NOVEL ARTEMISININ DERIVATIVES WITH PHEROID™ TECHNOLOGY FOR MALARIA TREATMENT

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Novel artemisinin derivatives with Pheroid™ technology for malaria treatment

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Destiny is not a matter of chance, it is a matter of choice.

It is not a thing to be waited for, it is a thing to be achieved.

William Jennings Bryan

Dedicated to my

parents,

Johan and Cobi Steyn

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ABSTRACT

Artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration. The poor solubility and erratic absorption usually translate to low bioavailability. Enzymatic degradation and physiological barriers are also amongst the challenges which must be overcome to ensure effective delivery. Artemisinin-based monotherapy and combination therapies are essential for the management and treatment of uncomplicated as well as cerebral malaria. Artemisone and artemiside are novel artemisinin derivatives, their antimalarial activity/efficacy was evaluated *in vitro* and *in vivo* in the presence and absence of Pheroid™ technology. Pheroid™ technology is a patented drug delivery system which has the ability to capture, transport and deliver pharmacologically active compounds. Pharmacokinetic models were also constructed for artemisone and artemiside, both in the presence and absence of Pheroid™ technology.

Results obtained with the *in vitro* antimalarial activity evaluation indicated that artemiside was slightly more potent than artemisone and much more potent than artesunate. Artemiside had IC_{50} values of 0.54 ± 0.03 nM (reference) and 0.10 ± 0.05 nM (Pheroid™) ($p = 0.009$) while artemisone had values of 0.94 ± 0.04 nM (reference) and 0.21 ± 0.04 nM (Pheroid™) ($p = 0.0001$). Artesunate had IC_{50} values of 29.65 ± 0.05 nM (reference) and 10.20 ± 0.04 nM (Pheroid™) ($p < 0.0001$).

Results obtained with the *in vivo* antimalarial activity evaluation indicated that artemisone led to more favourable treatment outcomes than artemiside. The Peters' 4-day suppressive test was used as a basis model. With artemisone treatment recrudescence occured at 16 days post infection at a dose of 20.0 mg/kg bodyweight and at 12 days post infection at 2.5 mg/kg bodyweight. With artemiside recrudescence occured at 8 days post infection with both the 10.0 mg/kg and 2.5 mg/kg bodyweight treatment regimens. When comparing the antimalarial effect of the drugs with and without Pheroid™ technology there was no significant difference in terms of parasite reduction or in the achieved treatment outcomes of either compounds.

The pharmacokinetic parameters were evaluated in a mouse model where C57 BL6 mice were used. The compounds were administered at a dose of 50.0 mg/kg bodyweight via an oral gavage tube at a volume of 200 µl. Blood samples were colleted by means of tail-bleeding. Sensitive and selective LC/MS/MS methods were developed to analyze the drug concentrations in the plasma samples. The relative bioavailability of artemisone was RA = 1.0 (reference) and RA = 4.57 (Pheroid™) ($p < 0.001$). The absolute bioavailability was

calculated as F = 0.10 (reference) and F = 0.48 (Pheroid™) ($p < 0.001$). The boiavailability of artemiside was not dramatically enhanced by the Pheroid™ delivery system.

Keywords: Malaria, Artemisone, Artemiside, Pheroid™ technology, *In vitro* efficacy, *In vivo* efficacy, Pharmacokinetic parameters.

UITTREKSEL

Artemisien derivate is bekend vir hul swak water-oplosbaarheid met gevolglike swak en wisselvallige absorpsie na orale toediening. Die swak oplosbaarheid en wisselvallige absorpsie lei tot lae biobeskikbaarheid. Ensiematiese afbraak en fisiologiese skanse dra ook by tot die uitdaging om hierdie middels suksesvol af te lewer. Artemisien gebasseerde mono- en kombinasie terapie word tans gebruik vir die behandeling van beide ongekompliseerde en serebrale malaria. Artemisoon en artemisied is nuwe artemisien derivate, hulle aktiwiteit/effektiwiteit teen malaria was ondersoek in *in vitro* sowel as *in vivo* modelle. Die middels was toegedien in 'n kontrole en 'n Pheroid™ formulering. Pheroid™ tegnologie is 'n gepatenteerde sisteem wat oor die vermoë beskik om geneesmiddels te enkapsuleer, te vervoer en af te lewer. Famakokinetiese modelle is ook saamgestel vir beide middels, geformuleer met en sonder Pheroid™ tegnologie.

Aktiwiteitsdata het aangetoon dat artemisied meer aktief was tydens die *in vitro* evaluering as artemisoon en baie meer aktief was as artesunaat. Artemisied het IC_{50} waardes opgelewer van 0.54 ± 0.03 nM (kontrole) en 0.10 ± 0.05 nM (Pheroid™) ($p = 0.009$) terwyl artemisoon waardes van 0.94 ± 0.04 nM (kontrole) en 0.21 ± 0.04 nM (Pheroid™) ($p = 0.0001$) opgelewer het. Artesunaat het IC_{50} waardes van 29.65 ± 0.05 nM (kontrole) en 10.20 ± 0.04 nM (Pheroid™) ($p < 0.0001$) opgelewer.

Die resultate van die *in vivo* evaluering het aangetoon dat artemisoon meer aktief was as artemisied. Die sogenaamde Peters' 4-dag toets was gebruik as basis-model. Na artemisoon behandeling het her-opvlamming van die infeksie plaasgevind 16 dae na infektering en behandeling teen 20.0 mg/kg liggaamsmassa en 12 dae na infektering en behandeling teen 2.5 mg/kg liggaamsmassa. Artemisied was minder effektief, her-opvlamming van die infeksie het plaasgevind 8 dae na infektering in beide die 10.0 mg/kg en die 2.5 mg/kg liggaamsmassa behandelingsgroepe. Daar was geen noemenswaardige verskille tussen die kontrole en Pheroid™ formulerings gewees in terme van behandelings-uitkomste nie.

Farmakokinetiese evaluerings was uitgevoer in 'n C57 BL6 muis-model. Die middels was oraal toegedien teen 50.0 mg/kg liggaamsmassa met behulp van 'n orale maagspoelbuis in 'n volume van 200 µl. Bloedmonsters was op voorafbepaalde tye geneem deur middel van stert-bloeding en die plasma was daarna ontleed met 'n sensitiewe en selektiewe LC/MS/MS metode. Die relatiewe biobeskikbaarheid van artemisoon was RA = 1.0 (kontrole) en RA =

4.57 (Pheroid™) ($p < 0.001$). The absolute biobeskikbaarheid was bereken as $F = 0.10$ (kontrole) en $F = 0.48$ (Pheroid™) ($p < 0.001$). Pheroid™ tegnologie het nie gelei tot enige noemenswaardige verhogings in die biobeskikbaarheid van artemisied nie.

Sleutelwoorde: Malaria, Artemisoon, Artemisied, Pheroid™ tegnologie, *In vitro* effektiwiteit, *In vivo* effektiwiteit, Farmakokinetiese waardes.

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INTRODUCTION AND AIM OF STUDY

South Africa has an estimated population of 40 – 50 million people and approximately 10%, or roughly 4 million, of these people live in malaria affected areas (WHO, 2006a). The effects of malaria presented much worse in the past, in comparison with current statistics. In 1932, for example, 22 132 deaths were recorded in KwaZulu-Natal with a population of 1 819 000 million people, effectively rendering a mortality of 1.2%. Disease incidence rates were equally devastating for Mpumalanga and the Limpopo province during that period (Hay *et al.,* 2004). Currently, South Africa's malaria status is well within containable boundaries. Control measures, including chemoprophylaxis and treatment regimes, are firmly in place and with the scientific resources and adequate funding available, the South African Department of Health can ensure that this deadly disease will be managed according to international standards (WHO, 2006a).

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 which 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 (World Health Organization (WHO), 2009).

Drug-resistant malaria is a huge threat and desperate measures should be taken to ensure the preservation of effectiveness of current antimalarial drugs (White, 2004). Drug-resistant malaria materializes with evolutionary, single or multiple, point-mutations in the *Plasmodium* genome rendering parasites that are drug insensitive (White, 2004). The emergence and spread of this phenomenon has greatly affected the control and treatment of malaria in endemic countries, specifically concerning *P. falciparum* infections which account for most of the disease burden (WHO, 2006a). Combination drug therapy is currently the mainstay approach in preventing the development of further resistance to current antimalarials. Artemisinin-based combination therapy is the treatment of choice and it is of great importance that the efficacy of those therapeutic regimens are maintained. There is presently no other effective alternatives to surmount the ever increasing problem of drug resistance, it is thus essential to focus all efforts on the research and development of novel antimalarial compounds (Baird, 2005; Mutabingwa, 2005).

Artemisinins are natural products and was first developed in China in the 1960's. Artemether, an artemisinin derivative, is known to be as effective as quinine in the treatment of severe *P. falciparum* malaria. Other artemesinin derivatives include artesunate, artemotil and dihydro-artemisinin. One key advantage of these agents is the fact that they are active against all of the red blood cell stages of *P. falciparum* (Krishna *et al.*, 2006). At this stage there is limited, if any, resistance to these agents. Due to the short elimination half-life of artemisinin-based drugs it is recommended that they are used in combination with other drugs such as mefloquine, lumefantrine or amodiaquine as first-line therapies for the treatment of uncomplicated malaria (White, 2004). The new artemisinin derivatives, artemisone and artemiside, are reported to be much more potent than the existing derivatives and it would be of great value to investigate both the *in vitro* and *in vivo* efficacy of these compounds against both *P. falciparum* and *P. berghei* (Haynes *et al.*, 2006). This thesis will describe studies in this regard in terms of novel drug delivery technology.

Alternative drug delivery options such as the Pheroid™ delivery system may play a key role in ensuring effective delivery and enhanced bioavailability of these novel antimalarial compounds. Pheroid™ technology is a patented, novel, colloidal type drug delivery system. It primarily consists of plant and fundamental fatty acids that have the ability to capture, transport and deliver pharmacologically active compounds and other valuable molecules (Grobler, 2004). The Pheroid™ delivery system is superior to most other delivery systems and is able to improve the delivery of dynamic complexes, reduce the time to onset of action, decrease the minimal effective drug concentration and enhance therapeutic efficacy. It is also able to indirectly decrease the cytotoxicity of therapeutic compounds and to infiltrate virtually all known barriers in the body. The system is further capable of targeting specific treatment areas, to transport genetic material to the cellular nucleus and to decrease drug resistance (Grobler, 2004). The Pheroid™ delivery system, based on Pheroid™ technology, is a patented system but will for ease of reading be referred to only as Pheroid(s) throughout the rest of this thesis.

The specific literature objectives of this study are to conduct literature studies on:

- malaria;
- artemisinin and derivatives thereof;
- Pheroid technology;

A literature overview in this regard will be presented in Chapters 1 – 3.

The specific experimental objectives are to:

- formulate suitable Pheroid vehicles for artemisone and artemiside;
- entrap the experimental compounds in the formulated Pheroid vehicle;
- conduct *in vivo* and *in vitro* experiments to evaluate the antimalarial activity of these novel compounds, alone and in combination with Pheroid technology;
- to construct pharmacokinetic models for artemisone and artemiside in the presence and absence of the formulated Pheroid vehicles.

The following pharmacokinetic parameters will be calculated using non-compartmental models:

- plasma peak concentration (C_{max}) in ng/ml;
- time to plasma peak concentration (T_{max}), only for oral dose experiments;
- apparent elimination half-life ($T_{1/2}$);
- area under the plasma concentration-time curve ($AUC_{0\text{-}last}$) in ng.h/ml;
- area under the plasma concentration-time curve ($AUC_{0\text{-}inf}$) in ng.h/ml;
- relative bioavailability and
- absolute bioavailability.

The experimental data will be presented and discussed in Chapters 4 – 5.

CHAPTER 1

MALARIA

1.1 INTRODUCTION

Malaria is a protozoan disease transmitted by the female anopheline mosquito. Transmission is accomplished by the bite of the blood-feeding female. Parasites from the genus *Plasmodium* are responsible for the greater majority of human infections. The most infectious are *P. falciparum* which account for the most instances of morbidity and mortality (World Health Organization (WHO), 2006a).

The exact incidence and prevalence are difficult to calculate but most statistics indicate that approximately 270 – 300 million people suffer from malaria and that at least 1 million people die from malaria annually. It is estimated that malaria is prevalent in 88 countries, this figure amounts to approximately 27% of the Earth's land surface (Hay *et al.*, 2004).

In previous years malaria case management has largely relied upon antimalarial drugs such as chloroquine and sulfadoxine-pyrimethamine combinations which are inexpensive and easy to obtain. The extensive deployment of these antimalarial drugs prompted evolutionary changes in the parasites which led to the emergence of various mechanisms of resistance (Hay *et al.*, 2004). Resistance has already developed to most of the major antimalarial drug classes. Artemisinin resistance has also been reported but to a much lesser extent (Dondorp *et al.*, 2009). This drug class are employed in combination therapies against multidrug-resistant malaria which increases the risk of *P. falciparum* developing more pronounced resistance against artemisinin therapy in the near future (WHO, 2001).

Against this background it is clearly essential that new artemisinin derivatives, and other promising compounds, should be critically evaluated in the ongoing fight against malaria. Novel delivery systems may also play a very valuable role in the effective delivery of these compounds and may be instrumental in the revival of some of the older and obsolete antimalarial compounds.

1.2 DISTRIBUTION AND ENDEMICITY

Female *Anopheles* mosquitoes are prevalent throughout Africa, Asia and Latin America. These mosquitoes act as both a vector and a secondary host for various *Plasmodium* species. Four species of the genus *Plasmodium* are responsible for the majority of human infections namely:

- *P. vivax*
- *P. ovale*
- *P. malariae*
- *P. falciparum*

Of these four species *P. falciparum* are the most infectious and is accountable for the most malaria related deaths on the African continent and elsewhere (Gkrania-Klotsas & Lever, 2006).

Malaria affects nearly 300 million people worldwide per annum and a further 2 billion more are presently susceptible to malaria (Hay *et al.*, 2004). The disease is responsible for a death toll of approximately 1 million people annually and children under the age of 5 years are very often among the victims (Root, 1999).

The measure of disease prevalence in a specific region is defined by the term endemicity. Endemicity is divided in four sub-categories and is defined by the World Health Organization (WHO) as follows:

- **holoendemic** - parasite rate in children of 2 – 9 years of age constantly above 75% but low in adults;
- **hyperendemic** - parasite rate in children of 2 – 9 years of age constantly above 50%;
- **mesoendemic** - parasite rate in children of 2 – 9 years of age between 11% and 50%;
- **hypoendemic** - parasite rate in children of 2 – 9 years of age do not exceed 10% (WHO, 2003).

A global model, divided in regional sub-levels, indicate that most *P. falciparum* attributable clinical events were concentrated in the African region in 2002. The African region amounted

to approximately 70% of the clinical events and stands in contrast to the densely populated region of South East Asia which only contributed to 25% of the total global clinical attacks (Snow *et al.*, 2005).

The following tables suggest a very strong correlation between the various endemicity classes and the incidence of clinical malaria attacks. Table 1.1 represents various regions, known to be affected by malaria, and its respective indemecity classification. Table 1.2 includes estimated data representing clinical malaria cases. In both tables the numerical values are represented in terms of million(s). These tables were adapted from Snow *et al.* (2005) and were originally compiled by the WHO.

Table 1.1: Populations at risk (Snow *et al.*, 2005)

Region	Population in *P. falciparum* endemicity classes				
	Unclassified	Hypo-endemic	Meso-endemic	Hyper-endemic and holo-endemic	Total population at risk
Africa	13.6	39.3	67.4	414.3	521.0
Americas	3.5	43.9	10.5	0	54.5
South East Asia	47.8	827.6	486.0	0.3	1 313.9
Western Pacific	22.4	77.6	63.4	1.0	142.0
Eastern Mediterranean	32.3	143.0	33.4	0	176.4
Europe	1.1	0.3	3.2	0	3.5
Total world	120.7	1 131.7	663.9	415.6	2 211.3

Table 1.2: Estimated data for *P. falciparum* clinical malaria cases (Snow *et al.*, 2005)

Parameter	Hypoendemic	Mesoendemic	Hyperendemic and holoendemic	Total *P. falciparum* cases
Attack rate (per 1 000 population per year) Cases per WHO region (millions)	43 (6 – 117)	171 (125 – 261)	849 [500]	-
Africa	1.69 (0.24 – 4.60)	11.52 (8.42 – 17.58)	351.77 (207.17 – 351.77)	364.98 (215.82 – 373.95)
Americas	1.89 (0.26 – 5.14)	1.80 (1.32 – 2.75)	0	3.69 (1.58 – 7.89)
South East Asia	35.59 (4.97 – 96.83)	83.11 (60.76 – 126.86)	0.24 (0.14 – 0.24)	118.94 (65.86 – 223.93)
Western Pacific	3.34 (0.46 – 9.08)	10.84 (7.93 – 16.55)	0.85 (0.50 – 0.85)	15.03 (8.89 – 26.48)
Eastern Mediterranean	6.15 (0.86 – 16.73)	5.71 (4.17 – 8.71)	0	11.86 (5.03 – 25.44)
Europe	0.01 (0.00 – 0.03)	0.54 (0.40 – 0.83)	0	0.55 (0.40 – 0.86)
Total world	48.67 (6.79 – 132.41)	113.52 (82.99 – 173.28)	352.86 (207.81 – 352.87)	515.05 (297.59 – 658.55)

The endemicity distribution of *P. falciparum*, within the global limits of risk, is depicted in figure 1.1.

