised by intense fragment ions at m/z 361.1 and m/z 282.8 respectively, which correlates well with the m/z obtained by Hodel *et al* (2009) which were 360.7 for mefloquine and 282.9 for the internal standard amodiaquine.

5.3.4.2 Calibration curves

The calibration standards were analysed in conjunction with the study samples. Representative calibration curves are presented in Figures 5.13 (artesunate), 5.14 (DHA) and 5.15 (mefloquine). The accuracy and precision statistics of the calibration curves are presented in Tables 5.3 (artesunate), 5.4 (DHA) and 5.5 (mefloquine).

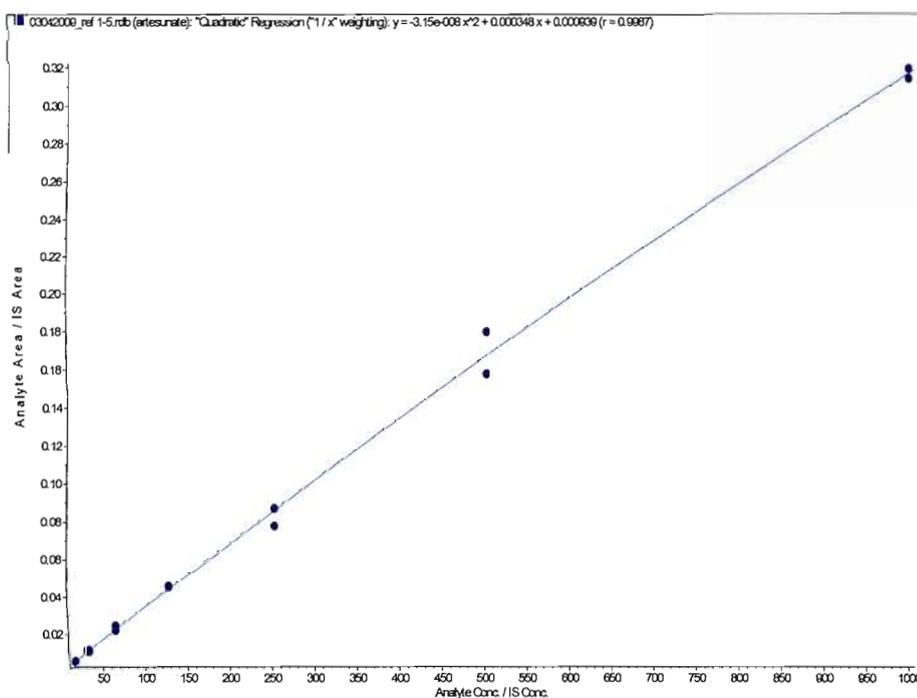


Figure 5.13: Representative calibration curve of artesunate ($r = 0.9987$).

Table 5.3: Accuracy and precision data of the representative calibration curve of artesunate

Expected concentration (ng/ml)	STD	Mean (ng/ml)	(% CV)	% Accuracy
15.6	STD10(n=2)	15.2	2.3	97.6
31.3	STD 9 (n=2)	30.8	4.9	98.3
62.5	STD 8 (n=2)	64.8	8.2	103.7
125.0	STD 7 (n=2)	129.0	0.6	103.6
250.0	STD 6 (n=2)	240.0	8.3	96.0
500.0	STD 5 (n=2)	505.0	9.7	101.0
1000.0	STD 4 (n=2)	1000.0	1.3	100.0

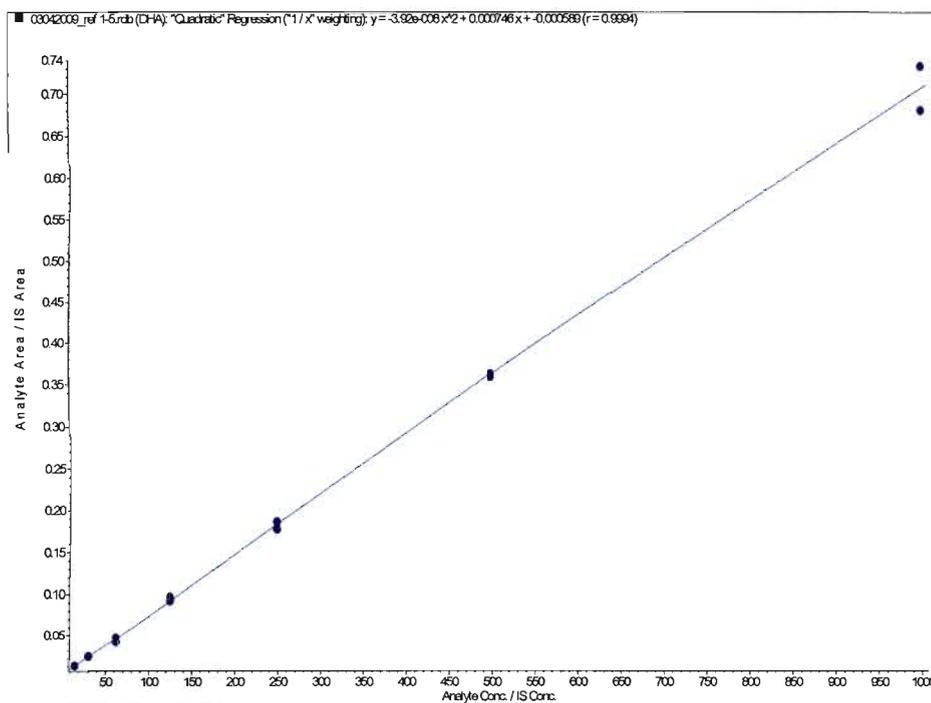


Figure 5.14: Representative calibration curve of DHA ($r = 0.9994$).

Table 5.4: Accuracy and precision data of the representative calibration curve of DHA.

Expected concentration (ng/ml)	STD	Mean (ng/ml)	(% CV)	% Accuracy
15.6	STD10(n=2)	15.7	1.3	100.3
31.3	STD 9 (n=2)	31.5	1.7	100.6
62.5	STD 8 (n=2)	60.5	9.5	96.8
125.0	STD 7 (n=2)	129.0	3.0	103.0
250.0	STD 6 (n=2)	248.0	3.3	99.3
500.0	STD 5 (n=2)	499.0	0.7	99.8
1000.0	STD 4 (n=2)	1001.0	5.5	100.1

The calibration curves were constructed by plotting the peak area ratios (y) versus the plasma concentrations (x) of the analytes. Good linearity was exhibited over the concentration range of 15.0 – 1000.0 ng/ml for artesunate ($r = 0.9987$) and DHA ($r = 0.9994$). According to the Food and Drug Administration (FDA) guidelines, the concentrations obtained should be between 85 - 115% of the actual values (% accuracy) and the precision determined at each concentration should not exceed 15% of the coefficient of variation (CV). The data obtained from the calibration curves of artesunate and DHA were thus well within these limits.

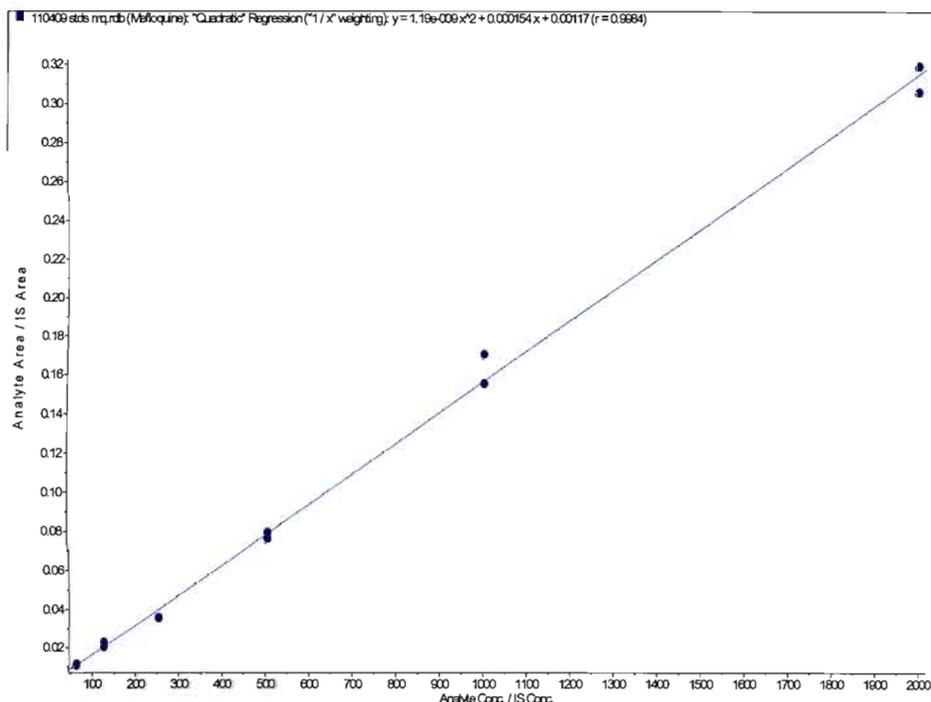


Figure 5.15: Representative calibration curve of mefloquine (r = 0.9984).

Table 5.5: Accuracy and precision data of the representative mefloquine calibration curve.

Expected concentration (ng/ml)	STD	Mean (ng/ml)	% CV	% Accuracy
62.5	STD 8 (n=2)	64.1	6.8	102.5
125.0	STD 7 (n=2)	133.0	9.1	106.5
250.0	STD 6 (n=2)	223.0	0.5	89.1
500.0	STD 5 (n=2)	494.0	2.9	98.7
1000.0	STD 4 (n=2)	1038.0	6.4	103.8
2000.0	STD 3 (n=2)	1985.0	2.9	102.5

Good linearity was exhibited over the concentration range of 60 - 2000 ng/ml for mefloquine with a correlation coefficient of $r = 0.9984$. The data obtained from the calibration curve of mefloquine was thus well within the limits stipulated by the FDA.

5.3.5 Discussion: method development

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites in guinea pigs are critical for the successful conduct of preclinical and/or biopharmaceutical and clinical pharmacological studies (FDA, 2001). Currently, liquid chromatography coupled with mass spectrometry techniques have been widely employed for the analysis of drug compounds in biological fluids because of their excellent specificity, high speed and sensitivity (Liu *et al.*, 2009). The selectivity of the method developed correlated well with chromatographic and mass spectrometry results obtained by Liu *et al.*, 2009 and Hodel *et al.*, 2009 from human plasma. This method was however only partially validated and is in effect a new method as it was specifically developed for analysis of mouse whole blood and plasma. There are however very few literature available of these specific studies in mice, as most studies are performed on either rat or human plasma.

5.4 Bioavailability evaluation

Artesunate and mefloquine were evaluated for their pharmacokinetic properties in a mouse model. The active dihydroartemisinin (DHA) metabolite of artesunate was also evaluated. The drugs were tested in both a reference and a Pheroid™ vesicle formulation. The drugs were manufactured in the oil phase of the Pheroid™ vesicles. The animals utilised were male C57 BL6 mice, weighing approximately 25 g each. The study was approved by the Ethics Committee of UCT with approval number 009/034. The drugs were administered via the oral route. An Agilent 1200 series HPLC system and an API 3200 triple quadrupole mass spectrometer were used to perform the analysis on all the samples. The reference standard, calibration standards and study samples were stored at - 20°C. No samples were stored for more than 7 days before performing the analysis.

5.4.1 Experimental design: artesunate and DHA

The time intervals were chosen based on the short half-life of artesunate (< 10 minutes) and DHA (~ 1 hour) (Krishna *et al.*, 2004). The following treatment groups were used:

Reference group (n = 5): Artesunate was administered orally at 20.0 mg/kg in water. The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding

before and at 10, 20, 30 and 40 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at – 20°C.

Pheroid™ group (n = 5): The artesunate Pheroid™ vesicle formulation was also administered orally at 20 mg/kg. The same protocol was used as described for reference group 1.

5.4.2 Experimentel design: mefloquine

The time intervals were chosen based on the long half-life of mefloquine of approximately 20 days. Mouse whole blood was used instead of plasma as mefloquine is extensively (98%) protein bound and extensively distributed into tissues (Rosenthal, 2004). The following treatment groups were used:

Reference group (n = 5): Mefloquine was administered orally at 20.0 mg/kg in water. The total volume per administration was 200 µl. Blood samples (10 µl) were collected before and at 1, 3, 6, 12, 24, 48, 72 and 96 hours after drug administration.

Pheroid™ group (n = 5): The mefloquine Pheroid™ vesicle formulation was also administered orally at 20.0 mg/kg. The same protocol was used as described for reference group 1.

5.4.3 Results: artesunate and DHA

➤ Reference group

The samples were analysed in one batch and the concentrations of artesunate and DHA are shown in Tables 5.6 and 5.7 respectively. Mean concentration versus time graphs of artesunate and DHA are presented in Figures 5.16 and 5.17 respectively.

Table 5.6: Plasma concentrations (ng/ml) of artesunate reference group, measured in individual mice over a time period of 40 minutes.

Time (minutes)	0	10	20	30	40
Mouse 1	0	BLQ	171.0	BLQ	BLQ
Mouse 2	0	BLQ	BLQ	BLQ	BLQ
Mouse 3	0	BLQ	BLQ	40.4	BLQ
Mouse 4	0	BLQ	266.0	16.4	BLQ
Mouse 5	0	BLQ	BLQ	BLQ	BLQ
Mean	0	N/A	218.5	28.4	N/A
SD	N/A	N/A	67.2	17.0	N/A

SD = standard deviation, BLQ = Below the limit of quantification, N/A = not applicable.

Table 5.7: Plasma concentrations (ng/ml) of DHA reference group, measured in individual mice over a time period of 40 minutes.

Time (minutes)	0	10	20	30	40
Mouse 1	0	62.9	25.4	BLQ	BLQ
Mouse 2	0	33.3	BLQ	BLQ	124.0
Mouse 3	0	108.0	52.9	105.0	19.4
Mouse 4	0	64.0	49.5	BLQ	BLQ
Mouse 5	0	BLQ	BLQ	BLQ	BLQ
Mean	0	67.1	42.6	105.0	71.7
SD	N/A	30.8	15.0	N/A	74.0

SD = standard deviation, BLQ = Below the limit of quantification, N/A = not appl

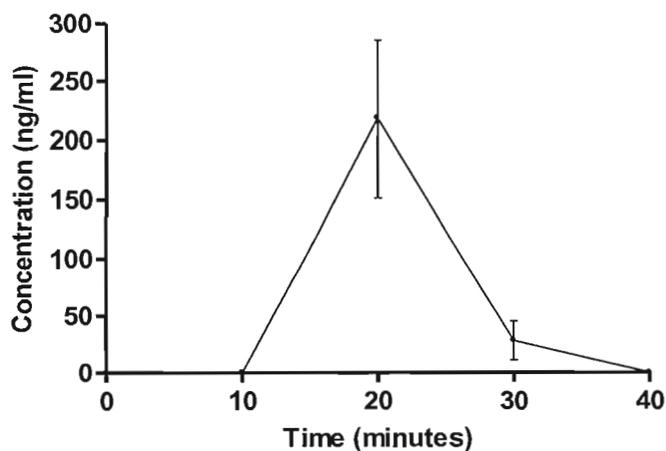


Figure 5.16: Mean plasma concentration (ng/ml) vs. time (minutes) graph of artesunate, reference group. The results are presented as mean \pm SD (n = 5).

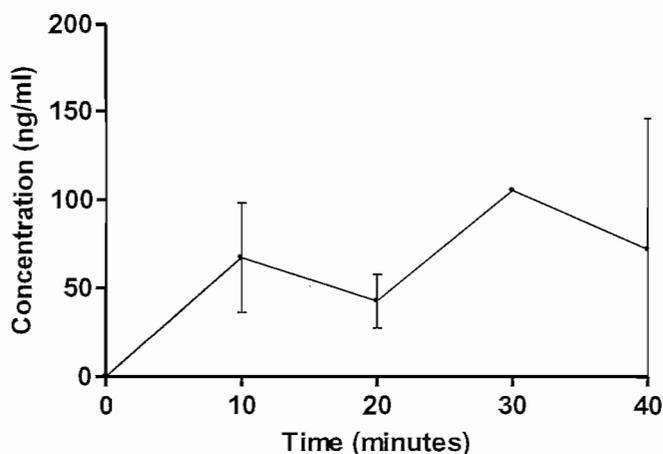


Figure 5.17: Mean plasma concentration (ng/ml) vs. time (minutes) graph of DHA, reference group. The results are presented as mean \pm SD (n = 5).

The first samples were collected just before drug administration, all of which tested negative for artesunate and DHA. Ten minutes post drug administration DHA reached a mean plasma drug concentration of 67.1 ± 30.8 ng/ml. Artesunate tested negative after 10 minutes. Twenty minutes post drug administration, artesunate reached a peak plasma drug concentration of 218.0 ± 67.2 mg/ml, where after a sharp decrease in plasma drug concentration could be observed at 30 and 40 minutes post drug administration. DHA decreased slightly after 20 minutes to 42.6 ± 15.0 ng/ml, where after it reached a peak plasma drug concentration of 105.0 ng/ml after 30 minutes. A slight decrease to 71.7 ± 74.0 ng/ml was observed for DHA after 40 minutes of drug administration.

➤ **Pheroid™ group**

The samples were analysed in one batch and the concentrations of artesunate and DHA are presented in Tables 5.8 and 5.9 respectively. Mean concentration vs. time graphs of artesunate and DHA are presented in Figures 5.18 and 5.19 respectively.

Table 5.8: Plasma concentrations (ng/ml) of artesunate Pheroid™ group, measured in individual mice over a time period of 40 minutes.

Time (minutes)	0	10	20	30	40
Mouse 1	0	93.6	BLQ	23.0	BLQ
Mouse 2	0	68.2	109.0	91.0	BLQ
Mouse 3	0	84.7	36.5	24.8	BLQ
Mouse 4	0	148.0	77.9	40.3	BLQ
Mouse 5	0	34.0	22.2	BLQ	46.0
Mean	0	85.7	61.4	44.8	46.0
SD	N/A	41.6	39.6	31.8	N/A

SD = standard deviation, BLQ = Below the limit of quantification, N/A = not applicable.

Table 5.9: Plasma concentrations (ng/ml) of DHA Pheroid™ group, measured in individual mice over a time period of 40 minutes.

Time (minutes)	0	10	20	30	40
Mouse 1	0	166.0	99.6	62.2	51.5
Mouse 2	0	215.0	179.0	98.4	54.9
Mouse 3	0	178.0	144.0	90.6	58.3
Mouse 4	0	197.0	135.0	88.3	59.3
Mouse 5	0	110.0	57.5	BLQ	BLQ
Mean	0	173.0	123.0	85.0	56.0
SD	N/A	39.9	46.3	15.7	3.5

SD = standard deviation, BLQ = Below the limit of quantification, N/A = not applicable.