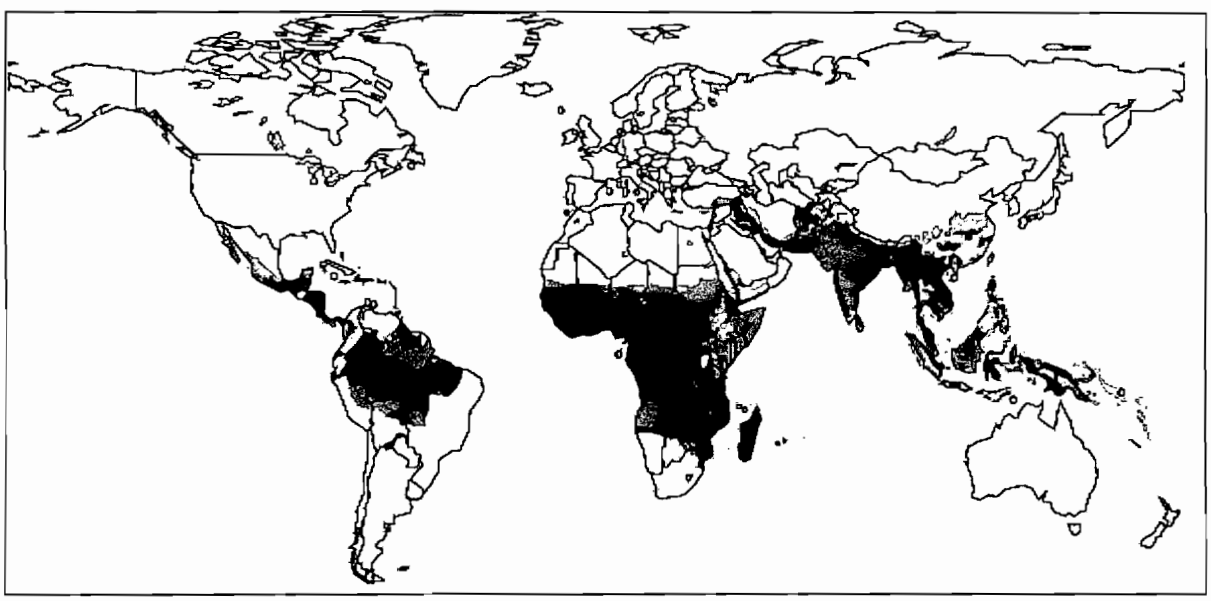

Figure 1.1: Endemicity classes (Snow *et al.*, 2005)

Hyperendemic and holoendemic = dark green
Mesoendemic = medium green
Hypoendemic = light green

1.3 LIFE CYCLE

As already mentioned, malaria is a vector reliant disease which is transmitted by the bite of an infected female *Anopheles* mosquito. Malaria is a parasitic, protozoan, disease which utilizes humans as primary hosts and *Anophelene* mosquitoes as secondary hosts (Hay *et al.*, 2004).

Sporozoites which are present in the salivary glands of the vector are injected while blood-feeding on a human host. These injected sporozoites remain in the bloodstream for approximately 30 minutes in their quest to migrate to, and invade, the liver. Upon reaching the liver these sporozoites are taken up by Kupffer cells and subsequently infect the hepatocytes where they mature and develop into schizonts. Upon maturation these schizonts rupture and release thousands of merezoites into the circulatory blood where they infect the erythrocytes (Jones & Good, 2006).

A dormant stage, called hypnozoites, can persist in the liver and cause relapses by invading the bloodstream weeks, or even years, later. These hypnozoites only occur in *P. vivax* and *P. ovale* malaria infections (Jones & Good, 2006).

After initial replication in the liver, called exo-erythrocytic schizogony, the parasites undergo further asexual multiplication in the erythrocytes (erythrocytic schizogony). The ring stage trophozoites mature into schizonts which rupture and subsequently release merezoites. Another fraction differentiates into sexual erythrocytic stages called gametocytes. The blood stage parasites are the main cause of the clinical manifestations of the disease and they will remain and multiply in the circulatory blood until eradicated by treatment or death of the primary host (Jones & Good, 2006).

Infection of a new secondary host is accomplished when a female *Anopheles* mosquito ingest micro- and macro gametocytes during blood-feeding on an infected host. Multiplication of the parasites in the mosquito is known as the sporogonic cycle and is responsible for the formation of sporozoites. The sporozoites now migrate to the mosquito's salivary glands, subsequent inoculation into a new human host perpetuates the life cycle (Jones & Good, 2006). The complete malaria life cycle is depicted in figure 1.2.

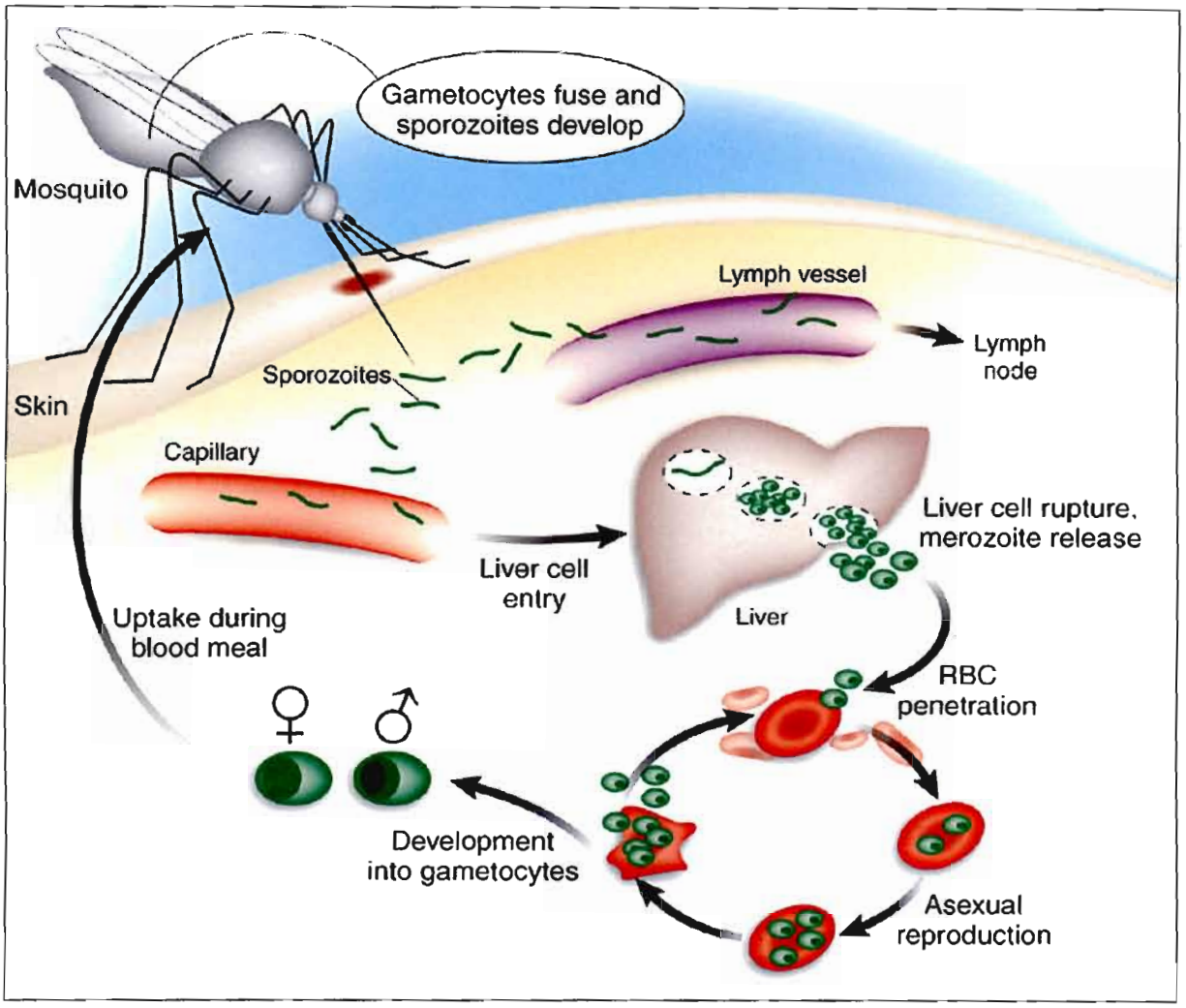

Figure 1.2: Life cycle of *P. falciparum* in both the primary and secondary host (Jones & Good, 2006)

1.4 CLINICAL ASPECTS OF THE DISEASE

The most important element in the diagnosis of malaria is to maintain a high index of suspicion. A review of the distribution data obviates the fact that the South African continent is greatly affected by malaria. Malaria affected areas in South Africa include North-Eastern KwaZulu Natal and the low altitude areas of both Mpumalanga and the Limpopo provinces. The regions bordering Zimbabwe, Mozambique and Swaziland are particularly high-risk areas. A limited number of cases have been reported in the North-West and Northern Cape provinces in areas bordering the Molopo and Orange rivers respectively. The seasonal peak of malaria incidences in Southern Africa is typically between September and May (WHO, 2006a).

Signs and symptoms of malaria usually occur between 10 – 21 days post infection but may even present as early as seven days after exposure. It is not uncommon for longer incubation periods to occur, especially in patients who have used chemoprophylaxis or selected antibiotics such as tetracycline, chloramphenicol, cotrimoxazole, macrolides or the quinolones. Some instances have even been recorded where symptoms of *P. falciparum* infections manifested 6 – 18 months post infection (WHO, 2006a).

The initial symptoms of malaria are non-specific and very similar to the symptoms presented during a minor systemic viral illness. The symptoms usually include:

- headache;
- lassitude;
- fatigue;
- abdominal discomfort;
- muscle aches, and
- joint aches.

As the magnitude of the parasitic infection progress the initial symptoms are followed by a combination of the following symptoms:

- fever;
- chills;
- perspiration;
- anorexia;
- vomiting, and
- malaise.

The above mentioned symptoms are very typical of uncomplicated malaria and are common among residents of endemic areas. In these endemic areas malaria is frequently over diagnosed on the basis of symptoms alone. Uncomplicated malaria, with no evidence of vital organ dysfunction, is relatively easy to treat with a low case-fatality rate when provided with prompt and effective treatment (WHO, 2006a).

In cases where the initial treatment has failed, or where no treatment was given at all, a patient may easily progress from having minor symptoms to having severe disease manifestations within a few hours. These severe malaria manifestations usually include:

- coma (associated with cerebral malaria);
- severe anaemia;
- metabolic acidoses;
- hypoglycaemia;
- acute renal failure, and
- acute pulmonary oedema.

When this stage is reached mortality in humans, receiving treatment, is usually between 15 – 20%. If severe malaria remains untreated it is almost always fatal (WHO, 2006a).

1.5 DIAGNOSTIC METHODS

It is essential to diagnose malaria promptly and accurately in order to effectively manage the disease, it is also crucial to prevent the unnecessary prescription of antimalarial treatment. High sensitivity of malaria diagnosis is critical, particularly in young children in which the disease can be rapidly fatal. The initial diagnosis of malaria is based on clinical criteria followed by parasitological or confirmatory diagnostic tests in an attempt to positively confirm the presence of parasites in the blood (WHO, 2006a).

1.5.1 Clinical diagnosis

The signs and symptoms of malaria are, to a large extent, non-specific. Malaria is mostly diagnosed on the basis of fever or a history of fever. The World Health Organization (WHO) has a few recommendations which should be considered when validating a clinical diagnosis:

- In settings where the risk of contracting malaria is low →
  diagnosis of uncomplicated malaria should be based on the degree of exposure to malaria and a history of fever in the previous three days with the absence of manifestations of any other severe diseases.
- In settings where the risk of contracting malaria is high →
  a history of fever in the previous 24 hours should be the basis of the diagnosis. There should also be looked for signs of anaemia while the most reliable sign in young children is pallor of the palms (WHO, 2006a).

1.5.2 Light microscopy

Microscopy provides a high degree of sensitivity and specificity for the diagnosis of malaria, and it is also possible to positively identify the infecting species and to quantify the magnitude of the infection. Microscopy is considered to still be the so-called "golden standard" against which other diagnostic methods are measured both in terms of specificity and sensitivity (Endeshaw *et al.*, 2008).

This method is inexpensive and robust and slides can be stored for future reference purposes for extended periods of time. It is possible to detect asexual parasites at densities of 10 or less parasites per µl of blood under ideal conditions. This is, however, not the norm since typical field conditions are usually far from ideal (Endeshaw *et al.*, 2008; WHO, 1988).

Light microscopy has a few distinct advantages:

- a high degree of sensitivity when operated by a skilled and experienced person;
- low direct costs, provided adequate infrastructure is already in place;
- differentiation between, and positive identification of, plasmodia species;
- determination of parasite densities, and
- diagnosis of many other conditions.

Giemsa-staining and oil-immersion microscopy are still considered to be the most reliable method of malaria diagnosis in typical health-care settings.

In order to maintain accurate microscopy it is important to consider a few important aspects, namely:

- adequate training and supervision of laboratory staff;
- maintaining of quality assurance and control of laboratory services, and
- electricity supply needs to be available from a reliable source in order to avoid interruptions due to power failure (Endeshaw *et al.*, 2008; Wongsrichanalai *et al.*, 2007).

1.5.3 Rapid diagnostic tests (RDTs)

The RDTs are also called immunochromatographic tests, these tests are used to detect parasite-specific antigens in a blood sample. The RDTs vary in their specificity, some only detect *P. falciparum* while others can detect one or more of the human malaria species such as *P. vivax*, *P. ovale* and *P. malariae.* RDTs are commercially available in varying formats such as cards, cassettes and dipsticks. Cards and cassettes are more robust and easier to use than dipsticks (Endeshaw *et al.*, 2008; WHO, 2006a).

These tests are easy to perform and interpret and no special equipment or electricity is required. Sensitivity rating of 95% or greater is recommended by WHO in order to detect *Plasmodia* at densities of 100 or more parasites per µl of blood.

Current RDTs are based on the detection of various target antigens such as:

- histidine-rich protein 2 (HRP_2) which is specific for *P. falciparum*;
- species-specific or pan-specific parasite lactate dehydrogenase (pLDH), and
- other pan-specific antigens like aldolase (Craig *et al.*, 2002).

The RDTs have quite a few advantages which include:

- results are obtained quickly;
- easy to use which implies that less training is needed to ensure that all general health workers is proficient in using the RDTs;
- reinforcement of patient confidence in the diagnosis and in the general health care system (Murray & Bennett, 2009).

Potential disadvantages of RDTs include:

- unpredictable sensitivity under field conditions due to adverse environmental conditions such as high humidity and extreme temperatures;
- false positive results due to the inability of some RDTs to distinguish between new, untreated infections, and previous effectively treated infections.

This can be attributed to the persistence of target antigens (HRP_2) which remain in the blood for 1 – 3 weeks post treatment (Endeshaw *et al.*, 2008; Craig *et al.*, 2002).

The sensitivity of RDTs for *P. falciparum* can be quite acceptable, greater than 90% at 100 – 500 parasites per 1 µl of blood. There are exceptions though; some widely used products may render a sensitivity as low as 40 – 50% at 100 – 500 parasites per 1 µl of blood.

Factors contributing to poor sensitivity are very speculative and may include:

- manufacturing flaws;
- damage due to high temperature or humidity exposure;
- geographical variation in the test antigen, and
- incorrect handling by end-users (Murray & Bennett, 2009).

It is important to ensure correct initial diagnosis by making use of a second confirmatory diagnosis with either microscopy or RDTs. This positive confirmation of diagnosis will reduce the unnecessary use of antimalarials which in turn will minimize the emergence of resistance (WHO, 2006a).

1.5.4 Immunodiagnosis and PCR-based molecular detection methods

The detection of antibodies in response to a parasitic infection may be useful for epidemiological studies; however, they are not sensitive enough to detect *Plasmodia* infections at low densities. Another drawback is the low specificity of the method and coupled with the fact that it is not able to generate a rapid result renders it useless in the management of patients suspected of having malaria (WHO, 2006a).

Techniques based on the polymerase chain reaction (PCR) are used to detect parasite DNA and are highly sensitive. These techniques are very useful in detecting infections of very low parasite densities and also for detecting mixed infections. This approach is quite handy in specialized epidemiological investigations and for studies concentrating mainly on drug resistance (WHO, 2006a).

1.6 CHEMOPROPHYLAXIS AGAINST MALARIA

1.6.1 Introduction

None of the existing prophylactic regimes are able to provide total protection against the contraction of malaria (Baird, 2005). The use of malaria chemoprophylaxis should be

approached carefully and the risk of contracting malaria should be weighed against the risk of experiencing adverse reactions due to the administered chemoprophylactic agent. The risk depends on various factors:

- host characteristics e.g. previous exposure history;
- the location visited;
- the duration of the visit;
- degree of exposure, and
- the level and type of drug resistance associated with the specific area (Weber *et al.*, 2003).

Areas associated with malaria risk are divided into various types by the WHO. These types and recommended prophylactic measures are presented in table 1.3.

Table 1.3: Malaria risk classification and recommended prophylactic approach (WHO, 2009)

	Malaria risk	Type of prevention
Type I	Very limited risk of malaria transmission	Mosquito bite prevention only
Type II	Risk of *P. vivax* malaria only; or fully chloroquine-sensitive *P. falciparum*	Mosquito bite prevention plus chloroquine chemoprophylaxis
Type III	Risk of *P. vivax* and *P. falciparum* malaria transmission, combined with emerging chloroquine resistance	Mosquito bite prevention plus chloroquine + proguanil chemoprophylaxis
Type IV	1. High risk of *P. falciparum* malaria, in combination with reported anti-malarial drug resistance, or 2. Moderate/low risk of *P. falciparum* malaria, in combination with reported high levels of drug resistance	Mosquito bite prevention plus mefloquine, doxycycline or atovaquone-proguanil chemoprophylaxis (select according to reported resistance pattern). Alternatively, when travelling to rural areas with multidrug-resistant malaria and only a very low risk of *P. falciparum* infection, mosquito bite prevention can be combined with stand-by emergency treatment.

Medication should be taken prior to departing for the intended destination. Atovaquone/proguanil should be taken 1 – 2 days before departure and chloroquine/ proguanil one week before travelling. Mefloquine is usually taken at least two and a half weeks before the trip. It is necessary to evaluate the patient for the occurrence of any

adverse effects and to take corrective measures if deemed necessary by the prescribing practitioner (Marra *et al.*, 2003).

The chemoprophylactic medication should be taken for the duration of the stay in the malaria risk area and its use should only be discontinued four weeks after vacating the area. Atovaquone/proguanil may be discontinued one week after leaving the area. Patient compliance with the prophylactic regimens are very critical (Marra *et al.*, 2003).

Prophylactic medication should be taken after meals with a fair amount of water. A brief summary of the most current prophylactic treatment regimens for adults are given in table 1.4, and for children in table 1.5.

Table 1.4: Chemoprophylactic medication for adults (adapted from Gkrania-Klotsas & Lever, 2007)

Drug	Application	Adult dose
Atovaquone/proguanil	Chloroquine or mefloquine-resistant *P. falciparum* areas	• 250 mg atovaquone and 100 mg proguanil hydrochloride • 1 tablet taken orally, daily
Chloroquine phosphate	Areas with chloroquine-sensitive *P. falciparum*	• 300 mg base (500 mg salt) taken orally once a week • Equivalent to two tablets taken orally once weekly (one tablet = 150 mg of the base)
Doxycycline	Areas with chloroquine-resistant *P. falciparum*	• 100 mg taken orally once a day (tablet or capsule)
Chloroquine + proguanil	Areas with little chloroquine resistance	• 2 tablets taken weekly (150 mg base) + 2 tablets daily (100 mg/tablet)
Hydroxychloroquine sulphate	Alternative to chloroquine for primary prophylaxis in areas with chloroquine-sensitive *P. falciparum*	• 310 mg base (400 mg salt) taken orally once a week
Mefloquine	Areas with chloroquine-resistant *P. falciparum*	• 228 mg base (250 mg salt) taken orally once a week
Primaquine	For primary prophylaxis in special circumstances	• 30 mg base (52.6 mg salt) taken orally once a day
Primaquine	Used to decrease the risk of relapses of *P. vivax* and *P. ovale*	• 30 mg base (52.6 mg salt), taken orally once a day, for 14 days after leaving the malarious area

Table 1.5: Chemoprophylactic medication for children (adapted from Gkrania-Klotsas & Lever, 2007)

Drug	Paediatric dose
Atovaquone/proguanil	• Paediatric tablets contain 62.5 mg atovaquone and 25 mg proguanil hydrochloride • Bodyweight: 11 – 20 kg – 1 tablet 21 – 30 kg – 2 tablets 31 – 40 kg – 3 tablets ≥ 40 kg – 1 adult tablet daily, 250 mg atovaquone and 100 mg proguanil hydrochloride
Chloroquine phosphate	• 5 mg/kg base (8.3 mg/kg salt) taken orally once a week, up to a maximum dose of 300 mg base • If chloroquine syrup is used: Under 4.5 kg – 2.5 ml 4.5 –7.9 kg – 5.0 ml 8.0 –10.9 kg – 7.5 ml 11.0 –14.9 kg – 10.0 ml 15.0 –16.5 kg – 12.5 ml
Doxycycline	• For children 8 years or older – 2 mg/kg up to 100 mg per day
Chloroquine + proguanil	• Chloroquine phosphate 5 mg/kg base (8.3 mg/kg salt) taken orally once a week up to a maximum of 300 mg base PLUS proguanil: < 6 kg – ¼ tablet 6 – 9.9 kg – ½ tablet 10.0 – 15.9 kg – ¾ tablet 16.0 – 24.9 kg – 1 tablet 25.0 – 44.9 kg – 1½ tablets ≥ 45 kg – adult dose
Hydroxychloroquine sulphate	• 5 mg/kg base (6.5 mg/kg salt) taken orally once a week, up to a maximum dose of 310 mg base
Mefloquine	• Recommended dose is 5 mg/kg bodyweight taken orally once a week • Bodyweight: 5 – 10 kg – ⅛ tablet 10 – 20 kg – ¼ tablet 20 – 30 kg – ½ tablet 30 – 45 kg – ¾ tablet > 45 kg – 1 tablet
Primaquine	• 0.6 mg/kg base (1 mg/kg salt) up to a maximum of 30 mg base (52.6 mg salt) taken orally once a day for primary prophylaxis. Continue with this regimen until 14 days after departure from the malarious area.

A few supplementary notes on the use of malaria chemoprophylactic medication:

- Primaquine is not recommended for use in individuals with a known Glucose-6-phosphate dehydrogenase (G6PD) deficiency (Baird *et al.*, 2003).
- Most antimalarial medications have a bitter taste and should be taken with a fair amount of water on a full stomach.
- Mefloquine is contra-indicated in individuals with a history of epileptic incidents (Bradley & Bannister, 2003).
- The pharmacokinetics of proguanil is affected by renal failure and should rather be substituted or the dose can be reduced in accordance with the severity of the renal impairment. In severe cases there are a high risk of acquiring haematological toxicity (Baird *et al.*, 2003).
- Antimalarials should not be prescribed to persons with severe liver failure. In patients with mild liver failure it is acceptable to use proguanil, chloroquine or atovaquone/proguanil with caution. Doxycycline and mefloquine are absolutely contra-indicated since both are exclusively excreted through the liver (Bradley & Bannister, 2003).

1.6.2 Antimalarial prophylaxis: precautions and adverse effects

- Atovaquone and proguanil are contra-indicated for use in infants, pregnant women, woman breast-feeding infants or in patients with severe renal impairment (creatinine clearance < 30 ml/min). The most common side effects that occur with this combination include nausea, abdominal pain, vomiting and headache (Camus *et al.*, 2004).
- Chloroquine phosphate and hydrochloroquine sulphate may both exacerbate psoriasis. They may also cause neurological side effects such as insomnia, headache and blurry vision. Retinopathy may also occur but is very uncommon when administered in small doses (Taylor & White, 2004).
- Mefloquine has been known to increase the risk of insomnia, depression, fatigue and anger and is subsequently contra-indicated in patients with a known history of psychiatric disorders or seizures. It is also not advisable for individuals with cardiac conduction abnormalities to use mefloquine as an antimalarial prophylactic drug (Gkrania-Klotsas & Lever, 2007).

- Doxycycline should not be prescribed to pregnant woman or young children. Photosensitivity and esophagitis are known side effects of doxycycline (Gkrania-Klotsas & Lever, 2007).

1.6.3 Chemoprophylaxis during pregnancy and breast-feeding

Malaria in a pregnant woman increases the risk of maternal death, stillbirth, neonatal death, miscarriage and low birth weight. Travelling to areas of known malaria transmission is not advised. When travel is unavoidable, it is absolutely critical to take effective preventative measures against malaria infection (WHO, 2009).

Pregnant woman are more susceptible to *P. vivax* and *P. falciparum* infections during pregnancy. These woman are particularly targeted by anopheles mosquitoes, this may be attributable to the suppression of pregnancy-specific immunological factors. Susceptibility is at its peak during the second trimester and to some extent during the puerperium (Coll *et al.*, 2008).

If malaria infection is suspected, pregnant woman should immediately seek medical attention. If medical help is not available, it is advisable to use standby emergency treatment. Very limited information exists concerning the safety and efficacy of most antimalarials in pregnancy (WHO, 2009).

➢ *Chloroquine*

In type II areas with predominantly *P. vivax* transmission or chloroquine sensitive *P. falciparum*, it is acceptable to use chloroquine as monotherapy for prophylaxis. Chloroquine is considered to be safe during all three trimesters of pregnancy for prophylactic applications (WHO, 2009).

The adult dosage for chloroquine phosphate is 500 mg/week taken on a full stomach. The first chloroquine dose should be taken one week before arrival, continue treatment until four weeks after departure from the risk area. The same instructions apply for hydroxychloroquine sulphate. The only difference being a lower dose of 400 mg/week (Coll *et al.*, 2008).

The most common adverse effects include: nausea, vomiting, dizziness, blurred vision and itching (Coll *et al.*, 2008).

➢ *Chloroquine plus proguanil*

This combination is effective in type III areas as a chemoprophylactic measure and can be safely prescribed during all three trimesters of pregnancy (WHO, 2009). Proguanil is administered, in conjunction with chloroquine, at a dose of 100 mg daily. Proguanil is a foliate antagonist and may cause or accentuate anaemia in pregnant woman. It is therefore common practice to prescribe folic acid supplementation at a daily dose of 5 mg/day (Taylor & White, 2004).

➢ *Mefloquine*

In areas classed as type IV areas, mefloquine prophylaxis may be given during the second and third trimesters. There is, however, limited information concerning the safety of mefloquine during the first trimester (WHO, 2009). The recommended prophylactic dosage is 250 mg once weekly (Taylor & White, 2004).

Most common adverse effects include: early drug induced vomiting, nausea, late vomiting and dizziness. Mefloquine may also cause various gastrointestinal and central nervous system symptoms (Taylor & White, 2004).

➢ *Breast-feeding*

There is currently no information documented concerning adverse events, in breast-fed infants, which can be directly linked to the intake of antimalarial drugs such as chloroquine, proguanil, amodiaquine or mefloquine by the mother. Small amounts of chloroquine and mefloquine are excreted in breast milk but to such a small extent that these drugs are not contraindicated during breast-feeding. These excreted amounts are not sufficient to protect a newborn against malaria (Coll *et al.*, 2008).

Woman breast-feeding children under 11 kg should not use atovaquone/proguanil combination therapy. Primaquine is contraindicated in those with G6PD deficiency. There is limited information regarding the administration of doxycycline during breast-feeding, but the little information that is available seems to suggest that it is safe to use (Coll *et al.*, 2008).

1.7 TREATMENT OF UNCOMPLICATED *P. FALCIPARUM* INFECTIONS

Uncomplicated malaria is defined as symptomatic malaria without signs of severity or evidence of vital organ dysfunction. In acute *P. falciparum* infections there is a continuum from mild to severe malaria. Young children and non-immune adults should be monitored very closely since their condition may deteriorate rapidly. To improve treatment outcomes, and to counter the threat of resistance of *P. falciparum* to monotherapies, the WHO strongly recommended the implementation of antimalarial combination therapy for the treatment of *P. falciparum* malaria (WHO, 2006a).

1.7.1 Antimalarial combination therapy

Antimalarial combination therapy can be defined as the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and with unrelated biochemical targets in the parasite. Combination therapy is of very significant value since this approach to malaria treatment greatly improves therapeutic efficacy and also delay the development of resistance to the individual components of the combination. The most important of these therapies is most certainly artemisinin-based combination therapies (ACT's) (WHO, 2006a).

1.7.2 Artemisinin-based combination therapy (ACT)

Artemisinin-based compounds are well known for their rapid clearance of parasitaemia in conjunction with a rapid relief of symptoms. The most well known drugs in this class include artesunate, artemether, artemotil, dihydroartemisinin and artemisone (Ramharter *et al.*, 2006). The artemisinins have a very rapid onset of action, the parasitaemia reduction factor is in the vicinity of 10^4 with each cycle. This reduction factor suggests that it is possible to totally abolish parasitaemia, with a parasite burden in the range of 10^{12}, within only three parasite cycles (WHO, 2006a).

Artemisinins are rapidly eliminated and daily administration is required over a period of seven days to effectively cover three parasite cycles. This seven day regimen is very impractical but briefer regimens are usually followed by episodes of recrudescence. The most practical approach to overcome this occurrence is to combine the artemisinin derivative with a slowly eliminated companion, blood-stage, schizonticide. This combination treatment regimen is then taken for only three days. The three day regimen is very effective, the artemisinin reduces the parasite burden by a factor 10^8 which in effect translates to

approximately 99.9% elimination. The remaining fraction of parasites is then subsequently eliminated by the slowly eliminated companion drug (Baird, 2005). The following ACTs, in random order, are currently recommended by the WHO:

- artesunate + amodiaquine;
- artesunate + mefloquine;
- artemether + lumefantrine, and
- artesunate + sulfadoxine – pyrimethamine (WHO, 2006a).

1.7.3 Malaria treatment with ACTs

➢ ***Artesunate + amodiaquine***

The recommended treatment is 4 mg/kg bodyweight for artesunate and 10 mg/kg, when in basic form, for amodiaquine. This is given once a day for three days. The combination is currently available only as separate tablets containing 50 mg artesunate and 153 mg amodiaquine base (WHO, 2006c).

The recommended dosing schedule for infants, children and adults are given in table 1.6. The dose is given in milligrams (mg) and the corresponding number of tablets is given in brackets (WHO, 2006a).

Table 1.6: Artesunate + amodiaquine dosing schedule (WHO, 2006a)

Age	Artesunate (50 mg)			Amodiaquine (153 mg)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
5 – 11 months	25 (½)	25	25	76 (½)	76	76
≥ 1 – 6 years	50 (1)	50	50	153 (1)	153	153
≥ 7 – 13 years	100 (2)	100	100	306 (2)	306	306
> 13 years	200 (4)	200	200	612 (4)	612	612

➢ ***Artesunate + mefloquine***

A total dose of 4 mg/kg bodyweight is recommended for artesunate and 25 mg base/kg bodyweight for mefloquine. Artesunate is given once daily for three consecutive days while mefloquine is usually split over two or three days. This combination is currently available

only as separate tablets containing 50 mg artesunate and 250 mg mefloquine base. Different doses of mefloquine had been evaluated, namely 15 mg base/kg bodyweight and 25 mg base/kg bodyweight. The doses were evaluated in terms of efficacy and induced adverse effects. The lower dose did not render satisfactory efficacy and is not recommended. The higher dose is usually associated with acute vomiting and subsequent poor absorption. To alleviate these pitfalls it is recommended that the 25 mg/kg dose should be administered as a split dose. The dose can be given as a 15 mg/kg dose on the second day followed by a 10 mg/kg dose on the third day, or as a 8.3 mg/kg dose once daily for a duration of three days. The most common adverse effects, attributed to the administration of mefloquine, include: nausea, vomiting, dizziness, dysphoria and insomnia (WHO, 2006a; WHO, 2006c).

Table 1.7 represents the recommended dosing schedule for artesunate + mefloquine combination therapy. The dose is given in milligrams (mg) and the corresponding number of tablets is given in brackets (WHO, 2006a).

Table 1.7: Artesunate + mefloquine dosing schedule (WHO, 2006a)

Age	Artesunate (50 mg)			Mefloquine (250 mg)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
5 – 11 months	25 (½)	25	25	–	125 (½)	–
≥ 1 – 6 years	50 (1)	50	50	–	250 (1)	–
≥ 7 – 13 years	100 (2)	100	100	–	500 (2)	250 (1)
> 13 years	200 (4)	200	200	–	1 000 (4)	500 (2)

➢ *Artemether-lumefantrine*

Artemether – lumefantrine is available as a fixed-dose combination of artemether (20 mg) and lumefantrine (120 mg). Lumefantrine is structurally very similar to quinine, mefloquine and halofantrine. This combination exhibits high efficacy against both *P. falciparum* and *P. vivax*. The recommended treatment comprises a six dose regimen of artemether-lumefantrine, twice a day for three days. Lumefantrine is not available as monotherapy nor has it ever been used by itself for the treatment of malaria. Very young children, weighing between 5 and 10 kg, can effectively be treated with this combination due to its favourable safety profile and therapeutic response. Lumefantrine is a lypophillic compound by nature and its absorption can be greatly enhanced by co-administration of fatty substances. Patients should be informed that it is essential to take this ACT with milk and/or fatty food, especially on the second and third days of treatment (WHO, 2006a; Taylor & White, 2004).

Table 1.8 represents the recommended dosing schedule for artemether–lumefantrine combination therapy.

Table 1.8: Artemether-lumefantrine dosing schedule (WHO, 2006a)

Bodyweight (kg)	Age	Number of tablets/time of dosing					
		0h	8h	24h	36h	48h	60h
5 – 14	< 3 years	1	1	1	1	1	1
15 – 24	≥ 3 – 8 years	2	2	2	2	2	2
25 – 34	≥ 9 – 14 years	3	3	3	3	3	3
> 34	> 14 years	4	4	4	4	4	4

➢ ***Artesunate + sulfadoxine-pyrimethamine***

Artesunate is currently available, as mentioned earlier in the text, as tablets containing 50 mg of the active. Sulfadoxine-pyrimethamine is a fixed-dose combination which contains 500 mg sulfadoxine and 25 mg pyrimethamine in each tablet. The recommended treatment is 4 mg/kg bodyweight of artesunate which is administered once daily for a duration of three days. Sufadoxine-pyrimethamine is only administered once and it is taken with the first artesunate dose. In most cases a single dose of sulfadoxine-pyrimethamine will be sufficient, while it is critical that artesunate should be given for three days to achieve the desired parasite reduction (WHO, 2006a; Taylor & White, 2004).

The sulphonamides have been associated with a broad spectrum of adverse effects, namely:

- **gastrointestinal toxicity** → stomatitis, pancreatitis, glossitis, melaena, psudomembranous colitis and salivary gland enlargement

- **skin reactions** → itching, urticaria, cutaneous vasculitis, photosensitivity, erythema nodosum, erythema multiforme, hair loss and lichen planus

- **CNS** → ataxia, benign intracranial hypertension, dizziness, aseptic meningitis, tinnitus, hearing loss and reversible peripheral neuropathy

- **Haematological** → agronulocytosis, megaloblastic anaemia, haemolytic anaemia and thrombocytopenia

- **renal effects** → haematuria, proteinuria, crystalluria and acute interstitial nephritis (Taylor & White, 2004)

Table 1.9 represents the recommended dosing schedule for artesunate + sulfadoxine-pyrimethamine combination therapy. The dose is given in milligrams (mg) and the corresponding number of tablets is given in brackets (WHO, 2006a).

Table 1.9: Artesunate + sulfadoxine-pyrimethamine dosing schedule (WHO, 2006a)

Age	Artesunate (50 mg)			Sulphadoxine-pyrimethamine (500/25)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
5 – 11 months	25 (½)	25	25	250/12.5 (½)	–	–
≥ 1 – 6 years	50 (1)	50	50	500/25 (1)	–	–
≥ 7 – 13 years	100 (2)	100	100	1 000/50 (2)	–	–
> 13 years	200 (4)	200	200	1 500/75 (3)	–	–

1.7.4 Monotherapy and non-artemisinin-based combination therapy

Monotherapy and non-artemisinin-based combination therapy imply that only a single drug is administered or a combination of drugs are administered with the exclusion of any artemisinin-based partner drug. Artemisinin-based drugs are not used as monotherapy since all of these compounds have a relatively short plasma half-life ($T_{½}$) which increases the risk of resistance development (WHO, 2006a). Since this study is mainly focused on artemisinin-based compounds, only a short overview will be given in the following pages concerning monotherapy and non-artemisinin-based combination therapy for the treatment of malaria.

➢ ***Treatment options***

It is important to determine if the patient had taken prophylactic treatment. If the individual did, the same regime should not be used during treatment. Always be aware of the possibility that mixed infections with both *P. falciparum* and *P. vivax* may occur and that treatment should be adjusted accordingly. The most common treatment given to travelers to

cure uncomplicated *P. falciparum* malaria is combinations of either atovaquone-proguanil, quinine + doxycycline or quinine + clindamycin (WHO, 2009).

The recommended treatment for *P. vivax* malaria infections are chloroquine combined with primaquine. This combination is usually very effective and is able to eliminate both blood stage and liver stage infections thereby preventing recrudescence and relapses. Another option is to combine amodiaquine with either primaquine or quinine. These combinations are especially useful in areas known to harbour chloroquine resistant *P. vivax* strains (WHO, 2009; Taylor & White, 2004).

In mixed infections with both *P. falciparum* and *P. vivax* it is customary to follow the standard regimen for *P. falciparum* infections and just add primaquine to achieve radical cure and prevent relapses. It is important to keep in mind that all patients must be tested for glucose-6-phosphate dehydrogenase (G6PD) deficiency before commencing with primaquine treatment. The primaquine regimen should be adjusted in cases of moderate G6PD deficiency. A dose of 0.75 mg base/kg bodyweight should be administered once weekly for a duration of eight weeks. In patients with severe G6PD deficiency it is not safe to include primaquine in the treatment regimen and should be excluded altogether (WHO, 2009; Taylor & White, 2004).