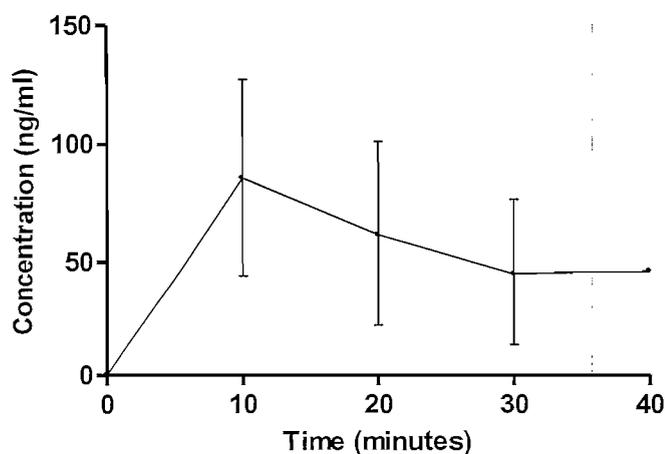


Figure 5.18: Mean plasma concentration (ng/ml) vs. time (minutes) graph of artesunate, Pheroid™ group. The results are presented as mean ± SD (n = 5).

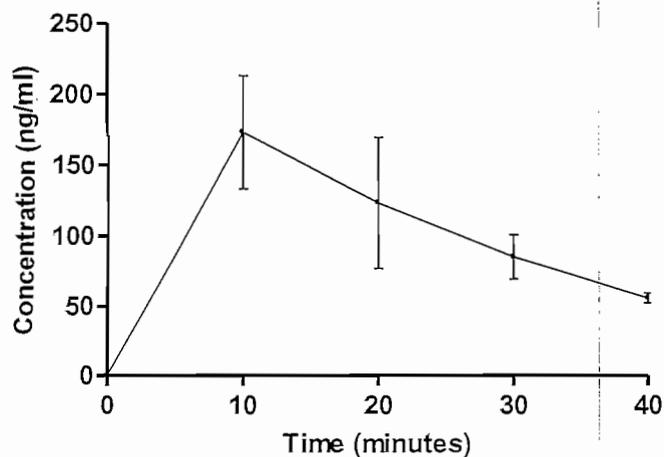


Figure 5.19: Mean plasma concentration (ng/ml) vs. time (minutes) graph of DHA, Pheroid™ group. The results are presented as mean \pm SD (n = 5).

The first samples were collected just before administering the Pheroid™ vesicle formulation, all of which tested negative for artesunate and DHA. After 10 minutes of drug administration, artesunate and DHA reached peak plasma drug concentrations of 85.7 ± 41.6 ng/ml and 173.0 ± 39.9 ng/ml respectively. DHA obtained a higher peak plasma drug concentration with the Pheroid™ vesicle group in relation to the reference group (105.0 ng/ml) at a much faster time (10 minutes in the Pheroid™ vesicles in contrast to 30 minutes of the reference group). A gradual decrease in mean plasma drug concentration of both drugs could be observed after 10 minutes of drug administration. The results of the reference and Pheroid™ groups are presented in the form of an overlay graph of the mean concentration versus time in Figures 5.20 and 5.21.

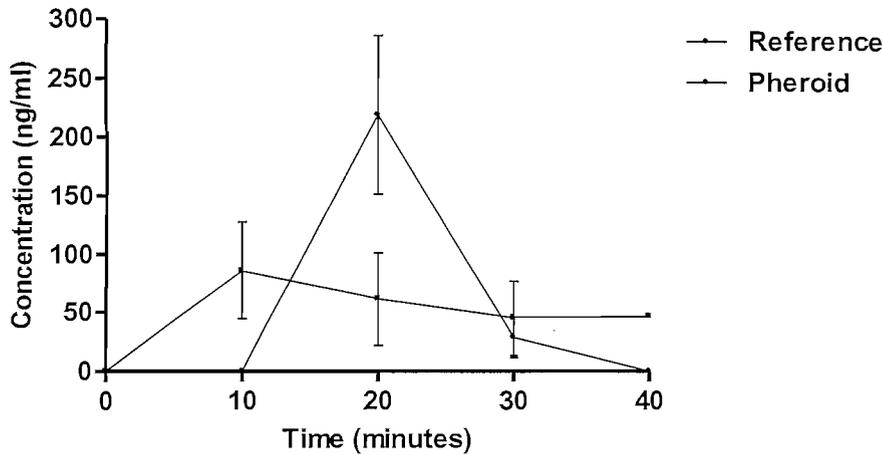


Figure 5.20: Mean plasma concentration (ng/ml) vs. time (minutes) overlay graph of artesunate reference and Pheroid™ groups. The results are presented as mean \pm SD (n = 5).

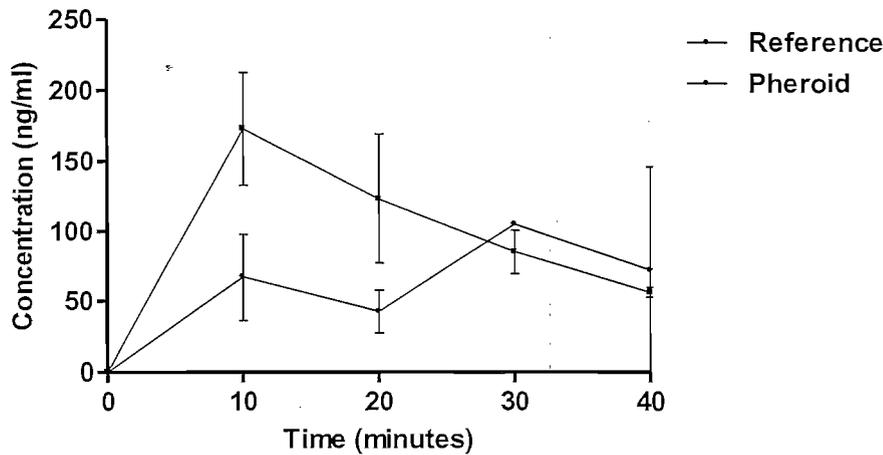


Figure 5.21: Mean plasma concentration (ng/ml) vs. time (minutes) overlay graph of DHA reference and Pheroid™ groups. The results are presented as mean \pm SD (n = 5).

Most of the artesunate plasma concentrations were below the limit of quantification in the reference group and some relatively high outliers were observed in some of the samples. The DHA levels were also irregular. The mean artesunate levels in the Pheroid™ group were lower compared to the reference group, but the variation within the Pheroid™ group lessened significantly. The mean DHA concentrations in the Pheroid™ group improved considerably and more than twofold higher levels were observed in this group. The artesunate and DHA plasma

concentrations of the Pheroid™ groups remained high and did not reach zero, as was the case with the artesunate reference group, which might indicate an increase in the plasma half-life, but this will have to be investigated further.

Pharmacokinetic models could not be constructed at this point and future experiments are planned with more saturated time points. However, it is clear that the Pheroid™ formulation improved the bioavailability of DHA.

5.4.4 Results: mefloquine

➤ Reference group

The samples were analysed in one batch and the results are presented in Table 5.10. The mean concentration vs. time graph of the mefloquine reference group 1 is presented in Figure 5.22.

Table 5.10: Blood concentrations (ng/ml) of mefloquine reference group, measured in individual mice over a time period of 96 hours.

Time (hours)	0	1.0	3.0	6.0	12.0	24.0	48.0	72.0	96.0
Mouse 1	0	865.0	1300.0	1530.0	1030.0	959.0	521.0	295.0	149.0
Mouse 2	0	1140.0	1780.0	1930.0	1300.0	1060.0	566.0	314.0	153.0
Mouse 3	0	1410.0	1770.0	1650.0	1600.0	1050.0	537.0	187.0	132.0
Mouse 4	0	932.0	1400.0	1320.0	1340.0	644.0	430.0	241.0	103.0
Mouse 5	0	1020.0	1740.0	1440.0	1220.0	820.0	405.0	173.0	88.30
Mean	0	1073.0	1598.0	1574.0	1298.0	907.0	492.0	242.0	125.0
SD	N/A	214.0	230.0	233.0	207.0	176.0	70.30	62.80	28.50

SD = standard deviation, N/A = not applicable.

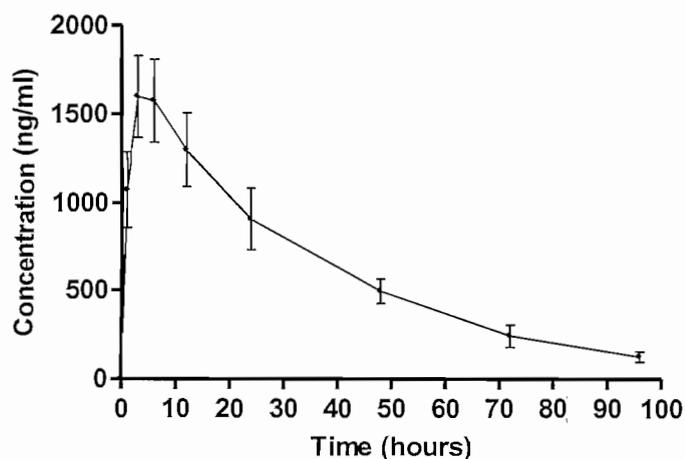


Figure 5.22: Mean blood concentration (ng/ml) vs. time (hours) graph of mefloquine, reference group. The results are presented as mean \pm SD (n = 5).