The treatment of falciparum malaria is becoming increasingly complex due to ongoing evolutionary changes in *P. falciparum* parasites and the subsequent emergence of resistance to various antimalarial drugs. Due to this increasing emergence of resistance it is not recommended by the WHO to blindly advocate the use of antimalarial monotherapy when more suitable combination regimens are close at hand. Nevertheless, it is still important to be well informed of all of the treatment options available which is why a few single drug options are also included in table 1.10.

Table 1.10 is a very cryptic summary of the most common monotherapies and non-artemisinin-based combination therapies currently in use (WHO, 2009; Gkrania-Klotsas & Lever, 2007; Taylor & White, 2004).

Table 1.10: Monotherapies and non-artemisinin-based combination therapies for antimalarial treatment (WHO, 2009; Gkrania-Klotsas & Lever, 2007; Taylor & White, 2004)

		Use in special groups				
Generic name	**Dosage regimen**	**Pregnancy**	**Breast-feeding**	**Children**	**Main contraindications**	**Remarks**
Amodiaquine	30 mg base/kg taken as 10 mg base/kg for 3 days	Limited data suggest that it is safe to use	Limited data suggest that it is safe to use	Safe	Amodiaquine hypersensitivity, hepatic disorders	Use only for *P. vivax, P. ovale* and *P. malariae* infections
Chloroquine	25 mg base/kg divided in daily dose 10, 10, 5 mg base/kg for 3 days	Safe	Safe	Safe	Chloroquine hypersensitivity, psoriasis and/or a history of epilepsy.	Use only for *P. vivax, P. ovale* or *P. malariae*. Concurrent use of chloroquine may reduce the antibody response to intradermally administered human diploid-cell rabies vaccine.
Clindamycin	Under 60 kg: 5 mg base/kg, 4 times daily for 5 days. Over 60 kg: 300 mg base 4 times daily for 5 days.	Limited data suggest that it is safe to use	Limited data suggest that it is safe to use	Limited data suggest that it is safe to use	Clindamycin hypersensitivity or hypersensitivity to lincomycin. History of intestinal disease such as colitis, severe liver and/or kidney impairment.	Used in **combination** with quinine in areas with emerging quinine resistance.
Doxycycline	Adults > 50 kg: 800 mg salt over 7 day period, taken as 2 tablets (100 mg salt each) 12 hours apart on day 1, followed by 1 tablet daily for 6 days	Contraindicated	Contraindicated	Contraindicated under 8 years of age	Hypersensitivity to tetracycline. Liver dysfunction.	Used in **combination** with quinine in areas with emerging quinine resistance.

Table 1.10: Monotherapies and non-artemisinin-based combination therapies for antimalarial treatment *(continued)* (WHO, 2009; Gkrania-Klotsas & Lever, 2007; Taylor & White, 2004)

Use in special groups						
Generic name	**Dosage regimen**	**Pregnancy**	**Breast-feeding**	**Children**	**Main contraindications**	**Remarks**
Mefloquine	25 mg base/kg as split dose (15 mg/kg plus 10 mg/kg) 6 – 24 hours apart	Not recommended in first trimester	Safe	Not recommended for children under 5 kg	Hypersensitivity to mefloquine. Psychiatric or convulsive disorders. History of severe neuropsychiatric disease. Concomitant halofontrine treatment. Mefloquine treatment in past 4 weeks.	Do not use mefloquine within 12 hours of last dose of quinine treatment. Mefloquine and related compounds (quinine, quinidine, chloroquine) may only be given concomitantly under close medical supervision due to possible additive cardiac toxicity and increased risk of convulsions. Co-administration with anti-arrhythmic agents, beta-adrenergic blocking agents, antihistamines which include H_1-blocking agents, calcium channel blockers and phenothiazines may all contribute to prolongation of QT interval. Mefloquine blood levels may increase when used in conjunction with metoclopramide, tetracycline and ampicillin.

Table 1.10: Monotherapies and non-artemisinin-based combination therapies for antimalarial treatment *(continued)* (WHO, 2009; Gkrania-Klotsas & Lever, 2007; Taylor & White, 2004)

Use in special groups						
Generic name	**Dosage regimen**	**Pregnancy**	**Breast-feeding**	**Children**	**Main contraindications**	**Remarks**
Primaquine	0.25 mg base/kg, taken with food once daily for 14 days. In South East Asia and Oceania the dose should be increased to 0.5 mg base/kg	Contraindicated	Safe	Generally contraindicated in young infants	G6PD deficiency, active rheumatoid arthritis, lupus erythematosus and conditions that predispose to granulocytopenia.	Used as an anti-relapse treatment for *P. vivax* and *P. ovale* infections.
Quinine	8 mg base/kg taken 3 times daily for 7 days	Safe	Safe	Safe	Hypersensitivity to quinine or quinidine, myasthenia gravis, optic neuritis, tinnitus. Use with caution in individuals with G6PD deficiency and also in patients with atrial fibrillation, cardiac conduction defects and/or heart block. Possible synergistic effect with cardio suppressant drugs. Use with caution in individuals using digoxin, Ca^{2+}-channel blockers, beta-blockers etc.	Use in **combination** with either tetracycline, clindamycine or doxcycline in areas with emerging resistance to quinine. Hypoglycaemia may also be induced by quinine treatment in malnourished children, pregnant woman and patients with severe disease.

Table 1.10: Monotherapies and non-artemisinin-based combination therapies for antimalarial treatment *(continued)* (WHO, 2009; Gkrania-Klotsas & Lever, 2007; Taylor & White, 2004)

Use in special groups						
Generic name	**Dosage regimen**	**Pregnancy**	**Breast-feeding**	**Children**	**Main contraindications**	**Remarks**
Atovaquone-proguanil combination tablet	One dose daily for 3 consecutive days **5 – 8 kg:** 2 paediatric tablets daily (62.5 mg atovoquone plus 25 mg proguanil per tablet) **9 – 10 kg:** 3 paediatric tablets daily **11 – 20 kg:** 1 adult tablet daily (250 mg atovaquone plus 100 mg proguanil) **21 – 30 kg:** 2 adult tablets daily **31 – 40 kg:** 3 adult tablets daily **> 40 kg:** 4 adult tablets (1 g atovaquone plus 400 mg proguanil) daily	Not recommended	Not recommended	Safe in children > 5 kg, but use with caution	Hypersensitivity to atovaquone and/or proguanil. Severe renal insufficiency, with a creatinin clearance of less than 30 ml/min.	Co-administration with rifampicin, rifabutin, tetracycline or metoclopramide will reduce atovaquone plasma concentrations.

1.8 TREATMENT AND SUPPORTIVE CARE OF PATIENTS WITH SEVERE MALARIA

1.8.1 Initial assessment and management

Urgent, appropriate, therapy is essential to ensure a favourable prognosis in patients with severe malaria. This is a medical emergency and treatment should not be delayed in patients with proven or strongly suspected malaria (Day & Dondorp, 2007).

The initial clinical assessment should concentrate on the airway and circulation, this includes examination of the conscious level, respiratory status and state of hydration of the individual. Hypoglycaemia should be ruled out or, if the patient is comatose, treated empirically. The presence of convulsions which present with subtle symptoms, especially in children, should be treated immediately. Treatment usually consists of intravenous rehydration and oxygen supplementation if there is clinical evidence of respiratory distress or hypoxia. An appropriate antimalarial agent should also be administered in conjunction with the abovementioned corrective measures (Day & Dondorp, 2007).

Parenteral treatment is the mainstay approach in the management and cure of patients diagnosed with severe malaria. Other indications for parenteral therapy in adults include:

- nauseous and/or vomiting patients which are unable to take or retain oral antimalarials;
- pregnant women, and
- patients with a parasitaemia of greater than 2% (Lalloo *et al.*, 2007).

The presence of severe malaria can be confirmed by the presentation of various clinical manifestations and laboratory tests (WHO, 2006a). These manifestations and tests are listed in table 1.11.

Table 1.11: WHO criteria for the diagnosis of severe malaria (WHO, 2006a)

One or more of the following clinical or laboratory features
Clinical manifestations • Prostration • Impaired consciousness • Respiratory distress or acidotic breathing • Multiple convulsions • Circulatory collapse • Pulmonary oedema (radiological) • Abnormal bleeding • Jaundice • Haemoglobinuria
Laboratory test • Severe malaria • Hypoglycaemia • Acidosis • Renal impairment • Hyperlactataemia • Hyperparasitaemia

1.8.2 Antimalarial treatment

➢ ***Quinine***

The current first line antimalarial treatment for severe malaria is intravenous quinine dihydrochloride. It should be administered as an intravenous infusion with a loading dose of 20 mg/kg in either 5% dextrose or dextrose/saline over a duration of 4 hours to rapidly achieve high blood levels and to prevent cardio toxicity. The initial dose is then followed by a lower dose of 10 mg/kg every 8 hours, it is paramount that these infusions are also administered over a 4 hour period (Lalloo *et al.*, 2007).

If quinine or mefloquine was administered to the patient during the past 12 hours, it is not safe to administer the above mentioned loading dose. An alternative regimen should be followed; rapid quinine loading in the form of quinine dihydrochloride (7 mg/kg) with the aid of an infusion pump should be infused over a 30 minute period. This loading dose is then followed by a 10 mg/kg dose over 4 hours (Day & Dondorp, 2007; Davis *et al.*, 1990).

In patients where intravenous infusion is not possible, quinine may be administered by means of a deep intramuscular injection. These injections have certain drawbacks such as the possibility of forming a sterile abscess which may lead to subsequent life-threatening tetanus. Injection into the buttocks can also lead to sciatic nerve damage (Day & Dondorp, 2007; Yen *et al.*, 1994).

When the patient is well enough to take oral medication, the patient should be switched to oral quinine at a dose of 600 mg, 3 times a day. This regimen should be followed for 5 – 7 days; doxycycline should be given in conjunction with quinine at a daily dose of 200 mg for a total of 7 consecutive days. Doxycycline should be interchanged with clindamycin when treating a pregnant woman, this should be administered at a dose of 450 mg three times a day (Lalloo *et al.*, 2007; WHO, 2006a).

Quinine is a relatively toxic compound with a narrow therapeutic index. A particularly common problem in patients with severe malaria is the occurrence of quinine-induced hyperinsulinemic hypoglycaemia. Thus occurrence is very problematic in patients with severe malaria, especially during pregnancy, since it is impossible to clinically diagnose this condition in an already unconscious patient (Newton & Krishna, 1998). The frequent monitoring of blood glucose concentrations is thus essential to ensure the safety of the patient.

The disease severity has a significant effect on the systemic clearance and the total apparent volume of distribution of quinine. As the disease severity increase, there is a proportional reduction in both the total apparent volume of distribution and the systemic clearance. Due to these effects it is essential to reduce the quinine dose by one-third after 48 hours of treatment with no clinical improvement (Krishna & White, 1996).

➢ *Artemisinin derivatives*

The most exciting recent development in the treatment of severe malaria is most certainly the introduction of artemisinin and various derivatives thereof. This class of drugs are rapidly parasitocidal and, in contrast to quinine, is able to eliminate young circulating parasites before they sequester in the deep microvasculature (Day & Dondorp, 2007).

Artesunate is available as a water-soluble intravenous formulation, this formulation was compared to quinine in the treatment of severe malaria. The comparative study was conducted in four South and South East Asian countries. It was titled "South and South East

Asian Quinine versus Artesunate in severe Malaria Trial" or SEAQUAMAT in short. The mortality in those individuals randomized to artesunate was 15% in comparison with 22% in quinine recipients. This amounts to a relative reduction of 34.7%. Based on these results of the SEAQUAMAT study, the WHO is now recommending parenteral artesunate as the drug of choice for the treatment of severe malaria in low transmission areas. Parenteral artesunate is also the drug of choice for the treatment of severe malaria in the second and third trimesters of pregnancy (WHO, 2006b).

Parenteral artesunate is administered in the form of an intravenous injection. The recommended adult dosage is 2.4 mg/kg, and this is administered at 0, 12 and 24 hours then once daily thereafter. A course of doxycycline should also be given, in conjunction with artesunate, for a period of 7 days (Lalloo *et al.*, 2007).

Artemether is an oil-based artemisinin derivative and is available in the form of an intramuscular preparation. Several studies and meta-analyses have not demonstrated a definite advantage of artemether over quinine in the management of severe malaria. This may be attributed to poor absorption properties of the intramuscular preparation in very sick individuals (Hien *et al.*, 2003).

Toxicity studies in animals have raised concerns that high doses of artemisinin derivative drugs may elicit neurotoxicity. In humans, however, despite widespread use, no neurotoxicity has been reported (Hien *et al.*, 2003).

The neurotoxicity which presented in the animal studies appear to be particularly related to the lipophilic artemisinin derivatives such as artemether. This may be attributed to high sustained plasma concentrations of the drug due to specific release properties from the intramuscular depot. Based on this evidence it was hypothesized that the water-soluble derivatives, such as artesunate, would exhibit a much better safety profile (Nontprasert *et al.*, 2002).

Animal studies have also revealed some evidence of reproductive toxicity in the form of foetal resorption in both rats and rabbits. To date there is no evidence of this occurring in humans. Viewed in light of the fact that severe malaria is a life-threatening illness for pregnant females the WHO regards artesunate as a feasible treatment option in the first trimester of pregnancy (WHO, 2006b).

A summary of the recommended treatment protocols for the treatment of severe malaria cases is given in table 1.12.

Table 1.12: Summary of recommendations on the treatment of severe malaria (WHO, 2006a; WHO, 2006b)

Recommendations
• Severe malaria is a medical emergency. After rapid clinical assessment and confirmation of the diagnosis, full doses of parenteral antimalarial treatment should be started immediately with whichever effective antimalarial is first available.
• Artesunate 2.4 mg/kg bodyweight (bw) i.v. or i.m. given on admission (time = 0), then at 12 hours and again at 24 hours, thereafter once daily. This is the recommended choice of treatment in low-transmission areas or outside malaria endemic areas.
• For children in high-transmission areas the following antimalarial medicines are recommended as there is insufficient evidence to recommend any of these antimalarial medicines over another for severe malaria: ❖ Artesunate 2.4 mg/kg bodyweight i.v. or i.m. given on admission (time = 0), then at 12 hours and again at 24 hours, then once a day; ❖ Artemether 3.2 mg salt/kg bodyweight i.m. given on admission, then 1.6 mg salt/kg bodyweight per day thereafter; ❖ Quinine 20 mg salt/kg bodyweight on admission (i.v. infusion or divided i.m. injection), then 10 mg/kg bodyweight every 8 hours – infusion rate should not exceed 5 mg salt/kg bodyweight per hour.
• Pre-referral treatment of severe *falciparum* malaria include: ❖ Artesunate or artemisinin by rectal administration; ❖ Artesunate or artemether via intramuscular administration; ❖ Quinine via intramuscular administration.
• Pregnant woman: ❖ Use the parenteral antimalarial treatment locally available for severe malaria, use the normal full doses; ❖ Artesunate is the first choice in the first, second and third trimesters of pregnancy; ❖ Artemether is the second choice for treatment during the second and third trimesters; ❖ Both quinine and artesunate may be used in the first trimester of pregnancy; however, further studies need to be conducted on this issue.

➢ *Supportive management and care of patients with complicated malaria*

All patients with severe or complicated malaria should be managed in an intensive care unit. Special attention should be given to patients presenting with severe acidosis, pulmonary oedema, acute respiratory distress syndrome, complicated fluid balance problems or renal impairment (Lalloo *et al.*, 2007).

➢ *Coma*

It is common practice to support the breathing of the unconscious cerebral malaria patient with a mechanical ventilator to protect the airway. The efficacy of this procedure has not yet been proven in terms of prevention of mortality and sequelae. Isolated trials have demonstrated the potential of mannitol, an anti-osmotic agent, to successfully reduce intracranial pressure for short periods of time; however, no convincing clinical evidence exists to support its routine use (Gerardin *et al.*, 2007).

➢ *Convulsions*

It is essential to ensure high-flow oxygen and appropriate airway management before commencing with anticonvulsive treatment. The seizures caused by cerebral malaria should be managed with rectal diazepam, intravenous lorazepam, paraldehyde or other standard anticonvulsants (Maitland *et al.*, 2005).

➢ *Acte renal failure*

This is a common complication in non-immune children and adults. The mortality of untreated acute renal failure is in the vicinity of 70% or greater and subsequently commands prompt renal replacement therapy. This replacement therapy is best accomplished by means of haemofiltration, this approach has been proven to be superior to peritoneal dialysis both in terms of mortality and cost-effectiveness (Phu *et al.*, 2002).

➢ *Acidosis*

A common complication of severe malaria is metabolic acidosis. This scenario is often associated with a fatal outcome in both children and adults (Day & Dondorp, 2007; Krishna *et al.*, 1994). A combination of lactic acid and impaired renal bicarbonate handling, together with other unidentified acids, play major roles in this occurrence. Dichloroacetate has been used to reduce plasma lactate but have no impact on pH. The greatest success in combating acidosis, in patients with renal failure, has been achieved by haemofiltration (Agbenyega *et al.*, 2003; Phu *et al.*, 2002).

➢ *Haemodynamic shock*

The mortality due to shock is relatively high in both children and adults (Gerardin *et al.*, 2007). The initial treatment should consist of oxygen supplementation and fluid replenishment with constant monitoring of central venous pressure. The possibility of bacterial sepsis should be covered by running a septic screen which includes blood cultures and appropriate broad-spectrum antibiotics. The use of inotropes such as dopamine, dobutamine and norepinephrine may also be considered. Epinephrine should be avoided as it can potentially induce serious lactic acidosis (Day & Dondorp, 2007; Day *et al.*, 1996).

➢ *Fluid resuscitation*

Fluid resuscitation is a very controversial topic in the treatment of severe malaria. It is currently unclear whether hypovolemia is responsible for poor tissue perfusion which in turn lead to anaerobic glycolysis and consequent acidosis. Aggressive fluid repletion tactics should be approached with caution as overzealous rehydration may lead to pulmonary and cerebral oedema (Maitland, 2006; Planche, 2005; Planche *et al.*, 2004).

➢ *Anaemia*

Anaemia is present in almost all patients diagnosed with severe malaria but especially in young children. The option of blood transfusion should be evaluated from all angles with consideration being given to the benefit versus risk factor. The specific haemoglobin cut-off levels are merely speculative. The threshold for blood transfusion in adults is usually set at a haematocrit of less than 20%. In children this threshold haemoglobin level is commonly set at 5 g/dL, especially in cases with co-existing respiratory distress, impaired consciousness or hyperparasitaemia (WHO, 2001).

➢ *Acute respiratory distress syndrome (ARDS)*

Acute respiratory distress syndrome (ARDS) is a life-threatening condition with mortality rates of between 40 – 60%. This complication can develop several days after admission and onset of treatment. The treatment of this condition in patients with severe malaria is based on expert opinion. This is due to a lack of knowledge on the etiology of ARDS (Day & Dondorp, 2007).

In limited studies it was found that corticosteroid therapy, given after the onset of ARDS, reduced the odds of mortality with 93.2%. However, these studies did not reveal a discernable time or dose effect on mortality with the therapeutic use of those steroids (Peter *et al.*, 2008).

1.9 EMERGENCE OF ANTIMALARIAL DRUG RESISTANCE

1.9.1 Introduction

Antimalarial drug resistance is defined by the WHO as "... the ability of a parasite strain to survive and/or multiply despite the proper administration and absorption of an antimalarial drug in the dose normally recommended" (WHO, 2006a).

The emergence of drug resistance in malaria is a vitally important public health concern. Resistance of *P. falciparum* to chloroquine was first observed approximately 50 years ago. At present chloroquine resistance has been documented in virtually all areas affected by *P. falciparum*. Most strains of *falciparum* parasites have developed resistance to either one or more of the commonly used antimalarials (Wongsrichanalai *et al.*, 2002).

The emergence of *P. falciparum* drug resistance is the most likely explanation for a doubling in malaria related mortality in children living in eastern and southern Africa (Korenromp *et al.*, 2003).

According to Wongsrichanalai *et al.* (2002) it is very common to encounter resistance to a specific antimalarial drug within 10 – 15 years from the date of initial introduction. Table 1.13 was adapted from Wongsrichanalai *et al.* (2002) and contain introduction dates, and also dates of first resistance reports of a few common antimalarials.

Table 1.13: Introduction dates and first reports of antimalarial drug resistance (Wongsrichanalai *et al.*, 2002)

Antimalarial agent	Introduced	First reports of resistance	Difference (years)
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulfadoxine-pyrimethamine	1967	1967	0
Mefloquine	1977	1982	5
Atovaquone	1996	1996	0

It is interesting to note that sufadoxine-pyrimethamine is also included in this table. This denotes the first evidence of emergence of multidrug resistance. The extensive deployment of these antimalarial drugs, in the past 50 years, has provided a tremendous selection pressure on malaria parasites to evolve various mechanisms of resistance (White, 2004).

1.9.2 Assessment of *P. falciparum* antimalarial susceptibility

Therapeutic response evaluation, by means of *in vivo* testing, is most commonly employed to assess *P. falciparum* susceptibility to antimalarial drugs. The WHO originally defined *in vivo* drug response in terms of parasite clearance and three degrees of resistance. Clearance is defined in terms of sensitivity (S) to the specific drug and the intensity of resistance is represented by the symbols RI, RII and RIII (WHO, 2001).

This classification is valid for areas with low transmission or no transmission at all. Its applicability is complicated in areas with intensive transmission where cases of new infections and recrudescence are both present. Due to these complicating elements the WHO introduced a modified protocol which is based on clinical outcomes, targeted at the practical assessment of therapeutic responses, in areas with intense transmission. In these areas it is common to encounter infected individuals with no clinical signs or symptoms of malaria (WHO, 2001).

The revised protocol has also been adapted to take the parasite clearance and resolution of symptoms into account, this revision enhances the adaptability of the model to areas with low to moderate endemicity (Wongsrichanalai *et al.*, 2002). A summary of both the original and revised protocols is presented in table 1.14.

Table 1.14: Classification of *in vivo* antimalarial drug sensitivity test outcomes, including both the original and modified classification (adapted from Wongsrichanalai *et al.*, 2002)

Classification	Definition
Original version • S (sensitive)	→ Reduction to less than 25% of original parasitaemia on day 2. → Smears negative for malaria from day 7 to end of follow-up (28 days or longer for drugs with a long half-life, e.g. mefloquine).
• RI response	→ Initial clearance of parasitaemia. → Smears negative for malaria on day 7. → Recrudescence 8 days or more after treatment.
• RII response	→ Initial clearance or substantial reduction of parasitaemia to less than 25% of initial count on day 2. → Persistence thereafter or recrudescence during days 4 – 7.
• RIII response	→ No significant reduction of parasitaemia.
Modified version • Early treatment failure (ETF)	→ Aggravation or persistence of clinical symptoms in the presence of parasitaemia during the first 3 days of follow up.
• Late treatment failure (LTF)	→ Reappearance of symptoms in the presence of parasitaemia during days 4 – 14 of follow-up.
• Adequate clinical response (ACR)	→ Absence of parasitaemia on day 14 irrespective of fever, or absence of clinical symptoms irrespective of parasitaemia, in patients not meeting ETF or LTF criteria.

In vitro assays can also be employed to determine the antimalarial drug susceptibility of *Plasmodium* parasites. This is accomplished by measuring the intrinsic sensitivity of *P. falciparum* based on the inhibition of growth or schizont maturation (Bloland, 2001).

The use of molecular markers has also been proposed as an additional element for the early detection of drug resistance in malaria (Wellems & Plowe, 2001).

Each of these methods has its own advantages and drawbacks, and the results may also not be directly comparable with each other. This variability in results can be attributed to the effect of host immunity during *in vivo* testing which cannot be mimicked during *in vitro* testing. Pharmacokinetic data should also be taken into account when attempting to differentiate between true resistance and failure to achieve adequate drug concentration profiles (White, 1999).

Drug resistance to an antimalarial compound results in a shift in the concentration-effect relationship. This shift is to the right on the X-axis of a corresponding dose-response curve. Figure 1.3 is an example of such a dose-response curve (WHO, 2006a).

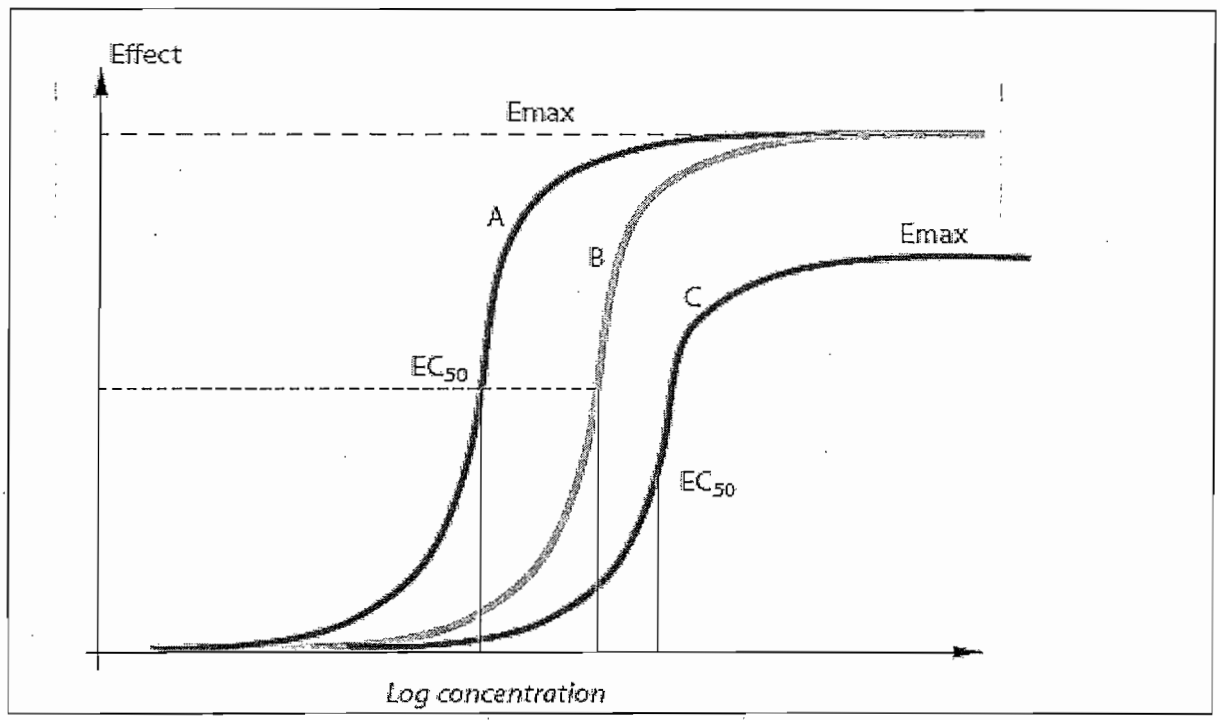

Figure 1.3: Demonstration of the rightward shift in the dose-response curve for a specific parasite population (WHO, 2006a)

The shift may be parallel (B) in respect to the commonly accepted curve (A) or in some circumstances the slope may change in conjunction with a reduction in the maximum achievable effect (C). The maximum achievable effect (Y-axis) represents the maximum possible parasite clearance.

It is important to distinguish between antimalarial drug resistance and malaria treatment failure. Treatment failure is defined as failure to clear malarial parasitaemia and/or resolve clinical symptoms despite the administration of an antimalarial. In view of this point, it can be concluded that drug resistance may lead to treatment failure, but all treatment failures cannot be attributed to drug resistance. Treatment failure may be a result of either incorrect dosing, poor patient compliance, poor drug quality, interactions with other drugs, compromised drug absorption or misdiagnosis of the disease. These factors may all contribute to inappropriate case management, but may also accelerate the spread of drug resistance due to the exposure of the parasites to subtherapeutic drug levels (WHO, 2006a).

1.9.3 Determinants of antimalarial resistance

Resistance development can be considered in two parts:

- the initial genetic event which produces the resistant mutant, and
- the subsequent selection process in which the survival advantage, in the presence of the drug, leads to preferential transmission of resistant mutants and in turn the spread of resistance (WHO, 2006a; White, 2004).

When there is an absence of antimalarial drugs, these resistant mutants may be at a survival disadvantage. This survival disadvantage is commonly referred to as the "fitness cost". The "fitness cost" of the resistance mechanism may result in a decline in the prevalence of resistance once drug pressure is removed (WHO, 2006a).

Cross-resistance should also be taken into consideration. This phenomenon occurs when resistance to one drug may select for resistance to another where the mechanisms of resistance are similar (Wongsrichanalai *et al.*, 2002).

Emergence of resistance can be classified as the product of the probabilities of *de novo* emergence and subsequent spread. When parasites are exposed to "selective" or subtherapeutic drug concentrations, the resistant parasites, if present, will be selected. In this context the term "selective" can be explained as a specific drug concentration that will eradicate the sensitive parasites but still allow growth of the resistant parasite population to such an extent that it will eventually be transmitted to another individual. *De novo* resistance is a random occurrence among malaria parasites, this is mostly encountered in non-immune patients infected with large numbers of parasites who receive inadequate treatment (WHO, 2006a; White, 2004).

The variant surface antigen, PfEMP1, is responsible for directing the principle specific immune response, this controls the primary symptomatic infection in *falciparum* malaria. The parasite population is able to evade this immune response by switching the specific sequence of its surface antigens. The probability of selecting a resistant parasite from the primary infection is influenced by two variables, namely the switch rate and also the rate of formation of viable, resistant, parasites (Miller *et al.*, 1994).

The resultant spread of resistant mutant malaria parasites is promoted by the use of drugs with long elimination phases. These drugs provide a "selective filter" effect which allows the resistant parasites to proliferate while the residual antimalarial activity prevents infection by sensitive parasites. This "selective filter" effect is in fact actually responsible for creating a temporary reservoir host for the resistant parasites from where future infections can spread. Slowly eliminated drugs, commonly associated with the "selective filter" effect, include mefloquine and chloroquine (Miller *et al.*, 1994).

According to White (2004), there are various factors which should be taken into account when determining the probability of selection of *de novo* antimalarial drug resistance, they are listed below:

- the frequency with which the resistance mechanism arises;
- the "fitness cost" to the parasite associated with the resistance mechanism;
- concentrations of the drug to which the parasites are exposed, this includes the administered doses and also the pharmacokinetic properties of the antimalarial drug;
- pharmacodynamic properties of the antimalarial drug;
- the degree of resistance that results from the genetic changes;
- the level of host immunity, both specific and nonspecific;
- the simultaneous presence of two or more antimalarial drugs or antiparasitic agents that will still eliminate the parasite if it develops resistance to the other drugs, e.g. the use of combination therapy (White, 2004).

Wongsrichanalai *et al.* (2002) concluded that the reasons for the development and spread of drug resistance involved a few other aspects in addition to the above selection criteria.

The characteristics of the specific drug are important determinants of resistance. The half-life of the drug plays a major role. Drugs with a long half-life, such as mefloquine, may exert notable residual selection due to new infections, contracted after treatment of the primary infection, when the drug persists at subtherapeutic concentrations in the plasma (Wernsdorfer, 1994). Secondly, it is paramount to maintain adequate drug concentrations over an extended period to ensure clearance of the entire parasite population within the individual. Subtherapeutic drug concentrations will eliminate most of the susceptible parasites and leave only those that are fit to recover and reproduce. To counter this scenario it may be necessary to increase the therapeutic dose. The downside of this approach is the possibility of exceeding the maximum tolerable dose. The widespread use of drugs at high

intensities is a determinant for selection of resistant populations since it leads to a resultant increase in drug pressure (Wongsrichanalai *et al.*, 2002).

Human hosts with more potent immune responses show increased efficacy with chemotherapy. Semi-immune patients might still be cured by an antimalarial despite the fact that a variable fraction of the infecting parasites might be partially drug resistant. Patients with non-specific immune responses increase the opportunity for manifestation and spread of resistance (Wongsrichanalai *et al.*, 2002).

The level of transmission is linked to the rate of development and spread of drug resistance but its exact role is complex and very likely multifactorial. Various studies revealed that the risk of drug resistance development is encountered in both low and high transmission areas but general observations suggest that low transmission areas are the main breeding ground for drug resistant mutants (Hastings & D'Alessandro, 2000).

Vector and environmental factors may also play an important role in the proliferation of resistant parasites. This is based on speculation that certain resistant parasites may be fit for reproduction in specific *Anopheline* mosquitoes than non-resistant strains (Wernsdorfer, 1994).

A summary of the above is given in table 1.15.

Table 1.15: Determinants of antimalarial-drug resistance (Wongsrichanalai *et al.*, 2002)

Factors and characteristics	Example
Drug	
• Half-life	→ Resistance to LAPDAP (short half-life) develops more slowly than that to sulfadoxine-pyrimethamine which has a longer half-life. (LAPDAP = chloproguanil plus dapsone)
• Dosing	→ Self-treatment with subtherapeutic doses, often encountered with antifolate drugs. → Poor drug compliance. → Use of chloroquine salt. → Mass drug administration with subtherapeutic doses.
• Non-target drug pressure	→ Presumptive administration of antimalarial drugs without laboratory diagnosis or for indications other than malaria.
• Pharmacokinetics	→ Use of drug formulations with reduced bioavailability.
• Cross-resistance	→ Sulfadoxine-pyrimethamine and sulfamethoxazole-trimethoprim.
Human	
• Host immunity	→ Non-immune, migrant, gem-miners and resistance to mefloquine on the Thai-Cambodian border.
• Maintenance of resistant parasite reservoir	→ Non-detection of drug failure.
Parasite	
• Genetic mutations	→ Molecular markers of antimalarial resistance will be discussed in 1.9.6.
• Transmission level	→ Whether low or high transmission has more influence on drug resistance is debatable. → Prevalence of drug resistance is greater in regions of low transmission, whereas a model suggests the benefits of transmission control in delaying resistance development.
Vector and environment	→ Increased infectivity and productivity of chloroquine – resistant parasites in *Anopheles dirus* and the propagation of chloroquine resistance in South East Asia and Western Oceania.

1.9.4 Transmission intensity and spread of resistance

The recrudescence and subsequent transmission of an infection that has generated a *de novo* resistant parasite is of paramount importance for the propagation of resistance (White, 1999). The resistant biomass need to expand to high parasite numbers (> 10^7 parasites) before the gametocytes, carrying the resistance genes, can reach transmissible densities. This implies that in order to prevent resistance spreading from a *de novo* resistance acquired infection, it is essential to prevent gametocyte production of the recrudescent resistant infection (Talisuna *et al.*, 2007).

Low transmission settings are the main contributor to the occurrence of drug resistant malaria. In these settings the majority of infections are symptomatic and selection is subsequently a product of inadequate treatment. Large numbers of parasites are usually encountered in these patients and antimalarials are administered at maximally effective concentrations. However, in a relatively small portion of these patients the antimalarial concentrations are sub-optimal due to interpatient variances which in turn may elicit the selection for resistance (WHO, 2006a).

In high transmission areas the majority of infections are asymptomatic and infections may be acquired repeatedly throughout life. This setting promotes a state of imperfect immunity or premonition in which the infection is controlled at levels low enough not to cause obvious symptoms. The intensity of transmission determines the rate at which premonition is acquired. During periods of intense transmission individuals will usually receive antimalarial treatment, but these treatments will mostly be unrelated to parasitaemia peaks, thereby reducing the probability of selection for resistance (WHO, 2006a).

The emergence of resistance is considerably reduced by host immunity. The defense of the host is a major contributor to the perceived antiparasitic effect, this means that any spontaneously generated drug-resistant mutant must not only contend with concentrations of antimalarials but also with host immunity. This situation reduces the probability of parasite survival at all stages of the transmission cycle (Dye & Williams, 1997).

1.9.5 Genetic basis of antimalarial drug resistance

The genetic conformations that translate to antimalarial drug resistance are spontaneous and rare and are thought to be independent of the drug treatment. Some of these genetic

events may lead to viability issues in the mutants which can be detrimental to successful proliferation. It can be described as mutations or changes in the copy number of genes encoding or relating to the specific parasite target of the drug. These mutations may also affect the influx pumps which in turn affect intraparasitic drug concentrations (Rathod *et al.*, 1997).

A single genetic event may be sufficient to produce a viable mutant, in other instances multiple unlinked events may be required to accomplish this end result. These additive multiple unlinked events are commonly referred to as epitasis. Multigenic resistance is the product of the individual component probabilities, this is a very rare event. Parasites originating from South East Asia, especially *P. falciparum,* have been shown to have an increased propensity to develop drug resistance (Rathod *et al.*, 1997).