The first samples were collected just before drug administration, all of which tested negative for mefloquine. One hour post drug administration the mean plasma drug concentration was 1073 ± 214 ng/ml. A peak plasma drug concentration of 1598.0 ± 230.0 ng/ml was reached after three hours post drug administration, where after a gradual decrease in the mean plasma drug concentration could be observed.

➤ Pheroid™ group

The samples were analysed in one batch and the results are presented in Table 5.11. The mean concentration vs. time graph of the mefloquine Pheroid™ group is presented in Figure 5.23.

Table 5.11: Blood concentrations (ng/ml) of mefloquine Pheroid™ group, measured in individual mice over a time period of 96 hours.

Time (hours)	0	1.0	3.0	6.0	12.0	24.0	48.0	72.0	96.0
Mouse 1	0	1100.0	1590.0	1500.0	1320.0	956.0	431.0	209.0	81.20
Mouse 2	0	882.0	1370.0	1450.0	1240.0	795.0	358.0	186.0	94.10
Mouse 3	0	1140.0	1610.0	1920.0	1490.0	1150.0	479.0	219.0	116.0
Mouse 4	0	1160.0	1620.0	1430.0	1146.0	694.0	412.0	203.0	100.0
Mouse 5	0	984.0	761.0	1260.0	1180.0	796.0	410.0	217.0	119.0
Mean	0	1053.0	1390.0	1512.0	1275.0	878.0	418.0	207.0	102.0
SD	N/A	118.0	367.0	245.0	137.0	179.0	43.60	13.30	15.70

SD = standard deviation, N/A = not applicable.

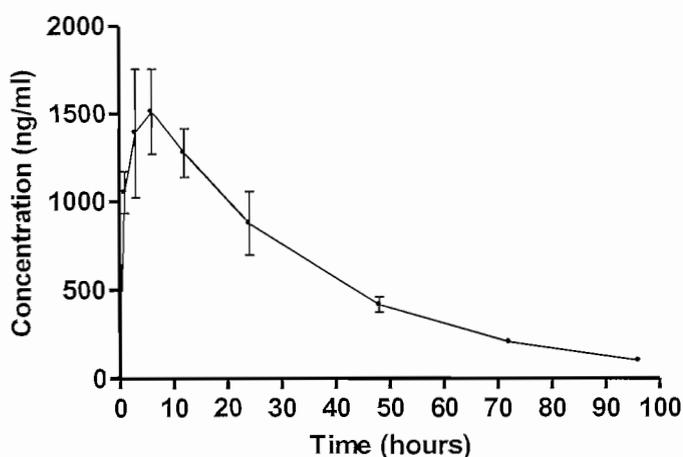


Figure 5.23: Mean blood concentration (ng/ml) vs. time (hours) graph of mefloquine, Pheroid™ group. The results are presented as mean ± SD (n = 5).

The first samples were collected just before administering the mefloquine Pheroid™ vesicle formulation, all of which tested negative for mefloquine. Six hours post drug administration a peak plasma drug concentration of 1512.0 ± 245.0 ng/ml was reached, where after a gradual decrease in mean plasma drug concentration could be observed.

5.4.4.1 Statistical evaluation of mefloquine

Noncompartmental analysis was used to calculate the pharmacokinetic parameters for mefloquine in mice. The data was analysed with WinNonlin Professional™ software, version 5.2 (Pharsight Corporation, California, USA). Linear interpolation was used to determine the area under the concentration time curve. AUC_{0-last} is defined as the AUC computed from time zero to the time of the last Y-value above the lower limit of quantification of the assay. All values below this limit were treated as "missing". AUC_{0-inf} was calculated by extrapolating the concentration time curve from time zero to infinity, using the last three concentration time points to estimate the observed elimination rate constant (λ_z). This constant was also used to determine the observed elimination half-life ($T_{1/2}$) of the compound. The summary statistics and Mann-Whitney non-parametric test calculations were performed using Prism version 5 (GraphPad Software Inc., California, USA). The pharmacokinetic parameters of the oral reference and the Pheroid™ vesicle group were calculated, the results are presented in the form of an overlay of the mean concentration versus time graphs in Figure 5.24. A summary of the pharmacokinetic data is presented in Table 5.12.

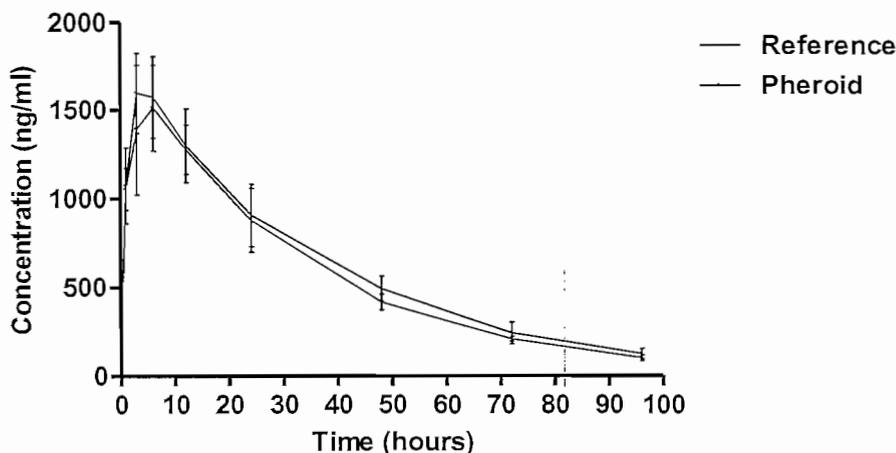


Figure 5.24: Mean blood concentration (ng/ml) vs. time (hours) overlay graph of mefloquine, reference and Pheroid™ groups. The results are presented as mean \pm SD (n = 5).

The incorporation of mefloquine in a Pheroid™ vesicle formulation did not seem to have improved results in relation to the reference group. The p-values depicted in Table 5.12 were scrutinised and subjected to the Mann-Whitney non-parametric test (two-tailed p-value). No statistical significant differences were observed in the pharmacokinetic parameters when comparing the mefloquine reference and Pheroid™ vesicle groups.

Table 5.12: Pharmacokinetic summary: mefloquine reference and Pheroid™ oral administration = 20.0 mg/kg.

Subject	T _{max} (hours)		C _{max} (ng/ml)		T _{1/2} (hours)		AUC _{0-last} (ng.h/ml)		AUC _{0-inf} (ng.h/ml)	
	Reference	Pheroid™	Reference	Pheroid™	Reference	Pheroid™	Reference	Pheroid™	Reference	Pheroid™
1	6.000	3.000	1,530.000	1,590.000	27.030	21.020	59,336.500	57,797.400	65,146.780	60,259.670
2	6.000	6.000	1,930.000	1,450.000	26.010	24.900	68,581.000	50,928.200	74,322.120	54,308.600
3	3.000	6.000	1,770.000	1,920.000	23.160	21.950	66,225.000	66,629.000	70,636.170	70,302.060
4	3.000	3.000	1,400.000	1,620.000	25.100	23.500	51,830.000	50,991.000	55,559.760	54,381.180
5	3.000	6.000	1,740.000	1,260.000	22.070	26.900	53,031.600	50,472.500	55,843.370	55,090.000
Arithmetic mean	4.200	4.800	1674.000	1568.000	24.670	23.650	59801.000	55364.000	64302.000	58868.000
Geometric mean	3.960	4.550	1663.000	1553.000	24.610	23.560	59420.000	55032.000	63849.000	58570.000
Arithmetic /Geometric	1.061	1.056	1.007	1.010	1.002	1.004	1.006	1.006	1.007	1.005
Lower 95% CI (Arithmetic)	2.471	2.838	1420.000	1282.000	22.190	20.850	50789.000	47374.000	54118.000	51043.000
Upper 95% CI (Arithmetic)	6.342	7.285	1948.000	1882.000	27.290	26.620	69518.000	63928.000	75330.000	67207.000
SEM	0.734	0.734	93.520	108.600	0.910	1.048	3376.000	3127.000	3803.000	3065.000
% CV	39.12%	34.23%	12.49%	15.49%	8.25%	9.90%	12.62%	12.63%	13.22%	11.64%
Mann-Whitney non-parametric test (two-tailed p-value)										
	0.6905		0.5476		0.4206		0.2222		0.1508	

5.4.4.2 Relative bioavailability

The relative bioavailability of mefloquine was also determined. The relative availability is the availability of the drug from a drug product as compared to a recognised standard. The availability of drug in the formulation is compared to the availability of drug in a standard dosage formulation, hence the two drug products are given at the same dosage level and by the same route of administration (Shargel *et al.*, 2005).

The relative bioavailability of mefloquine was determined by using the calculated arithmetic mean of the area under the curve (AUC_{0-last}) values of both the reference and the Pheroid™ vesicle data, as depicted in Table 5.12. The AUC_{0-inf} for the reference was calculated as 59801.0 ng.h/ml and 55364.0 ng.h/ml for the Pheroid™ vesicle formulation. The following equation was used to calculate the relative bioavailability:

$$\text{Relative bioavailability (RA)} = \frac{[AUC]_A}{[AUC]_B}$$

where B is represented by the arithmetic mean of the AUC_{0-last} values of the reference and A is represented by the arithmetic mean of the AUC_{0-last} values of the Pheroid™ vesicle formulation.

The relative bioavailability of the Pheroid™ vesicle incorporated mefloquine was RA = 0.93 in comparison to the reference which was represented by RA = 1.00. When converted to percentage values, reasoning that the reference is represented by 100%, the Pheroid™ vesicle entrapped mefloquine was 7% less bioavailable.