1.9.6 Molecular markers of antimalarial resistance

- ***Chloroquine***

The molecular basis of chloroquine resistance studies are focused on polymorphisms in two genes of the *P. falciparum* genome. The *pfcrt* gene is located on chromosome 7, this gene codes for the vacuolar membrane transporter protein namely PfCRT (Wellems *et al.*, 1991). The substitution of threonine for lysine in codon 76 was recently proven to associate with resistance in various *P. falciparum* isolates. Most studies concluded that although the Thr 76 mutation may be essential for the resistance phenotype, it is also present in the chloroquine-sensitive strains but to a much lesser extent. This suggests that other additional *pfcrt* polymorphisms are necessary to achieve a viable mutant or that a combination of several genes is involved (Wongsrichanalai *et al.*, 2002).

The *pfmdr1* gene which is located on chromosome 5 and which codes for P-glycoprotein homologue may also play a role in chloroquine resistance. The point mutation of aspartic acid to tyrosine in codon 86 has also been associated with resistance in some clinical and *in vitro* studies. Other *pfmdr1* mutations include Phe184, Cys1034, Asp1042 and Tyr1246. Recent studies found that *pfmdr1* gene polymorphisms do not only modulate susceptibility to chloroquine but also to mefloquine, quinine and halofantrine (Ward & Bray, 2000).

➢ *Sulfadoxine-pyrimethamine*

The molecular basis of resistance to sulfadoxine-pyrimethamine is very well defined. There are specific mutations in *P. falciparum* that lead to resistance to both sulphadoxine and pyrimethamine, this combination act synergistically against *P. falciparum.* Sulphadoxine is known to inhibit dihydropteroate synthetase (DHPS) while pyrimethamine inhibits dihydrofolate reductase (DHFR). Both of these enzymes are involved in folate synthesis (Wongsrichanalai *et al.*, 2002).

Point mutations in five codons of the *dhps* gene are implicated in conferring resistance by decreasing the binding affinity of the enzyme. The point mutations in the five codons are:

- serine at codon 436 to alanine or phenylalanine;
- alanine at 437 to glycine;
- lysine at 540 to glutamic acid;
- alanine at 581 to glycine, and
- alanine at 613 to serine or threonine.

Point mutations have also been reported at Gly437 and Glu540. They can occur together or as single entities. Mutations at Gly581 have also been observed, either alone or with Gly437 (Wongsrichanalai *et al.*, 2002).

Specific point mutations, associated with pyrimethamine resistance, in the *dhfr* gene have also been reported. The mutations are responsible for reducing the drug-binding affinity of DHFR. These mutations include:

- alanine at 16 to valine;
- asparagine at 51 to isoleucine;
- cysteine at 59 to arginine;
- serine at 108 to aspargine or threonine, and
- isoleucine at 164 to leucine (Sibley *et al.*, 2001).

The key mutation for pyrimethamine resistance is a change from serine at codon 108 to aspargine. The degree or resistance was found to increase progressively with additional point mutations in three other codons, namely Ile51, Arg59 and Leu164. Quadruple mutants

with the Leu164 mutation are reported to be more resistant than triple mutants with Ile51 and Arg59 (Sibley *et al.*, 2001).

➢ *Quinine*

It has previously been suggested that *pfmdr1* mutations, also associated with chloroquine resistance, may be the cause for reduced susceptibility to quinine. Studies implicated *pfmdr1* mutations: Asn86, Phe184, Cys1034, Asp1042 and Tyr1246 to low quinine susceptibility. In an isolated incedent *pfmdr1* Tyr86 was also associated with a small decrease in sensitivity (Zalis *et al.*, 1998).

➢ *Mefloquine*

Possible molecular markers of mefloquine resistance may also be related to polymorphisms of the *pfmdr1* gene. There is also evidence on increased *pfmdr1* copy number as a possible molecular marker for mefloquine resistance, but evidence is still very conflicting. In a parasite transfection experiment it was found that Ser1034, Asn1042 and Asp1246 mutations were the main culprits in initiating resistance to mefloquine, quinine and halofontrine (Wongsrichanalai *et al.*, 2002).

➢ *Artemisinin*

Artemisinin resistance has been associated with poor drug uptake and also with over expression of translationally controlled tumor protein which may be a potential artemisinin target. Studies suggest that the *pfmdr1* Tyr86 variant may also play a role in increased sensitivity to artemisinins. Additional mutations of Ser1034, Asn1042 and Asp1246 may lead to a further alteration of *P. falciparum* sensitivity to artemisinin (Ward & Bray, 2000).

Decreased *in vitro* sensitivity to artemether was reported and associated with the *PfSERCA* gene and a mutation at S769N. This finding could, however, not be confirmed in *in vivo* studies (Jambou *et al.*, 2005). Another *in vivo* study associated high failure rates of artesunate with an increased number of copies of the *pfmdr1* gene (Wongsrichanalai & Meshnick, 2008). A summary of the mentioned molecular markers and genetic mutations which select for antimalarial resistance is given in table 1.16. Table 1.17 was adapted from Wongsrichanalai *et al.* (2002), various sources were consulted to compile the table. It

contains specific molecular markers of antimalarial resistance and the global distribution of the reported genetic mutations.

Table 1.16: Molecular markers of antimalarial resistance (Babiker *et al.*, 2001; Basco & Ringwald, 2001; Djimde *et al.*, 2001; Mawili-Mboumba *et al.*, 2001; Urdaneta *et al.*, 1999; Zalis *et al.*, 1998; Von Seidlein *et al.*, 1997)

Drug	Parasite related genetic mutations
• Chloroquine	→ *pfcrt* Th76 strongly associated. → *pfmdr1* Tyr86, Phe184, Cys1034, Asp1042, Tyr1246 possible associated.
• Sulphadoxine-pyrimethamine	→ Principal mutations in codons 108, 51, 59 and 64 of *dhfr* gene and codons 436, 437, 540, 581 and 623 of *dhps* gene. → *Dhfr* Asn108 essential for pyrimethamine resistance. → Degree of resistance increases with additional mutations, Leu51 and Arg59 or triple mutations. → Absolute resistance conferred by the addition of Leu164, thus quadruple mutation, irrespective of *dhps* mutations.
• Mefloquine, Quinine	→ Genetic basis of resistance not yet clearly understood *pfmdr1* polymorphism may modulate mefloquine and quinine susceptibility. → Point mutations being investigated at positions 86, 184, 1034, 1042 and 1246 of *pfmdr1*.
• Artemisinins	→ Translationally controlled tumor protein is possible artemisinin target. → *pfmdr1* Tyr86 possible increased sensitivity, additional mutations of Ser1034, Asn1042 and Asp1246 further alteration in sensitivity. → *PfSERCA* gene, 5769N mutation, *in vitro* sensitivity decreased.

Table 1.17: Drug specific polymorphisms in *P. falciparum* (Wongsrichanalai *et al.*, 2002)

Drug and polymorphism	Location
Chloroquine	
pfcrt	
Thr76, Ile74, Glu75	Asia, Africa
Thr76	Africa
Thr76	Africa
Thr76, Ile74, Glu75, Ser220, Ile371	Africa
Thr76, Ser72 (one isolate)	S America
pfmdr1	
Tyr86	Asia
Tyr86, Asp1042	Indonesia
Tyr86	Africa
Tyr86	Africa
Tyr86	Africa
Tyr86, Asp1042, Tyr1246	Africa
Tyr86, Asp1042, Tyr1246, Phe184, Cys1034	S America
Asp1042, Tyr1246	S America
Sulfadoxine-pyrimethamine	
dhps	
Gly437, Glu450	Asia
Gly437, Glu450	Indonesia
Gly437	Africa
Glu450	Africa
Gly437, Gly581	S America
Gly581	S America
Gly437, Glu450, Gly581	S America
dhfr	
Asn108, Ile51, Arg59	Asia
Asn108, Arg59	Indonesia
Asn108, Ile51, Arg59	Africa
Asn108, Arg50, Ile51, Leu64	S America
Asn108, Arg50, Ile51	S America
Asn108, Arg50, Ile51, Leu161	S America

Table 1.17: Drug specific polymorphisms in *P. falciparum (continued)*
(Wongsrichanalai *et al.*, 2002)

Drug and polymorphism	Location
Mefloquine	
pfmdr1	
Tyr86*	Thailand
Copy number	Thailand
Copy number	Thailand
Tyr86*	Africa
Quinine (decreased sensitivity)	
pfmdr1	
Tyr86, Phe184, Cys1034, Asp1042, Tyr1246	S America
Tyr86	Africa
Artesunate	
pfmdr1	
Copy number	Thailand
Dihydroartemisinin	
pfmdr1	
Tyr86*	Africa

* Increases sensitivity

1.10 MULTIDRUG RESISTANCE

1.10.1 Introduction

Multidrug resistance of *P. falciparum* can be defined as resistance to more than two operational antimalarial compounds of different chemical classes (Wernsdorfer, 1994). Wongsrichanalai *et al.* (2002) modified this previous definition by further specifying the extent of resistance to a third drug class. The first two classes are 4-aminoquinolones, such as chloroquine, and the antifolates which include sulfadoxine-pyrimethamine. In areas where the effectivity of the third drug is compromised to such an extent that treatment failure occurs in 25% or more of treated cases, it is reasonable to suspect multidrug resistance (WHO, 2001).

1.10.2 Established multidrug resistance

This phenomenon occurs mainly in the border regions of Thailand in South East Asia. High levels of mefloquine resistance started to occur between 1994 – 1996 in these regions which prompted the use of artesunate in combination with an increased mefloquine dose. Two issues that are of particular relevance when following this approach is firstly the maintenance of this therapeutic regimen and secondly to limit the multidrug resistance to its present area. A common problem which promotes the further spread of resistance in these areas is self-treatment with artesunate and mefloquine at subtherapeutic concentrations (Wongsrichanalai *et al.*, 2002).

Areas in the Amazon Basin also fall in this category with sporadic reports of reduced quinine susceptibility and mefloquine resistance. The first-line treatment regimen in the border regions of Brazil and Peru is also a combination of artesunate and mefloquine (Wongsrichanalai *et al.*, 2002).

1.10.3 Areas of emerging multidrug resistance

The term "emerging multidrug resistance" is defined as a widespread loss of clinical efficacy of chloroquine and sulfadoxine-pyrimethamine with a potential emergence of resistance to a third drug class. This seems to be limited to areas such as Tanzania and Kenya situated in East Africa. Another area with a reported high-degree of sulfadoxine-pyrimethamine resistance is the Amazon Basin. Drug-susceptibility surveillance data is very limited and incomplete which makes it very difficult to determine exact boundaries of emerging multidrug resistance (Wongsrichanalai *et al.*, 2002).

Tropical Africa has been associated with sulfadoxine-pyrimethamine resistance and a subsequent progression to severe or complicated manifestations with increased mortality. This area is known for its lack of affordable alternative medication and urgent counter measures are required to counteract this potential threat. The artesunate-mefloquine regimen employed in Thailand is expensive and may not be as effective in these African settings with differing malaria epidemiology and poor peripheral health-care systems (Wongsrichanalai *et al.*, 2002).

Figure 1.4 is a map which indicate the various areas of resistance as described in the text.

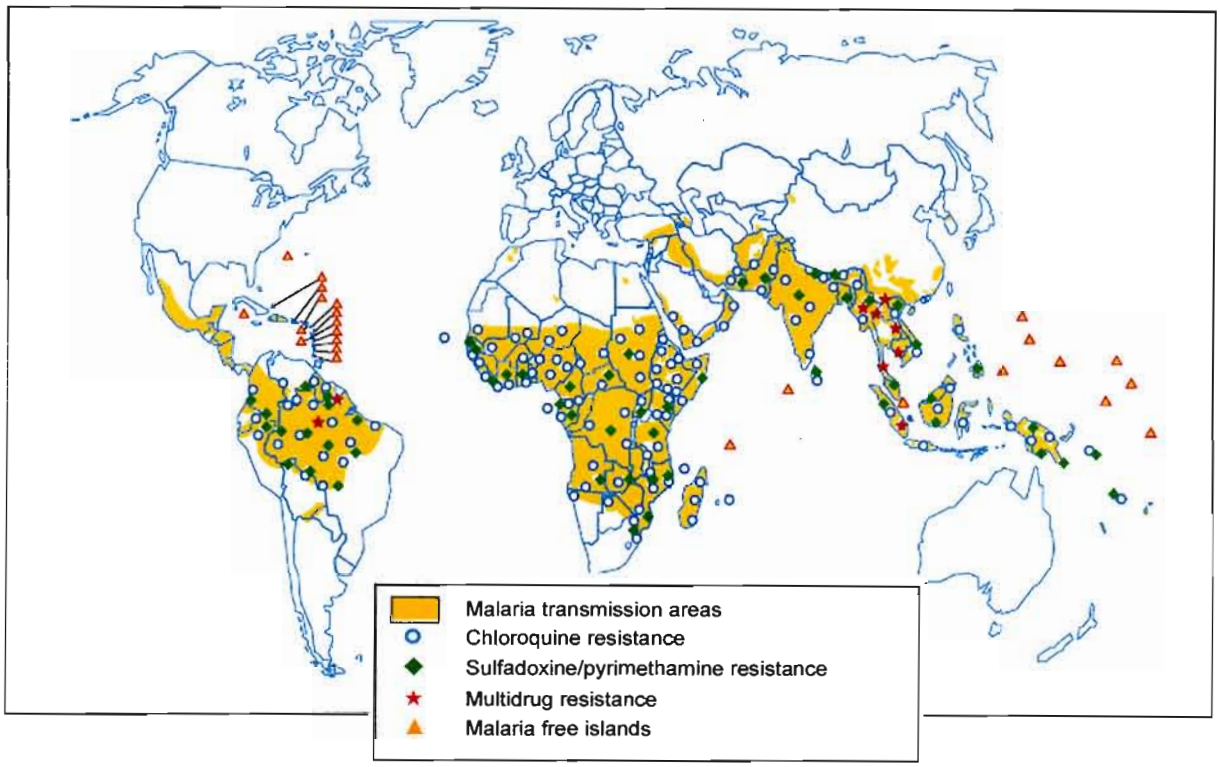

Figure 1.4: Areas with reduced susceptibility of *P. falciparum* to chloroquine and sulphadoxine-pyrimethamine and also areas of multidrug resistance (WHO, 2001)

1.10.4 Future prospects

In order to combat drug resistant malaria in the years to come, it is of paramount importance to continue the search for novel antimalarial agents and to utilize molecular information to constrain the development of further drug resistance. Suggested measures to limit the emergence of resistance are listed below.

- In areas with no drug resistance it is important to extend chloroquine efficacy as long as possible.
- Areas with no multidrug resistance need constant surveillance for early detection of development of multidrug resistance.
- Areas with emerging multidrug resistance calls for prevention and rapid detection of new foci development. New regimens and alternative drugs are needed with a shift to drugs other than 4-aminoquinolines and antifolates. Drug pressure must be limited in these areas by promoting rational drug use.
- In areas with established multidrug resistance it is important to make an effort to limit the geographical spread and to implement new drug combinations in order to

preserve efficacy of current drugs. The development of new drug candidates is also a definite requirement (Wongsrichanalai *et al.*, 2002).

➢ ***Utilization of molecular information***

Future studies will lead to the identification of more drug resistance causing alleles. This information will be helpful in identifying new drug targets, designing tools for rapid malaria diagnosis and perhaps the construction of an effective vaccine. Genetic markers can be implemented for the surveillance of potential drug resistance, and the *pfcrt* Thr76 mutation, for example, can be utilized in the prediction of chloroquine treatment outcomes (Djimde *et al.*, 2001).

1.11 CONCLUSION

Drug resistance is a major problem and presents a great challenge to all malaria-control programmes. The problem of drug-resistant malaria is a worldwide occurrence but Africa is the focal point of concern. Sulfadoxine-pyrimethamine resistance development is a reality in Africa and provides a pressing need for new affordable antimalarial treatment regimens. There is no clear answer to the resolution of multidrug-resistant malaria. Combination therapy has long been the focus for the management of multidrug-resistant malaria. The combination of a drug with an extended half-life such as mefloquine with a drug with a short half-life such as artesunate is relatively effective. The main problem with this is that mefloquine resistance is already present and reports of possible artemisinin resistance in *Plasmodium falciparum* has also been posted by Dondorp *et al.* (2009).

Resistance has already developed to all the antimalarial drug classes with the only exception, until recently, being the artemisinins. These drugs are already an intricate component in the management of multidrug-resistant *falciparum* malaria. If we lose the artemisinins we may be faced with untreatable malaria. It is thus essential to implement rational treatment regimens and to ensure proper patient compliance.

From a research point of view it is paramount to continue the search for novel antimalarial compounds and to evaluate already existing compounds for possible antimalarial activity. Novel delivery systems should also be evaluated in an effort to revive some of the already existing antimalarials by enhancing their bioavailability and by protecting these drugs against enzymatic degradation.

CHAPTER 2

THE ARTEMISININS

2.1 INTRODUCTION

Artemisia annua has been used in China for more than 2000 years before the active compounds were isolated and identified. Artemisinin is a sesquiterpene lactone and was first isolated by Chinese scientists in 1971. This was accomplished by extraction of *Artemisia annua*, more commonly known as the sweet wormwood plant, into diethyl ether at a low temperature. This extracted "active principle" was subsequently proven to cure mice infected with *Plasmodium berghei*. During 1972, further research culminated in the isolation of a crystalline compound that was named qinghaosu or artemisinin. This compound demonstrated antimalarial activity attributed to a peroxide bond within its structure (Meshnick *et al.*, 1996). Artemisinin can be converted into semi synthetic derivatives, artesunate and artemether, which are more active against malaria than their parent compound (Woodrow *et al.*, 2005). Artemisinin derivatives are potent and rapidly acting against multidrug-resistant *Plasmodium falciparum* and are used in combination with other antimalarials. In countries where standard antimalarials are ineffective, due to drug resistance, the WHO recommends artemisinin-based combination therapies for the treatment of uncomplicated malaria (Mutabingwa, 2005).

Artemisone is a new semi-synthetic 10-alkylamino-artemisinin which can be synthesized in a one-step process from dihydroartemisinin. This compound was found to exhibit increased antiplasmodial activity, improved oral bioavailability, improved metabolic stability, extended *in vivo* half-life and no neurotoxicity. Artemiside is also a novel artemisinin derivative which is synthesized in a very similar manner as artemisone. Artemiside was reported to be a very active antimalarial agent and will also be evaluated in this thesis (Haynes *et al.*, 2006).

2.2 SYNTHESIS OF ARTEMISININ DERIVATIVES

According to Haynes *et al.*, (2006) the first methods of synthesis have been published in the mid 1980's. The parent compound, artemisinin (1), is converted by reduction into dihydroartemisinin (DHA, 2). This DHA is then further converted by reduction to yield 65% of the hemi-ester, artesunate (ATS, 3). Artemether (4) and arteether (5) are obtained from

DHA, methanol and an acid catalyst. This produces an approximate yield of 60% after fractional crystallization to remove the α-epimers (Haynes *et al.* 2006).

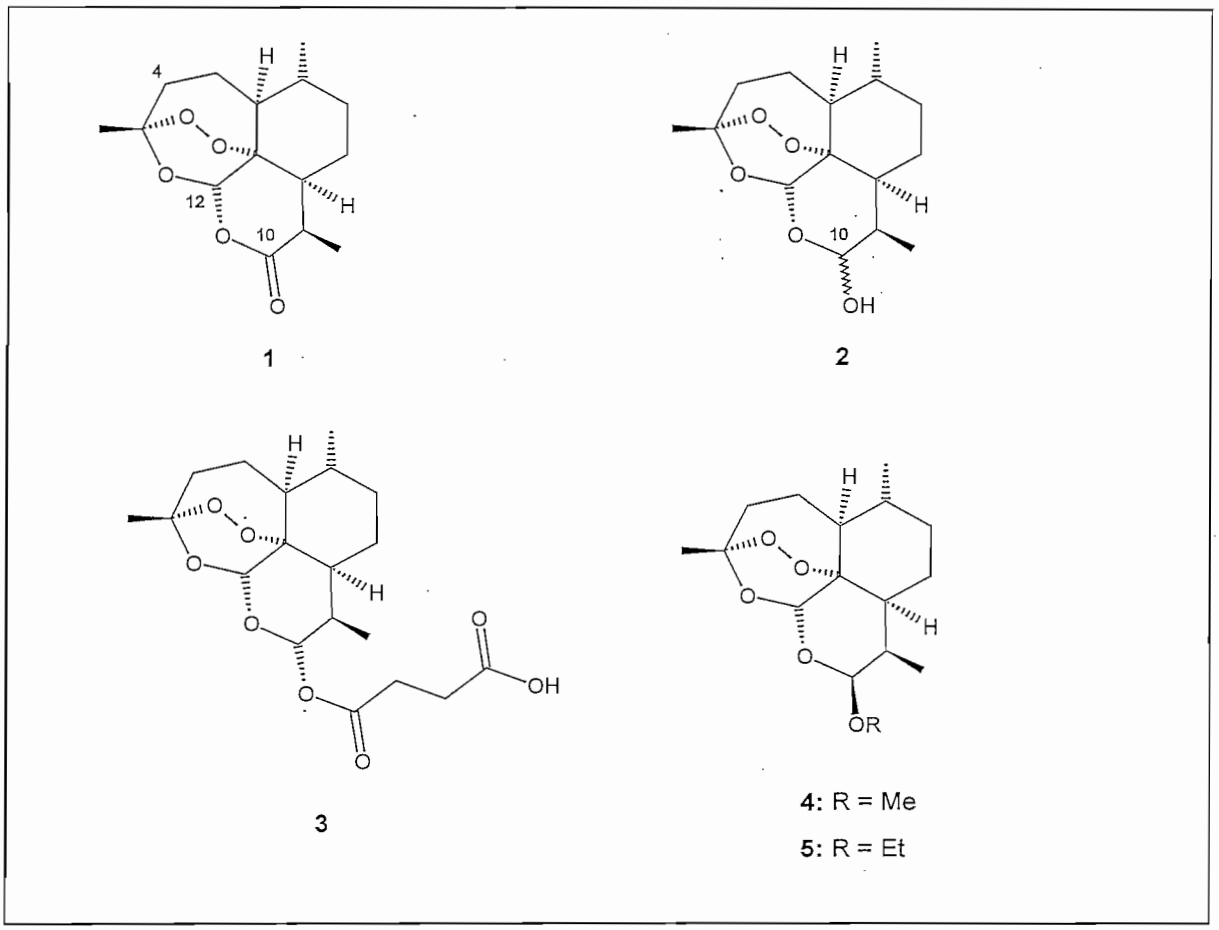

Figure 2.1: Molecular structure of artemisinin (1), DHA (2), artesunate (3), artemether (4) and arteether (5) (Haynes *et al.*, 2006)

Artemiside (12) and artemisone (14), which is the main focus of this study, were obtained from DHA α-trimethylsilyl ether (7) and trimethylsilyl bromide (TMSBr), followed by treatment of the intermediate bromide (8), formed *in situ*, with the amine nucleophile (figure 2.2, route a). Oxidation of artemiside (12) produced artemisone (14) and also a sulfoxide (13). An easier route to follow involves the treatment of DHA (2) with a mixture of NaBr and then with TMSCl in toluene followed by the amine (figure 2.2, route b). Both artemiside (12) and artemisone (14) were isolated by crystallization of the crude product mixtures and are subsequently relatively accessible and produced as isomerically pure, air-stable, substances (Haynes *et al.*, 2006).

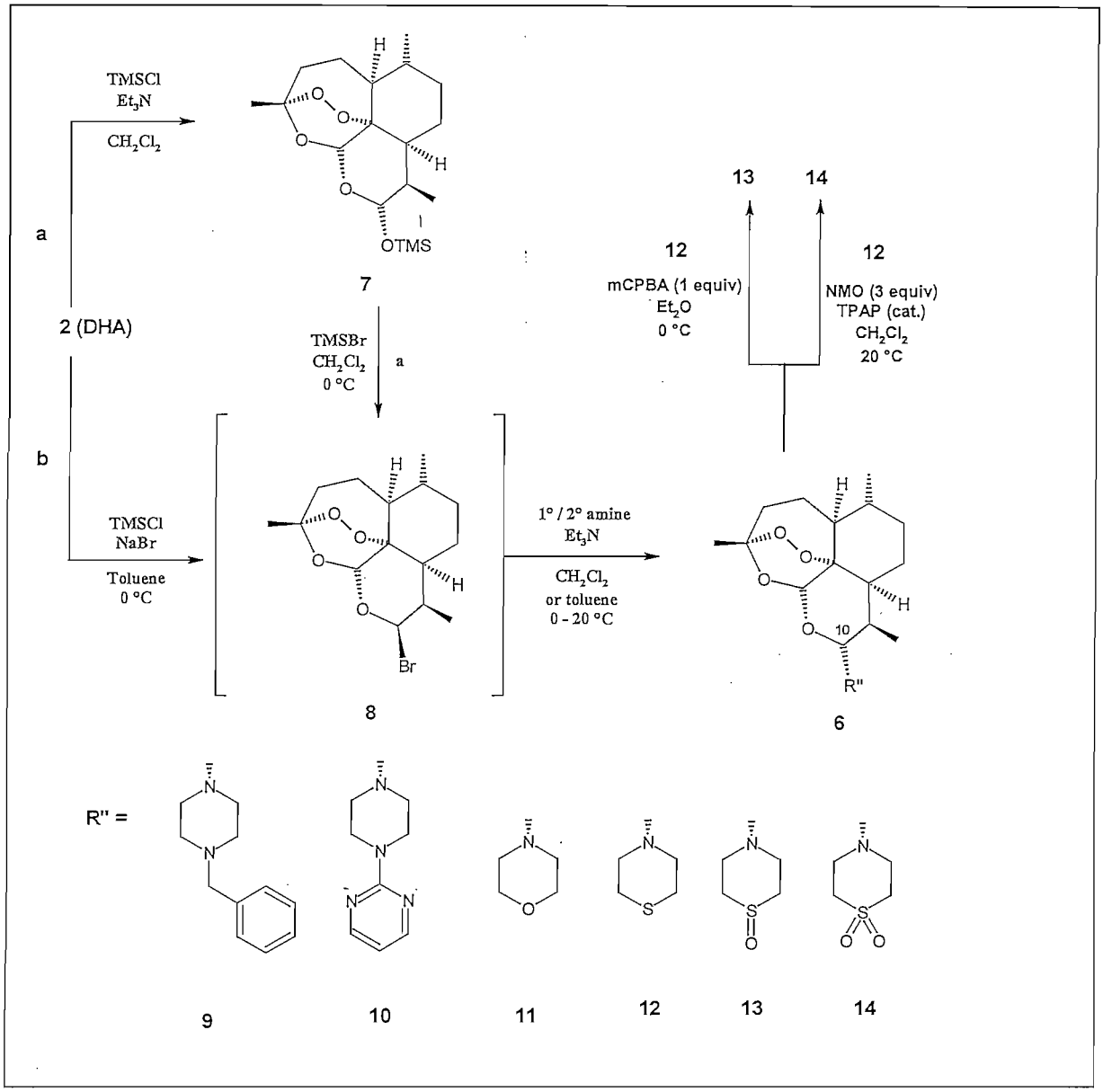

Figure 2.2: Preparation of 10-alkylamino derivatives (Haynes *et al.*, 2006)

Artemisone (14) is obtained by either treatment of (8) with thiomorpholine-5,5-dioxide or by oxidation of artemiside (12). Artemside is obtained from (8) and thiomorpholine. Oxidation of artemiside also produces a sulfoxide (13) (Haynes *et al.*, 2006).

2.3 PHYSICOCHEMICAL PROPERTIES

Artesunate is the hemisuccinate salt of DHA. This compound has a calculated log *P* value of 3.04 and a measured value of 2.77 and can be classified as being water soluble (Meshnick *et al.*, 1996).

According to Haynes *et al.* (2006) artemether is relatively lipophilic. Its calculated log *P* value is 3.51 while the measured log *P* value was 3.98. DHA on the other hand exists as two epimers in solution, two log *P* values have been recorded, namely 2.35 and 2.73. The solubility of DHA could not be measured due to decomposition problems. Artemiside is lipophilic by nature and has a measured log *P* value of 4.97 and a calculated value of 3.98. Artemiside is virtually insoluble in an aqueous medium. Artemisone is very crystalline by appearance and is easily purified, it further displays adequate aqueous solubility which exceeds the limiting requirements for a drug (Lipinski *et al.*, 2001). Table 2.1 contains both measured and calculated log *P* values for artemisinin and a few of its derivatives.

Table 2.1: Aqueous solubility and octanol-water partition coefficients (Haynes *et al.*, 2006)

	Artemisinin	Artesunate	Artemether	Artemiside	Artemisone
Solubility [mg/L]	63	565	117	< 2	89
Log *P*	2.94	2.77	3.98	4.97	2.49
Log *P* (calculated)	3.15	3.04	3.51	3.98	2.08

- Solubility = aqueous solubility at pH 7.2
- Log *P* = octanol-water partition coefficient for the neutral compound determined at pH 7.4, except artesunate which was determined by HPLC at pH 2
- Log *P* (calculated) = calculated log *P* value (Ghose & Crippen, 1987)

2.4 PHARMACOKINETIC PROPERTIES

Artemisinins can be administered via several routes. Artesunate is a water soluble derivative and can be administered via the oral, intramuscular, intravenous and intrarectal routes (Krishna *et al.*, 2001). The most clinically useful artemisinins are metabolized to DHA, they include artemether, artesunate and arteether (Alin *et al.*, 1996). Artemisone on the other hand is not converted to DHA; this occurrence will be discussed in more detail under the headings of neurotoxicity and metabolism. Artemiside is a novel compound and very little data is available. It is included in this study to investigate its antimalarial activity and bioavailability.

Artesunate has a very short elimination half-life of approximately 10 minutes. Its metabolite (DHA) is more important in exerting antimalarial activity due to a longer elimination half-time

of approximately one hour. This, still relatively short, half-life of DHA confers the theoretical advantage that selection for drug resistant parasites is less likely to occur. The disadvantage of this is, however, a higher associated risk of recrudescence when these drugs are used in monotherapy regimens. Oral artesunate is rapidly absorbed, with good bioavailability, in patients with uncomplicated malaria (Alin *et al.*, 1996).

According to Krishna *et al.* (2001) it is unlikely that normal artemisinin clearance parameters are adversely affected by increasing disease severity. However, the absorption of artemisinin may be altered by variables such as the specific formulation, solubility characteristics and the chosen route of administration.

Artemisinins are currently used almost exclusively to treat severe malaria which is resistant to chloroquine therapy. Artesunate bioavailability is acceptable when administered via the intramuscular route in patients with severe malaria with no local toxicity symptoms (Nontprasert *et al.*, 2002).

2.5 PHARMACODYNAMIC PROPERTIES

The endoperoxide moiety of artemisinin derivatives is essential for antimalarial activity. The potency can be markedly increased by specific substitutions on the lactone carbonyl group. These compounds act very rapidly upon asexual erythrocytic stages of *P. vivax* and *P. falciparum.* It is effective against chloroquine sensitive, chloroquine-resistant, and multidrug-resistant strains of *P. falciparum.* The *in vivo* potency of the artemisinins is rated 10 – 100 fold greater than that of other antimalarial drugs (White, 1997). Artemisinins act upon the broadest age range of parasites, from the smallest rings which have very recently invaded erythrocytes to more mature parasite stages such as developing trophozoites and schizonts (Angus *et al.*, 1997).

Due to this broad stage-specificity of action the artemisinins are also able to impede the development of gametocytes. This stands in stark contrast to other antimalarial classes such as the antifolates or the 4-aminoquinolines. The antifolates and 4-aminoquinolines do not have the ability to interrupt transmission of malaria. These favourable properties enable the artemisinins to remove ring stage parasites from within red blood cells without destruction of the cells (Angus *et al.*, 1997; White, 1997).

This property translates to a rapid reduction in the circulating parasitaemia, sometimes by up to 2 – 3 log orders of magnitude (White, 1999). Another advantage of the artemisinins is their

ability to inhibit the cytoadherence to endothelial cells of maturing parasites. This efficient inhibition may confer to a comparative advantage over other drug classes (Udomsangpetch *et al.*, 1996).

2.6 MECHANISM OF ACTION

Artemisinins act via specific mechanisms distinct from other antimalarial classes. Artemisinins do not inhibit specific target enzymes of folate biosynthesis or the 1-Deoxy-D-Xyluose 5-Phosphate (DOXP) reductase pathway, nor does it act upon the cytochrome electron transport system. The peroxide, essential for antimalarial activity, is located within the 1, 2, 4-triozane system of the artemisinins. These artemisinins include artesunate, artemether and arteether. The 10-alkylamino derivative, which also includes artemiside and artemisone in which the peroxide is intact, was more active than their parent compound (artemisinin) against the malaria parasite during *in vitro* studies (Jung *et al.*, 1990). The peroxide structure has subsequently become a focus for considerable analysis aimed at trying to understand the mechanism of action of artemisinins. A few mechanistic possibilities are presented in the following pages.

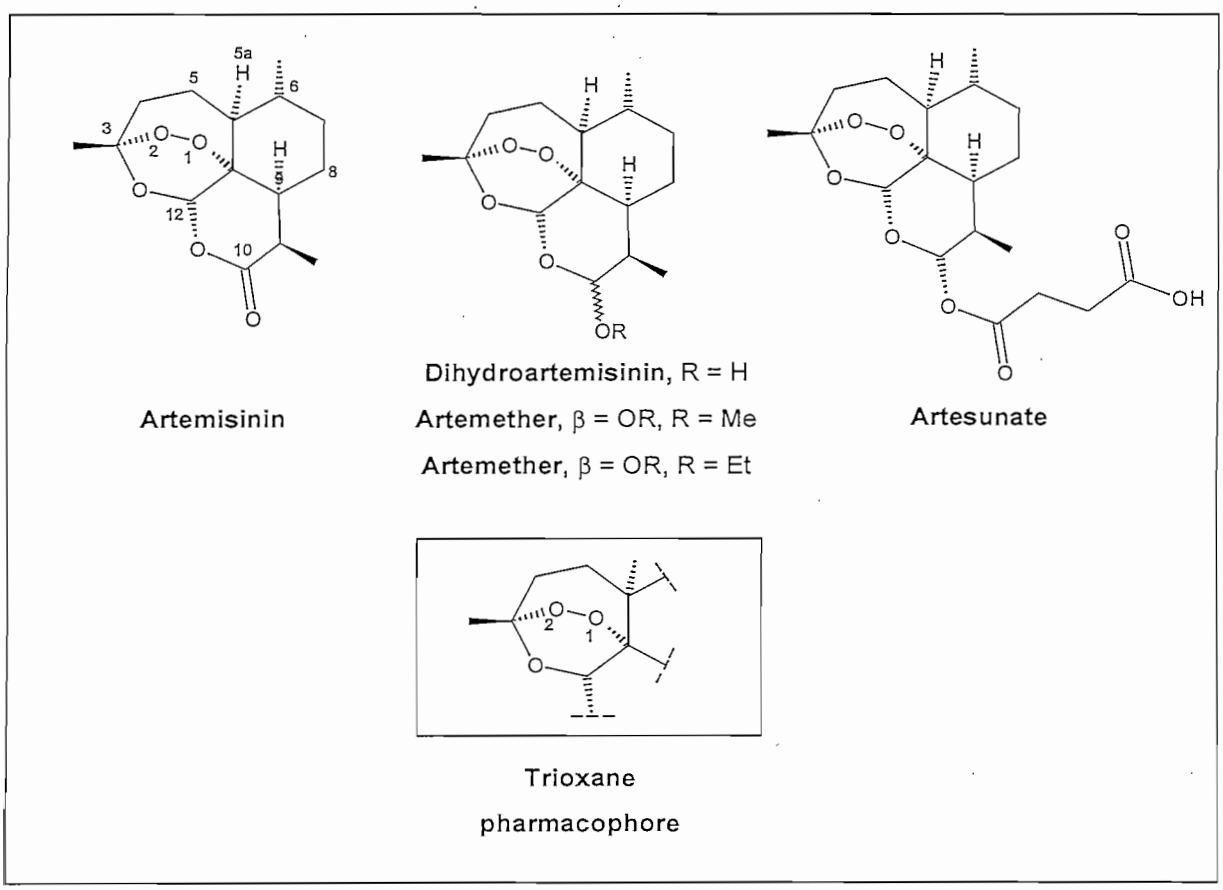

Figure 2.3: Artemisinins currently in use and the active trioxane pharmacophore (Olliaro *et al.*, 2001)

2.6.1 Peroxides as "prodrugs"-putative action via reactive oxygen species

Peroxides are generally reactive entities. A connection has been drawn between the mechanism of action of artemisinins and the generation of reactive oxygen species (ROS). The ROS can be represented by hydroxyl, alkoxyl, and protonated superoxide or peroxyl radicals within the parasitized erythrocyte. Studies suggest that infected erythrocytes may be haemolyzed due to solutes, such as sorbitol, which enter through novel permeation pathways caused by oxidant stress of proteins induced by exposure to *tert*-butyl hydroperoxide (Huber *et al.*, 2002). The formation of ROS may also be enhanced through the Fe^{2+}-dependent Fenton process, conversely desferrioxamine inhibits the parasiticidal activity of peroxides. This is apparently accomplished by scavenging the intracellular Fe^{2+} iron required for the Fenton process through its redox equilibration with Fe^{3+} and formation of an extremely stable F^{3+} complex (Vennerstrom & Eaton, 1989).