5.5 Conclusion

The Pheroid™ drug delivery system was tested in this study in an attempt to improve the pharmacokinetic profile of the selected antimalarial drugs artesunate and mefloquine. As an artemisinin derivative, the efficacy of artesunate is impaired by its short plasma

half-life, its low solubility in water or oil, the requirement of a high dosage with oral administration and a high rate of recrudescence when used alone as monotherapy (Kongpatanakul *et al.*, 2007). Fatty food has been implicated to improve the oral bioavailability of several lipophilic antimalarial drugs (Crevoiser *et al.*, 1997). As Pheroid™ technology consists primarily of essential fatty acids, this drug delivery system is expected to improve the bioavailability and thus efficacy of the lipophilic antimalarial drugs, mefloquine and artesunate.

The oral artesunate in Pheroid™ vesicles rendered much improved results. The peak plasma concentration of DHA in Pheroid™ vesicles was reached after 10 minutes of drug administration in relation to the reference group which was reached only after 30 minutes and the mean DHA peak plasma concentration of the Pheroid™ group (173.0 ng/ml) was greater than that of the reference group (105.0 ng/ml). The incorporation of mefloquine in a Pheroid™ vesicle formulation did not seem to have improved results in relation to the reference group. No statistical significant differences were observed in the pharmacokinetic parameters when comparing the mefloquine reference and Pheroid™ vesicle groups. The relative bioavailability (%) of the Pheroid™ vesicle incorporated mefloquine was 7% less bioavailable than the reference group. This observation is however in accordance with the observation made by Van Hoang Dao *et al* (2005), who observed that fatty food does not significantly improve the absorption of mefloquine.

SUMMARY AND FUTURE PROSPECTS

Many antimalarial drugs currently used for malaria chemotherapy are limited in efficacy due to poor aqueous solubility, low bioavailability, high toxicity and/or multiple drug resistance (Santos-Magalhães & Mosqueira, 2009). As an artemisinin derivative, the efficacy of artesunate is impaired by its rapid onset of action and fast elimination time, its low aqueous solubility and resultant poor and erratic absorption upon oral administration, as well as a high rate of recrudescence when used alone as monotherapy (Kongpatanakul *et al.*, 2007). Mefloquine is eliminated slowly with a terminal elimination half-life of approximately 20 days, which leads to increased toxicity. Mefloquine is associated with central nervous system side-effects, such as dizziness, headache and insomnia, which lead to poor patient compliance. Moreover, resistance to mefloquine was reported as early as five years after its introduction as a prophylactic agent in parts of Thailand (Wongsrichanalai *et al.*, 2002). There is thus an increasing need for the development of new antimalarial compounds, or optimisation of antimalarial therapy with existing drugs by formulation strategies.

Any possible improvement in the delivery of these antimalarial drugs must be exploited to improve the efficacy of current treatment regimens and to enhance the delivery of future antimalarial compounds. Alternative drug delivery options such as the nanosized lipid-based colloidal Pheroid™ drug delivery system may play a key role in ensuring effective delivery and enhanced bioavailability of current antimalarial compounds. Pheroid™ technology consists primarily of plant and fundamental fatty acids which have the ability to entrap, transport and deliver pharmaceutical compounds. The Pheroid™ drug delivery system has high affinity for cell membranes, thus resulting in effective and fast delivery of a drug beyond the mechanism of drug efflux pump often found in drug resistance (Grobler, 2004).

The first objective of this study was to investigate the potential efficacy enhancement of current antimalarial drugs, namely artesunate and mefloquine in combination with Pheroid™ vesicles against a chloroquine sensitive (3D7) strain of *P. falciparum*. *In vitro* antimalarial efficacy studies were conducted with both artesunate and mefloquine at various concentrations and under different experimental conditions, namely different Pheroid™ vesicle formulations, incubation periods and DNA-binding fluorescent dyes.

Artesunate evaluated against the chloroquine-sensitive (3D7) strain decreased parasite levels by ~51%, ~32% and ~80% in the reference groups of Experiments 1, 2 and 3 respectively. In

combination with Pheroid™ vesicles, parasite levels were decreased by ~93%, ~65% and ~85% for Experiments 1, 2 and 3 respectively. A statistical significant difference between the reference and Pheroid™ vesicle groups was obtained for Experiments 1 and 2, but not for Experiment 3. The IC₅₀ values decreased by approximately 90%, 72% and 29% for Experiments 1, 2 and 3 respectively. These results indicated that artesunate was most efficacious when co-formulated with Pheroid™ vesicles and evaluated over an assay incubation period of 48 hours.

Mefloquine evaluated against the chloroquine-sensitive (3D7) strain decreased parasite levels by ~78%, ~72% and ~63% in the reference groups of Experiments 1, 2 and 3 respectively. In combination with Pheroid™ vesicles, parasite levels were decreased by ~104%, ~88% and ~84% for Experiments 1, 2 and 3 respectively. A statistical significant difference between the reference and Pheroid™ vesicle groups was obtained for Experiments 2 and 3, but not for Experiment 1. The IC₅₀ values decreased by approximately 36%, 51% and 66% for Experiments 1, 2 and 3 respectively. These results indicated that the extended incubation period and 24 hour entrapment after manufacturing markedly improved the efficacy of mefloquine in the reference as well as Pheroid™ vesicles. In contrast with the results obtained for artesunate, the type of drug formulation, together with the Pheroid™ does not seem to influence the efficacy of mefloquine on 3D7 parasites.

An *in vivo* bioavailability study of artesunate and mefloquine in combination with Pheroid™ vesicles was conducted using a mouse model. A LC-MS/MS method was developed for analysing the pharmacokinetic properties of the study samples. The method was found to be sensitive, selective and accurate for the determination of artesunate and DHA in mouse plasma and mefloquine in mouse whole blood. The calibration curves had correlation (*r*) values of > 0.999, thus indicating their linearity over the concentration range. The chromatograms were found to be free from interferences. The extraction procedure yielded high recovery of the analytes, thus enabling the compounds to be quantified. Accuracy and precision of the method was found to be within the acceptable criteria as set by the US FDA guidelines for the validation of a bioanalytical method, where the determined drug concentrations should be within 85 – 115% of the actual values and the CV's should be < 15%.

Most of the artesunate plasma concentrations were below the limit of quantification in the reference group and some relatively high outliers were observed in some of the samples. The DHA plasma concentrations were also irregular. The mean artesunate levels in the Pheroid™ group were lower compared to the reference group, but the variation within the Pheroid™ group lessened significantly. The mean DHA concentrations in the Pheroid™ group improved

considerably and higher DHA levels were achieved at a faster time interval in this group. Pharmacokinetic models could not be constructed due to blood sampling per animal limitation. However, it is clear that the Pheroid™ formulation improved the bioavailability of DHA.

The incorporation of mefloquine in a Pheroid™ vesicle formulation did not seem to have improved results in relation to the reference group. No statistical significant differences were obtained in the pharmacokinetic parameters when comparing the mefloquine reference and Pheroid™ vesicle groups. The relative bioavailability (%) of the Pheroid™ vesicle incorporated mefloquine was 7% less bioavailable than the reference group. This observation is however in accordance with the observation made by Van Hoang Dao *et al* (2005), who observed that fatty food does not significantly improve the absorption of mefloquine. Different results might be obtained when an intravenous (IV) formulation of mefloquine in Pheroid™ vesicles are tested to determine absolute bioavailability. Although no differences in the bioavailability were observed, it is important to note that bioavailability is not indicative of *in vivo* efficacy. Pheroid™ vesicles may still play a role in the enhancement of *in vivo* efficacy.

Recommendations to consider for future studies regarding artesunate and mefloquine, as well as other antimalarial drugs in combination with Pheroid™ technology, may include the following:

- optimisation and standardisation of a flow cytometric method, investigating different DNA- binding fluorescent dyes to quantify parasite densities;
- development and validation of possible alternative methods to determine parasitemia in malaria cell cultures;
- combination therapy of artesunate and mefloquine should be investigated in an *in vitro* and *in vivo* model;
- optimisation and standardisation of a LC-MS/MS method specifically for application to pharmacokinetic studies in a mouse model;
- considering the short half-life of artesunate (< 10 minutes) and its active metabolite DHA (45 minutes to 1 hour) and the *in vivo* results obtained in this study, experiments with more saturated time points should be evaluated in an attempt to be able to construct pharmacokinetic models of these compounds;
- considering formulation of an IV formulation for evaluation *in vivo* to determine the absolute bioavailability of the experimental drugs;
- an *in vivo* efficacy study using a *Plasmodium berghei* mouse model to evaluate the effect of artesunate and mefloquine in combination with Pheroid™ technology.

When evaluating this project in its entirety, it can be considered to be a stepping stone with the aim of formulating a new dosage form for the treatment of malaria in the near future with mono- and combination therapy strategies.

ANNEXURE A

ETHICAL APPROVAL



NORTH-WEST UNIVERSITY
YUNIBESITHI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

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South Africa 2520

Tel: (018) 299-1111/2222
Web: <http://www.nwu.ac.za>

**UNIT FOR DRUG RESEARCH AND
DEVELOPMENT**

Tel: (018) 018 299-2274
Fax (018) 018 293-5219
EMail jeanetta.duplessis@nwu.ac.za

Research Ethics Committee

North-West University

Potchefstroom Campus

15 April 2008

Dear Ms Botha

FINAL RESPONSE: ETHICS APPLICATION: NWU-0008-08-S5

The abovementioned application has reference.

We have received satisfactory answers to all the questions posed by the Ethics panel and has therefore found the ethical aspects to be in order.

A handwritten signature in black ink, appearing to read 'J. du Plessis'.

**PROF. J. DU PLESSIS
DIRECTOR**

ANNEXURE B

PARTICLE SIZE ANALYSIS REPORTS



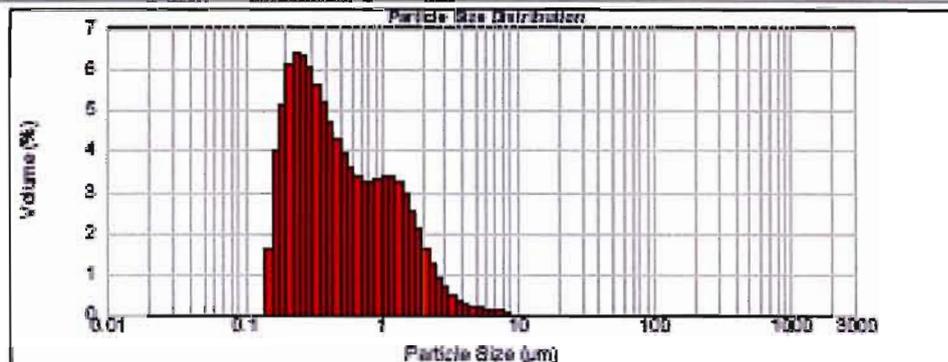
MASTERSIZER 2000

Result Analysis Report

Sample Name: Pharoid vesicles	SCP Name: Pharoid vesicles using Hydro 2000 MU	Measured: 13 March 2009 02:22:45 PM
Sample Source & type: V39004	Measured By: Liz-Marie	Analysed: 13 March 2009 02:22:45 PM
Sample bulk lot ref:	Result Source: Measurement	

Particle Name: Citic Acid	Accessory Name: Hydro 2000MU (A)	Analysis mode: General purpose	Sensitivity: Enhanced
Particle ID: 1.435	Absorption: 0.1	Size range: 0.020 to 2000.000 μm	Obscuration: 14.43 %
Dispersant Name: Water	Dispersion ID: 1.330	Weighted Residual: 10.054 %	Result Emulation: Off
Concentration: 0.0032 %Wol	Span : 3.355	Uniformity: 1.11	Result units: Volume
Specific Surface Area: 15.5 m^2/g	Surface Weighted Mean D(3,2): 0.367 μm	Vol. Weighted Mean D(4,3): 0.746 μm	

d(0.1): 0.197 μm d(0.5): 0.432 μm d(0.9): 1.549 μm



Pharoid vesicles, 13 March 2009 02:22:45 PM

Bin Cent	Bin Width	Sample Volume %	Bin Cent	Bin Width	Sample Volume %	Bin Cent	Bin Width	Sample Volume %	Bin Cent	Bin Width	Sample Volume %	
0.020	0.005	0.142	0.150	0.030	1.002	3.20	7.000	0.10	50.250	0.00	200.000	0.00
0.025	0.005	0.150	0.165	0.030	1.125	3.30	7.800	0.10	55.500	0.00	220.000	0.00
0.030	0.005	0.158	0.180	0.030	1.260	3.40	8.600	0.10	61.200	0.00	241.500	0.00
0.035	0.005	0.166	0.195	0.030	1.410	3.50	9.400	0.10	67.300	0.00	265.250	0.00
0.040	0.005	0.174	0.210	0.030	1.575	3.60	10.200	0.10	73.800	0.00	291.250	0.00
0.045	0.005	0.182	0.225	0.030	1.765	3.70	11.000	0.10	80.700	0.00	319.500	0.00
0.050	0.005	0.190	0.240	0.030	1.980	3.80	11.800	0.10	88.000	0.00	350.000	0.00
0.055	0.005	0.198	0.255	0.030	2.220	3.90	12.600	0.10	95.700	0.00	382.750	0.00
0.060	0.005	0.206	0.270	0.030	2.490	4.00	13.400	0.10	103.800	0.00	417.750	0.00
0.065	0.005	0.214	0.285	0.030	2.790	4.10	14.200	0.10	112.200	0.00	455.000	0.00
0.070	0.005	0.222	0.300	0.030	3.120	4.20	15.000	0.10	120.900	0.00	494.750	0.00
0.075	0.005	0.230	0.315	0.030	3.480	4.30	15.800	0.10	130.000	0.00	537.000	0.00
0.080	0.005	0.238	0.330	0.030	3.870	4.40	16.600	0.10	139.500	0.00	581.750	0.00
0.085	0.005	0.246	0.345	0.030	4.290	4.50	17.400	0.10	149.400	0.00	629.000	0.00
0.090	0.005	0.254	0.360	0.030	4.740	4.60	18.200	0.10	159.600	0.00	678.750	0.00
0.095	0.005	0.262	0.375	0.030	5.220	4.70	19.000	0.10	170.100	0.00	731.000	0.00
0.100	0.005	0.270	0.390	0.030	5.730	4.80	19.800	0.10	180.900	0.00	785.750	0.00
0.105	0.005	0.278	0.405	0.030	6.270	4.90	20.600	0.10	192.000	0.00	843.000	0.00
0.110	0.005	0.286	0.420	0.030	6.840	5.00	21.400	0.10	203.400	0.00	902.250	0.00
0.115	0.005	0.294	0.435	0.030	7.440	5.10	22.200	0.10	215.100	0.00	963.500	0.00
0.120	0.005	0.302	0.450	0.030	8.070	5.20	23.000	0.10	227.100	0.00	1026.750	0.00
0.125	0.005	0.310	0.465	0.030	8.730	5.30	23.800	0.10	239.400	0.00	1093.000	0.00
0.130	0.005	0.318	0.480	0.030	9.420	5.40	24.600	0.10	252.000	0.00	1162.250	0.00
0.135	0.005	0.326	0.495	0.030	10.140	5.50	25.400	0.10	264.900	0.00	1234.500	0.00

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



MASTERSIZER 2000

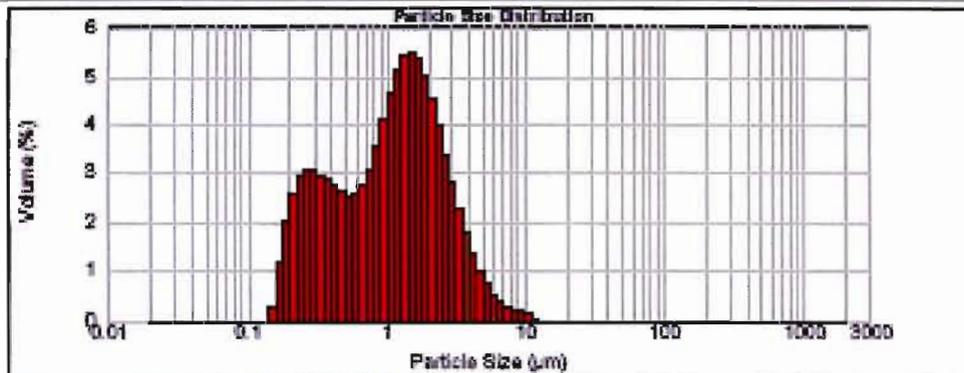
Result Analysis Report

Sample Name: Phorbol vesicles
Sample Source & type: V09006
Sample bulk lot ref:
SDP Name: Phorbol vesicles using Hydro 2000 MU
Measured by: Lion-Maria
Result Source: Measurement
Measured: 13 March 2009 02:35:18 PM
Analyzed: 13 March 2009 02:35:18 PM

Particle Name: Clioquinol
Particle RI: 1.458
Dispersion Name: Water
Accessory Name: Hydro 2000MU (A)
Absorption: 0.1
Dispersion RI: 1.330
Analyse model: General purpose
Size range: 0.020 to 2000.000 um
Weighted Residual: 0.079 %
Sensitivity: Enhanced
Obscuration: 17.05 %
Result Evaluation: Off

Concentration: 0.0043 %Vol
Specific Surface Area: 0.00 m²/g
Span : 2.444
Surface Weighted Mean D[0,3]: 0.882 um
Uniformity: 0.81
Vol. Weighted Mean D[4,3]: 1.465 um
Result units: Volume

d[0.1]: 0.281 um d[0.5]: 1.153 um d[0.9]: 3.029 um



Phorbol vesicles, 13 March 2009 02:35:18 PM

Size (um)	Volume (%)								
0.020	0.00	0.142	0.26	1.002	4.87	7.088	0.00	57.252	0.00
0.022	0.00	0.156	1.10	1.125	0.14	7.882	0.24	63.586	0.00
0.025	0.00	0.170	2.00	1.262	0.48	8.804	0.19	65.292	0.00
0.028	0.00	0.185	2.81	1.410	0.86	9.858	0.10	68.000	0.00
0.032	0.00	0.201	2.81	1.566	0.92	11.047	0.16	71.827	0.00
0.036	0.00	0.218	2.95	1.732	0.76	12.378	0.00	76.887	0.00
0.040	0.00	0.237	3.07	1.907	0.75	13.858	0.00	83.237	0.00
0.045	0.00	0.257	3.07	2.090	4.00	15.492	0.00	90.970	0.00
0.050	0.00	0.279	3.07	2.281	4.00	17.287	0.00	99.997	0.00
0.056	0.00	0.302	2.80	2.480	3.40	19.250	0.00	110.347	0.00
0.062	0.00	0.328	2.74	2.687	2.81	21.390	0.00	122.380	0.00
0.069	0.00	0.356	2.80	2.902	2.26	23.714	0.00	136.133	0.00
0.077	0.00	0.387	2.90	3.133	1.11	26.229	0.00	151.630	0.00
0.085	0.00	0.420	2.90	3.381	1.31	28.942	0.00	168.890	0.00
0.094	0.00	0.455	2.75	3.647	1.00	31.862	0.00	187.936	0.00
0.103	0.00	0.493	2.80	3.930	1.00	35.000	0.00	208.780	0.00
0.112	0.00	0.534	2.80	4.231	0.71	38.367	0.00	231.430	0.00
0.121	0.00	0.578	2.85	4.550	0.74	41.974	0.00	255.890	0.00
0.131	0.00	0.625	4.10	4.886	0.40	45.830	0.00	282.160	0.00

Operator notes: Add 2ml vesicle and allow the obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30% before measurement is accepted.

ANNEXURE C

CLSM MICROGRAPHS

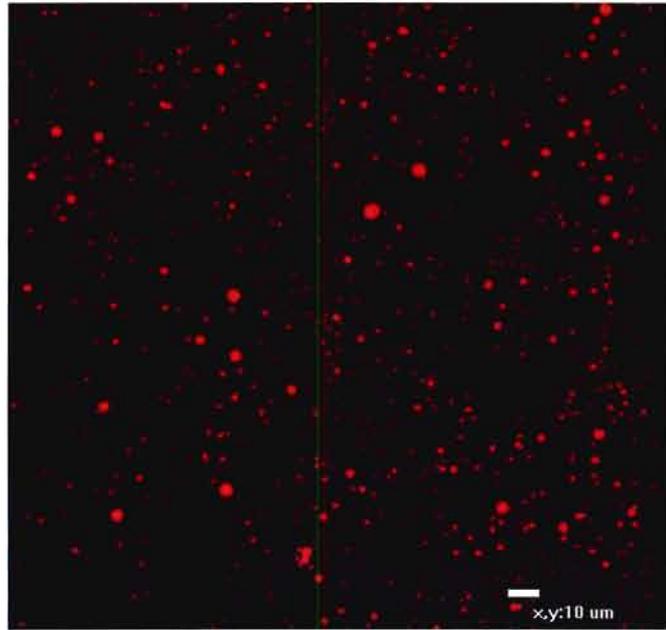


Figure C.1: CLSM micrograph of Pheroid vesicles (Batch number: V09004).

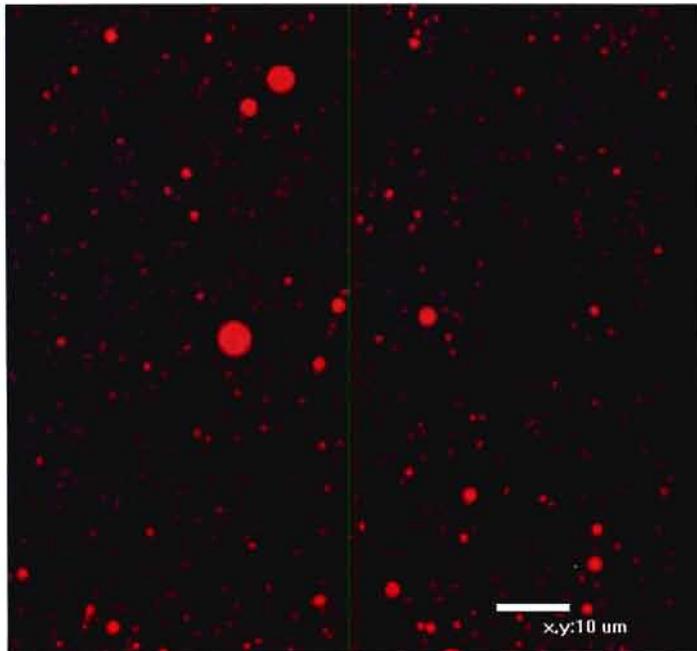


Figure C.2: CLSM micrograph of Pheroid vesicles (Batch number: V09006).

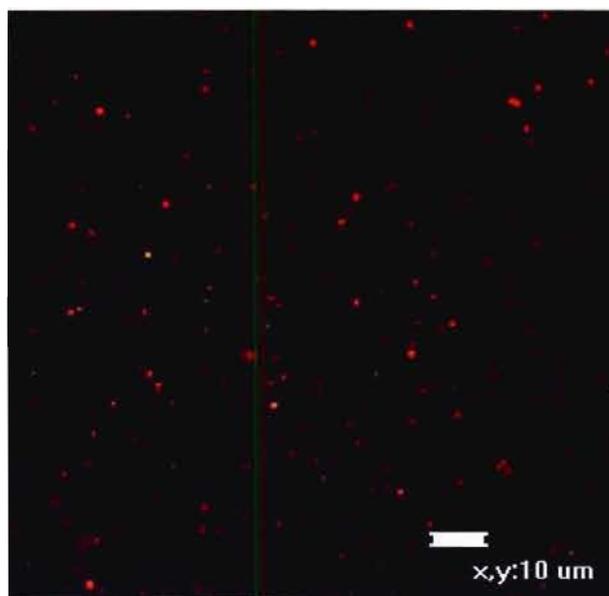


Figure C.3: CLSM micrograph of artesunate entrapped (green fluorescence) in Pheroid vesicles (red fluorescence) (Batch number: V09004).

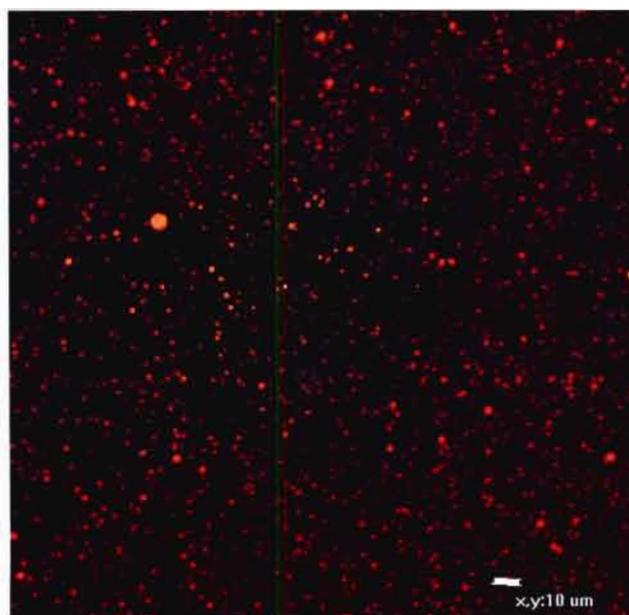


Figure C.4: CLSM micrograph of mefloquine entrapped (green fluorescence) in Pheroid vesicles (red fluorescence) (Batch number: V09006).

ANNEXURE D

CERTIFICATES OF ANALYSIS



益飞(香港)有限公司

IFFECT CHEMPHAR (HK) COMPANY LIMITED

Address: 6TH FLOOR, FLAT A, BLOCK F, SUNNING BUILDING, SHATIN, N.T., HONGKONG, P.R.CHINA.
E-mail: sziffect@iffchem.com.cn iffect@iffchem.com.cn sziffect@hotmail.com
Website: www.iffchem.com.cn

CERTIFICATE OF ANALYSIS

PRODUCT NAME:	ARTESUNATE	BATCH NO.:	IF-AR-080830
QUANTITY:	1KG	CAS NO.:	88495-63-0
MFG. DATE:	AUG.30, 2008	EXPIRY DATE:	AUG.29, 2011
ITEMS	SPECIFICATION	RESULTS	
DESCRIPTION	WHITE, FINE CRYSTALLINE POWDER	CONFORMS	
IDENTIFICATION	HPLC	CONFORMS	
MELTING POINT	130°C ~ 135°C	133°C	
SPECIFIC ROTATION	+10° ~ +14°	CONFORMS	
PH (1% SUSPENSION)	3.5 ~ 5.0	4.1	
LOSS ON DRYING	NOT MORE THAN 0.5%	0.13%	
RELATED SUBSTANCES	OTHER RELATED SUBSTANCES: NOT MORE THAN 1.0% DIHYDROARTEMISININ: NOT MORE THAN 1.0%	<1.0% <1.0%	
HEAVY METALS	NOT MORE THAN 20PPM	<20PPM	
SULFATE ASH	NOT MORE THAN 0.1%	0.04%	
CHLORIDE	NOT MORE THAN 0.02%	<0.02%	
ASSAY	98.0% ~ 102.0% (C ₁₅ H ₂₈ O ₈)	99.87%	
CONCLUSION:	THE RESULTS CONFORM TO THE ENTERPRISE STANDARD.		

DIRECTOR OF QC. SECTION: ANALYSED BY: CHECKED BY:
CATHERINE SPRING VIVIAN

STORAGE: PRESERVE IN WELL-CLOSED, LIGHT-RESISTANT AND TIGHT CONTAINERS.

STORE IN COOL & DRY PLACE

Iffect Chemphar (HK) Company Limited



益飞(香港)有限公司

IFFECT CHEMPHAR (HK) COMPANY LIMITED

Address: 6TH FLOOR, FLAT A, BLOCK F, SUNNING BUILDING, SHATIN, N.T., HONGKONG, P.R.CHINA.
E-mail: sziffect@iffchem.com.cn iffact@iffchem.com.cn sziffect@hotmail.com
Website: www.iffchem.com.cn

CERTIFICATE OF ANALYSIS

PRODUCT NAME:	MEFLOQUINE HYDROCHLORIDE	BATCH NO.:	IF-ME-080830
QUANTITY:	1KG	CAS NO.:	51773-92-3
MFG. DATE:	AUG.30, 2008	EXPIRY DATE:	AUG.29, 2011
ITEMS	SPECIFICATION	RESULTS	
DESCRIPTION	WHITE OR SLIGHTLY YELLOW CRYSTALLINE POWDER	CONFORMS	
SOLUBILITY	FREELY SOLUBLE IN METHANOL, SOLUBLE IN ALCOHOL, VERY SLIGHTLY SOLUBLE IN WATER	CONFORMS	
IDENTIFICATION	A. IR B. IT RESPONDS TO THE TESTS FOR CHLORIDE	CONFORMS	
OPTICAL ROTATION	-0.2° ~ +0.2°	-0.18°	
WATER	NOT MORE THAN 3.0%	2.28%	
RESIDUE ON IGNITION	NOT MORE THAN 0.1%	<0.1%	
HEAVY METALS	NOT MORE THAN 20PPM	<20PPM	
RELATED COMPOUNDS	RELATED COMPOUND A: NOT MORE THAN 0.2% ANY OTHER INDIVIDUAL IMPURITIES: NOT MORE THAN 0.1% TOTAL IMPURITIES: NOT MORE THAN 0.5%	0.04% 0.07% 0.12%	
RESIDUAL SOLVENTS	ETHANOL: NOT MORE THAN 5000PPM METHANOL: NOT MORE THAN 3000PPM ACETIC ETHER: NOT MORE THAN 5000PPM ACETONE: NOT MORE THAN 5000PPM	NOT DETECTED NOT DETECTED NOT DETECTED NOT DETECTED	
ASSAY	99% ~ 101.0% (C ₁₇ H ₁₆ F ₆ N ₂ O·HCl)	100.78%	
CONCLUSION	THE RESULTS CONFORM TO THE STANDARD OF USP29.		

DIRECTOR OF QC. SECTION:
CATHERINE

ANALYSED BY:
SARAH

CHECKED BY:
VIVIAN

STORAGE: PRESERVE IN WELL-CLOSED, LIGHT-RESISTANT AND TIGHT CONTAINERS.

STORE IN COOL & DRY PLACE.

Iffect Chemphar (HK) Company Limited

ANNEXURE E

DATA TABLES

Table E.1: *In vitro* activity of artesunate against a 3D7 chloroquine-sensitive strain, Experiment 1.

Conc. [nM]	Reference			Mean	SEM	Pheroid vesicles			Mean	SEM
0.0	100.00	100.00	100.00	100.00	0.00	42.03	153.96	145.37	113.78	35.96
2.5	104.75	94.82	87.32	95.63	5.04	121.01	128.27	115.76	121.68	3.62
5.0	89.07	101.75	96.58	95.80	3.68	58.88	53.54	52.71	55.04	1.93
10.0	59.04	91.65	84.48	78.39	9.89	49.70	53.79	61.05	54.85	3.31
25.0	99.08	94.32	93.16	95.52	1.81	65.22	46.70	50.12	54.01	5.68
50.0	84.65	68.89	77.56	77.03	4.55	45.37	41.95	38.53	41.95	1.97
75.0	84.48	81.98	67.63	78.03	5.24	45.53	35.69	43.28	41.50	2.97
100.0	77.14	62.46	68.39	69.33	4.26	44.62	37.03	28.10	36.58	4.77
200.0	42.28	46.03	57.29	48.54	4.51	25.93	19.59	16.76	20.76	2.71

Table E.2: *In vitro* activity of artesunate against a 3D7 chloroquine-sensitive strain, Experiment 2.

Conc. [nM]	Reference			Mean	SEM	Pheroid vesicles			Mean	SEM
0.0	38.97	70.86	105.51	71.78	19.21	53.14	111.02	82.28	82.15	16.70
1.0	180.31	113.38	131.49	141.73	19.98	118.50	91.73	67.32	92.51	14.77
5.0	147.63	122.04	117.71	129.13	9.33	72.83	90.15	102.75	88.58	8.67
15.0	105.90	98.03	85.03	96.32	6.08	60.23	50.78	58.66	56.56	2.92
25.0	100.00	100.00	33.85	77.95	22.04	63.38	76.37	48.81	62.86	7.95
50.0	77.55	48.03	64.56	63.38	8.54	44.09	27.16	35.03	35.43	4.89
75.0	51.18	59.84	15.74	42.25	13.48	22.83	44.09	39.37	35.43	6.44
100.0	41.73	39.76	55.51	45.66	4.95	25.59	20.86	41.73	29.39	6.31
200.0	41.73	40.94	44.09	42.25	0.94	14.96	9.84	9.84	11.54	1.70
500.0	42.12	40.55	37.79	40.15	1.26	21.25	16.14	14.96	17.45	1.93

Table E.3: *In vitro* activity of artesunate against a 3D7 chloroquine-sensitive strain, Experiment 3.

Conc. [nM]	Reference		Mean	SEM	Pheroid vesicles		Mean	SEM
0.00	100.00	100.00	100.00	0.00	88.38	125.46	106.92	18.53
0.30	71.16	96.62	83.89	12.73	79.40	110.86	95.13	15.73
0.60	65.54	63.67	64.60	0.93	75.65	64.04	69.85	5.80
1.25	45.31	61.79	53.55	8.23	46.44	59.17	52.80	6.36
2.50	55.05	47.19	51.12	3.93	62.92	48.68	55.80	7.11
5.00	43.07	40.44	41.76	1.31	40.44	41.19	40.82	0.37
10.00	36.32	38.95	37.64	1.31	33.33	35.95	34.64	1.31
20.00	27.71	25.46	26.59	1.12	33.70	23.22	28.46	5.24
40.00	28.08	25.09	26.59	1.49	23.22	22.47	22.84	0.37
80.00	20.97	19.10	20.03	0.93	18.72	13.48	16.10	2.62

Table E.4: *In vitro* activity of mefloquine against a 3D7 chloroquine-sensitive strain, Experiment 1.

Conc. [nM]	Reference			Mean	SEM	Pheroid vesicles			Mean	SEM
0.0	100.00	100.00	100.00	100.00	0.00	90.29	100.00	159.22	116.50	21.54
2.5	133.00	165.04	109.70	135.92	16.04	123.30	45.63	102.91	90.61	23.24
5.0	87.37	56.31	37.22	60.30	14.61	86.40	76.69	47.57	70.22	11.66
10.0	70.87	38.83	73.78	61.16	11.19	52.42	45.63	21.35	39.80	9.42
20.0	36.89	44.66	49.51	43.68	3.67	28.15	42.71	42.71	37.86	4.85
50.0	38.83	33.00	37.86	36.56	1.80	24.27	41.74	30.09	32.03	5.13
100.0	33.98	37.86	30.09	33.98	2.24	17.47	12.62	13.59	14.56	1.48
200.0	29.12	20.38	36.89	28.80	4.76	16.50	24.27	15.53	18.77	2.76
500.0	24.27	19.41	23.30	22.33	1.48	11.65	18.44	6.79	12.29	3.37

Table E.5: *In vitro* activity of mefloquine against a 3D7 chloroquine-sensitive strain, Experiment 2.

Conc. [nM]	Reference			Mean	SEM	Pheroid vesicles			Mean	SEM
0.0	68.33	107.77	137.77	104.62	20.10	63.33	128.33	140.55	110.74	23.96
5.0	122.22	72.22	96.66	97.03	14.43	92.22	110.55	118.33	107.03	7.74
10.0	63.88	71.66	85.00	73.51	6.16	147.22	104.44	153.33	135.00	15.37
25.0	68.88	59.44	60.00	62.77	3.05	82.77	101.11	98.33	94.07	5.70
50.0	53.88	61.66	70.00	61.85	4.65	105.55	82.77	65.55	84.62	11.58
75.0	67.77	34.44	45.00	49.07	9.83	24.44	66.66	33.88	41.66	12.79
100.0	48.33	31.66	55.00	45.00	6.93	48.88	21.11	31.11	33.70	8.12
200.0	53.88	52.77	46.66	51.11	2.24	42.77	27.22	25.55	31.85	5.48
500.0	60.55	62.77	60.55	61.29	0.74	43.88	33.88	35.00	37.59	3.16
1000	20.55	46.111	30.55	32.40	7.43	32.77	22.77	13.88	23.14	5.45

Table E.6: *In vitro* activity of mefloquine against a 3D7 chloroquine-sensitive strain, Experiment 3.

Conc. [nM]	Reference		Mean	SEM	Pheroid vesicles		Mean	SEM
0.0	100.00	100.00	100.00	0.00	109.36	129.21	119.28	9.92
1.0	65.16	97.75	81.46	16.29	92.50	111.23	101.87	9.36
3.0	98.12	88.76	93.44	4.68	79.40	65.91	72.65	6.74
6.0	61.42	72.28	66.85	5.43	58.80	73.03	65.91	7.11
12.0	77.52	74.53	76.02	1.49	72.65	54.68	63.67	8.98
25.0	76.77	67.04	71.91	4.86	63.29	53.18	58.23	5.05
50.0	71.16	56.92	64.04	7.11	44.19	53.93	49.06	4.86
100.0	56.92	67.41	62.17	5.24	46.44	38.20	42.32	4.11
200.0	48.68	59.55	54.11	5.43	44.56	37.45	41.01	3.55
400.0	40.82	33.33	37.07	3.74	35.20	34.45	34.83	0.37

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