Based on these observations it is commonly excepted that parasite death and heamolysis are mediated by reactive oxygen species whose presence eventually destroys the parasite's anti-oxidant defense systems. The exogenous peroxide is instrumental in further enhancing the activity of the reactive oxygen species (Erel *et al.*, 1997).

2.6.2 Artemisinins as "prodrugs"-activation via C-centered radicals or carbocations

Many studies concluded that Fe^{2+} is important in activating peroxides. The most commonly accepted source of Fe^{2+} in an infected erythrocyte is considered to be the food vacuole of the parasite. The food vacuole is responsible for digestion of haemoglobin and also for detoxification of free iron by means of deposition in haemozoin (Krishna *et al.*, 2004). This free iron is chelatable and is probably instrumental in free radical formation and might subsequently activate artemisinin. The peroxide ring is opened by protonation or by complexation with Fe^{2+}, resulting in an open hydroxyl-peroxide or metal-peroxide. The positive charge of the open peroxide is in turn stabilized by the oxygen atom which is not in the ring. This is responsible for lowering the energy and opening the ring (Golenser *et al.*, 2003).

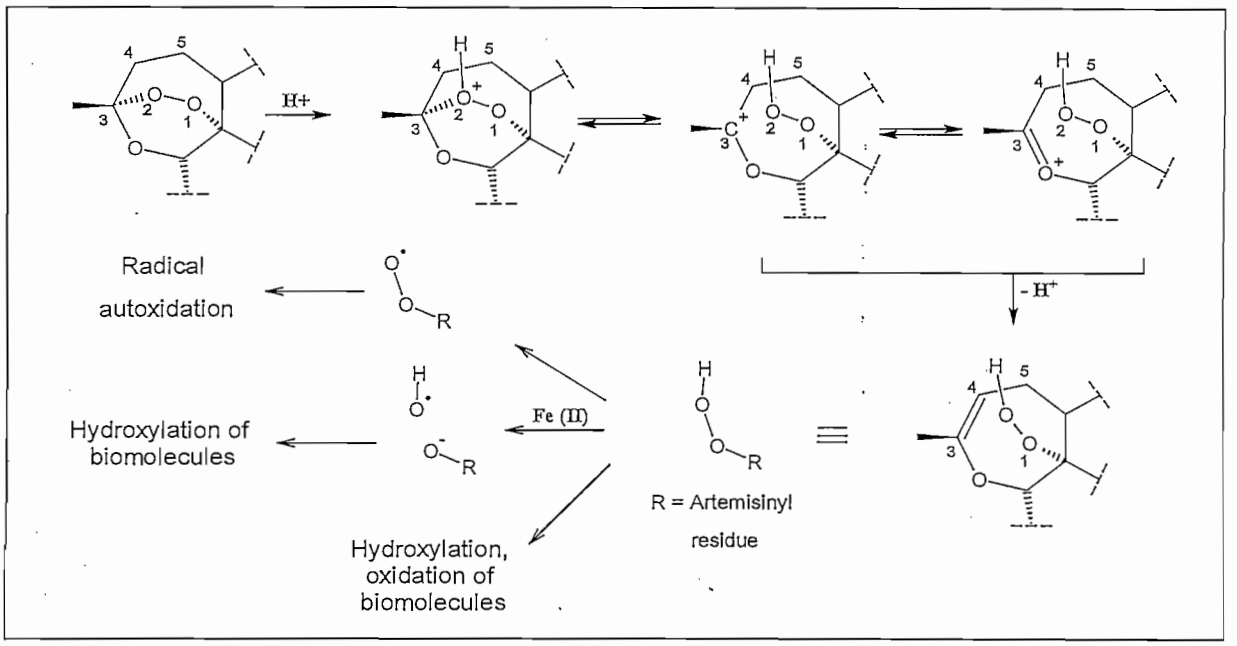

Figure 2.4: Proposed mechanism of hydroperoxide generation by means of opening the peroxide bond (Olliaro *et al.*, 2001)

In figure 2.4 the subsequent decomposition pathways are also included. In this case a proton is illustrated, but an oxophilic metal ion M^{n+}, such as Fe (II), would have achieved the same result (Olliaro *et al.*, 2001).

The oxygen-centered radical attracts hydrogen ions which in turn causes the formation of carbon-centered radicals. These carbon-centered radicals are thought to target haeme-binding proteins and proteinases which are instrumental in haemoglobin degradation (Haynes & Krishna, 2004).

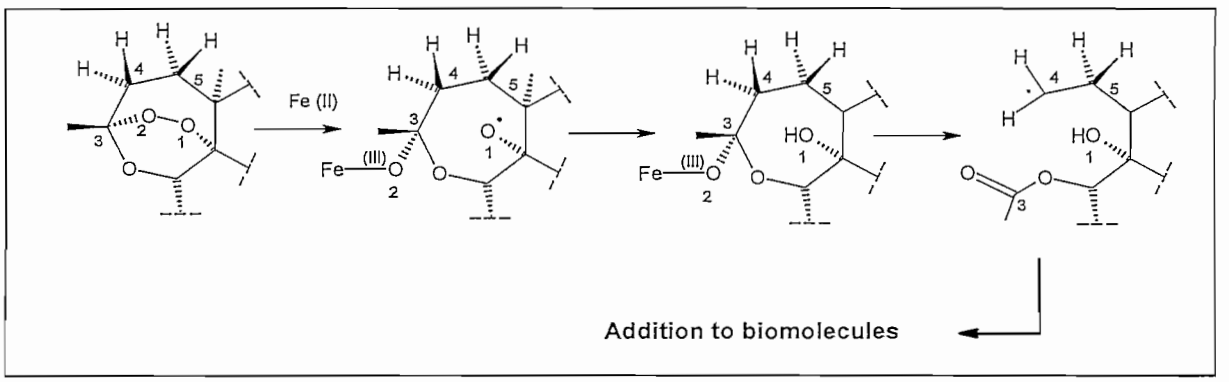

Figure 2.5: Proposed mechanism of reductive scission of the peroxide bond and the formation of carbon-centered radicals. Fe (II) is either iron (II) in ferrous haem, or exogenous iron (II) (Olliaro *et al.*, 2001)

In stark contrast to the above hypothesis, is evidence from *in vivo* studies where covalent binding to haemoglobin has been demonstrated. The conclusion was that malaria parasites could be eliminated by a joint effect of stopping haemozoin formation while simultaneously creating a more reducing milieu which is not conducive to the further formation of haemozoin (Kannan *et al.*, 2005).

The antimalarial efficiency of artemisinins and its derivatives has been found proportional to their binding of haemin. This finding ads further support to the iron-dependent proposed mechanism of action. Molecular docking simulations between artemisinin analogs and free haemin (Fe^{2+}-haeme) were followed by three- dimensional quantitative structure-activity analysis of the formed complexes. The formed complexes were evaluated and it was found that the global energy-minimum configurations of the complexes indicated that the endoperoxide bridge of the drug pointed directly in the direction of the iron molecule. The two carboxyl ethyl groups in haemin are perpendicular to the porphyrin ring, and their carboxyl groups interacting with the hydrogen atoms on both C1 and C9 of the artemisinin analogs. Studies revealed that these complexes were primarily formed as a result of electrostatic interactions between the peroxy group in the analogs and the iron ion in haemin. The transfer of an electron from the peroxy group to the iron may be enabled by this configuration, cleavage of the peroxide bond and formation of a free radical is a direct result of this occurrence (Cheng *et al.*, 2001).

More recent *in vitro* experiments characterized the complexes formed between F^{3+}-haeme and artemisinin-type drugs. The artemisinin formed a noncovalent complex and in addition to this, DHA formed a unique complex with haeme in which a hydrogen atom was replaced by a sodium atom. The formed artemisinin-haeme complex was not very stable while the Na^{+}-containing DHA-haeme complex exhibited great stability. The efficiency of the artemisinins in forming noncovalent complexes with Fe^{3+}-haeme is very much comparable to that of quinine, which is known to bind to haeme, indicating a possible similar mechanism of action (Pashynska *et al.*, 2004).

This haeme-dependent activation theory has two main flaws. The first being the fact that artemisinins are localized in the red blood cell tubovesicular membrane network which transports the drug into both the parasite and parasite membranes but usually not into the parasite food vacuole membranes where there is a reported increased chance for availability of labile iron (Olliaro *et al.*, 2001). The second flaw being the fact that artemisinins are also active against the ring forms of *P. falciparum*. These juvenile forms do not yet metabolize haemoglobin and therefore lack haemozoin (Eckstein-Ludwig *et al.*, 2003).

The theory concerning the mediation of DHA activity by means of free radical formation and potentiation of DHA's effects by the addition of iron was evaluated. The theory was examined by exposing *L. major* promastigotes to varying concentrations of Fe-nitrilotriacetic acid (NTA), in addition to DHA (Golenser *et al.*, 2006). The rationale behind the addition of NTA was to induce a chelating effect where NTA bind the iron and prevent the formation and subsequent precipitation of iron complexes in the culture medium (Fischer *et al.*, 1998). The separate addition of either 0.11 µM or 50 µM Fe – NTA had no effect, but their combination resulted in a 55% cell growth inhibition. This result supports the theory of an iron-mediated non-specific free radical mechanism of damage by DHA (Golenser *et al.*, 2006).

2.6.3 Effect of immune system functions

Artemisinin and its derivatives have been shown to affect immune responses of the primary host. It has further been demonstrated that artemisinins are able to suppress nitric oxide (NO) synthesis. This finding stands in contrast to the iron-mediated formation of free radicals theory (Aldieri *et al.*, 2003). The inducible nitric oxide synthase (iNOS) gene in macrophages catalyzes the generation of NO. NO is involved in inflammatory reactions and immune responses. Nitrite and nitrate is the end products when NO is converted in living systems. The conversion process is quite rapid. Human astrocytoma T67 cells, which contain iNOS, were studied. The study revealed a significant reduction in nitrite levels with the addition of 10 µM artemisinin. This occurrence suggests an inhibition of iNOS activity and consequently suppression of NO synthesis. Closer inspection led to the conclusion that this iNOS activity inhibition by artemisinins occurred at the transcription level. Artemisinin prevents the LPS-induced proteolytic degradation of I_xB and the linked activation and translocation of NF-$_xB$, which under normal circumstances would have led to the promotion of iNOS transcription. This translocated NF-$_kB$ is implicated in the transcription of genes involved in cytokine production, cellular adhesion, inflammation and apoptosis (Pahl, 1999).

Artemisinin is considered to be highly effective in the treatment of human cerebral malaria (CM) (WHO, 2006). Tumor necrosis factor (TNF) or the closely related lymphotoxin α (LTα) was proven to play a key role in the pathogenesis of experimental CM (Hunt *et al.*, 2006). A study was conducted to assess the potential role of DHA on *in vivo* TNF production and subsequently on CM. The production of TNF was assessed in both LPS-challenged and malaria-infected mice. The administration of DHA, for a period of 3 days, to the infected mice led to significantly lower TNF levels in comparison to the untreated control group. All of the DHA treated mice had lower TNF levels and survived the experiment. The control group did

not survive for more than 7 days. TNF concentrations were assessed by bioassay and by ELISA methods. The samples from the DHA treated group indicated a reduced TNF mRNA accumulation in both the spleen and lungs in comparison with the control group. The results provides evidence that a significant part of the effect of artemisinin may be attributed to the downregulation of both TNF and NO (as mentioned earlier) (Golenser *et al.*, 2006).

2.6.4 Effect on angiogenesis

Studies indicate that DHA is able to inhibit the binding of vascular endothelial growth factor (VEGF) to its receptors and in turn lower VEGF receptor expression. This causes inhibition of angiogenesis. The effect of DHA on VEGF function may have an influence on the implications of CM. VEGF is implicated in the activation of vascular and endothelial cells which instigate disruption of the blood-brain barrier function. It is also responsible for angiogenesis in blood vessels where parasite sequestration is prevalent. The severity of malarial infection is correlated to both endothelial cell activation and blood-brain barrier disruption. This results in fatal brain oedema and haemorrhages and is most probably the source of neurological impairments in CM survivors (Deininger *et al.*, 2003). These findings suggest that the down regulation of VEGF, with the aid of DHA administration, may alleviate symptoms of CM and also reduce neurological damage in those who survive the disease (Golenser *et al.*, 2006).

2.6.5 Interference with plasmodial sarcoplasmic/endoplasmic calcium ATPase (SERCA)

Artemisinin was shown to be sensitive to steric effects during *in vitro* antimalarial activity experiments. It was suggested that the molecule was activated after binding to a specific site (Haynes & Krishna, 2004; Krishna *et al.*, 2004). Thapsigargin is an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and is structurally very similar to artemisinin. It was hypothesized that artimisinins specifically inhibit the *P. falciparum* SERCA (Eckstein-Ludwig *et al.*, 2003).

The calcium ion concentrations are maintained by the SERCA. This maintenance is crucial for the generation of calcium-mediated signaling and the correct folding and post-translational processing of proteins. The only SERCA-type Ca^{2+} ATPase encountered in *P. falciparum* is PfATP6. Tests confirmed that artemisinin completely inhibited PfATP6 activity in *Xenopus laevis* oocysts. A concentration of 50 µM artemisinin was sufficient to obviate the

highly specific nature of this inhibition. No other transporters, including the non-SERCA Ca^{2+}-ATPase PfATP4 were affected. The addition of both thapsigargin and artemisinin to *P. falciparum* cultures rendered an antagonistic effect. This occurrence suggests that these drugs have a common target (Eckstein-Ludwig *et al.*, 2003). Three-dimensional modeling of the PfATP6 amino acid sequence and subsequent docking simulation demonstrated that artemisinins bind to the protein target by means of hydrophobic interactions while leaving the peroxide bonds exposed (Jung *et al.*, 2005). It is then possible for iron to cleave the exposed peroxide bridge and consequently generate carbon-centered radicals. This leads to enzyme inactivation with resultant parasite death (Uhlemann *et al.*, 2005).

Point mutations in PfATP6 have been found in field isolates. These isolates exhibited reduced *in vitro* sensitivity to artemether (Jambou *et al.*, 2005). This finding may imply that these point mutations prevent artemisinin derivatives from interfering with the conformational changes required for protein function and multiplication. The exact mechanism by which these point mutations induce the suggested stereo chemical effect has not yet been elucidated. Jambou *et al.* (2005) suggested that PfATP6 could be a protein target for artemisinins and that there was a possibility of resistance development to these drugs in the near future due to alterations in the PfATP6 sequence.

It has been reported that artemisinins are able to inhibit red blood cell endocytosis in the parasite. This effect of artemisinin on *P. falciparum* endocytosis of red blood cell cytoplasmic macromolecules were investigated. Erythrocytes were preloaded with biotinylated dextran and infected with parasites. The cells were then incubated for 12 hours in the presence of 110 nM artemisinin. Although changes in cytosolic Ca^{2+} levels, as a result of SERCA inhibition, may have had a major regulatory effect on endocytosis, no direct link could be established during that investigation (Hoppe *et al.*, 2004).

2.6.6 Mitochondrial electron transport interference

Recent studies suggest that artemisinin and its various derivatives are activated by components of the electron transport chain of the parasite mitochondria and also subsequently interfere with the electron transport chain. Yeast preferentially utilize glycolysis to generate energy in the presence of a fermentable carbon source, *Saccharamyces cerevisiae* is able to grow with minimal mitochondrial activity and was used to investigate the above theory. Mitochondrial activity is required by *S. cerevisiae* for growth in the presence of a non-fermentable carbon source. *S. cerevisiae* cultures were grown on either glycerol or ethanol non-fermentable carbon sources, addition of artemisinin to these cultures strongly

inhibited yeast growth. The IC_{50} was achieved with 10 nM artemisinin. This concentration was comparable with previous results achieved during *in vitro* experiments on *P. falciparum*. The authors concluded that the addition of artemisinin caused a depolarization of the mitochondrial membrane (Krungkrai *et al.*, 1999).

Genetic studies demonstrated that deletion of yeast genes NDE1 and NDI1, which encode NADH dehydrogenases in the mitochondrial electron transport chain, led to artemisinin resistance. The *P. falciparum* database contains a gene which is homologous to NDI1. It was suggested that the mitochondria may play a role in the activation of artemisinin. The activation of artemisinin causes a local production of reactive oxygen species and depolarization of the mitochondrial membrane (Li *et al.*, 2005). It has been shown that it was possible for artemisinins to achieve this respiratory chain inhibition in both sexual and asexual stages of *P. falciparum* (Krungkrai *et al.*, 1999).

The loss of membrane potential has an adverse effect on Pyrimidine biosynthesis. Pyrimidine biosynthesis is a key metabolic process for nucleic acid production and interruption of this process may readily cause parasite death (Li *et al.*, 2005).

2.7 METABOLISM

2.7.1 Metabolism of the most common artemisinin derivatives

Artemisinin and most of its derivatives are metabolized by cytochrome P450 enzymes, CYP2 C19 and CYP2 B6. These are both associated with first pass drug metabolism. Artemisinin and most of its derivatives, including artesunate, artemether and arteether are metabolized to DHA. DHA has an effect at least as potent as its parent compounds (Burk *et al.*, 2005). Artemisinin class drugs are known for their relatively short elimination half-lives. Artesunate is almost immediately metabolized while the lipophilic derivatives, such as artemether and arteether, are metabolized more slowly. DHA has a half-life of approximately 1 hour. Artemisinin treatment causes a rapid reduction in parasite count, to such an extent that it is usually below detectable limits after initial treatment. The main problem with artemisinin monotherapy is the occurrence of recrudescence due to a few parasites remaining in detected in the host after initial treatment. This can be prevented by combining the preferred artemisinin drug with a longer acting drug such as atovaquone. In healthy individuals, orally administered artemisinin is metabolized in the liver, due to poor and erratic absorption and to the first pass effect, bioavailability is only 32% (Meshnick *et al.*, 1996).

2.7.2 Metabolism of artemisone

Artemisone is not metabolized to DHA. To demonstrate this fact and to identify metabolites formed *in vitro* and *in vivo*, labeled artemisone (14*), incorporating either ^{14}C or ^{2}H in the metabolically inert 15-methyl group was used (Haynes *et al.*, 2006). Exocyclic methylidene was cleaved from artemistene (19) to give the known dicarbonyl compound (16), treatment of this compound (16) with ^{2}H- or ^{14}C-methylidene triphenylphosphorane provided labeled artemistene (19*). The deuteriated reagents are shown in figure 2.6.

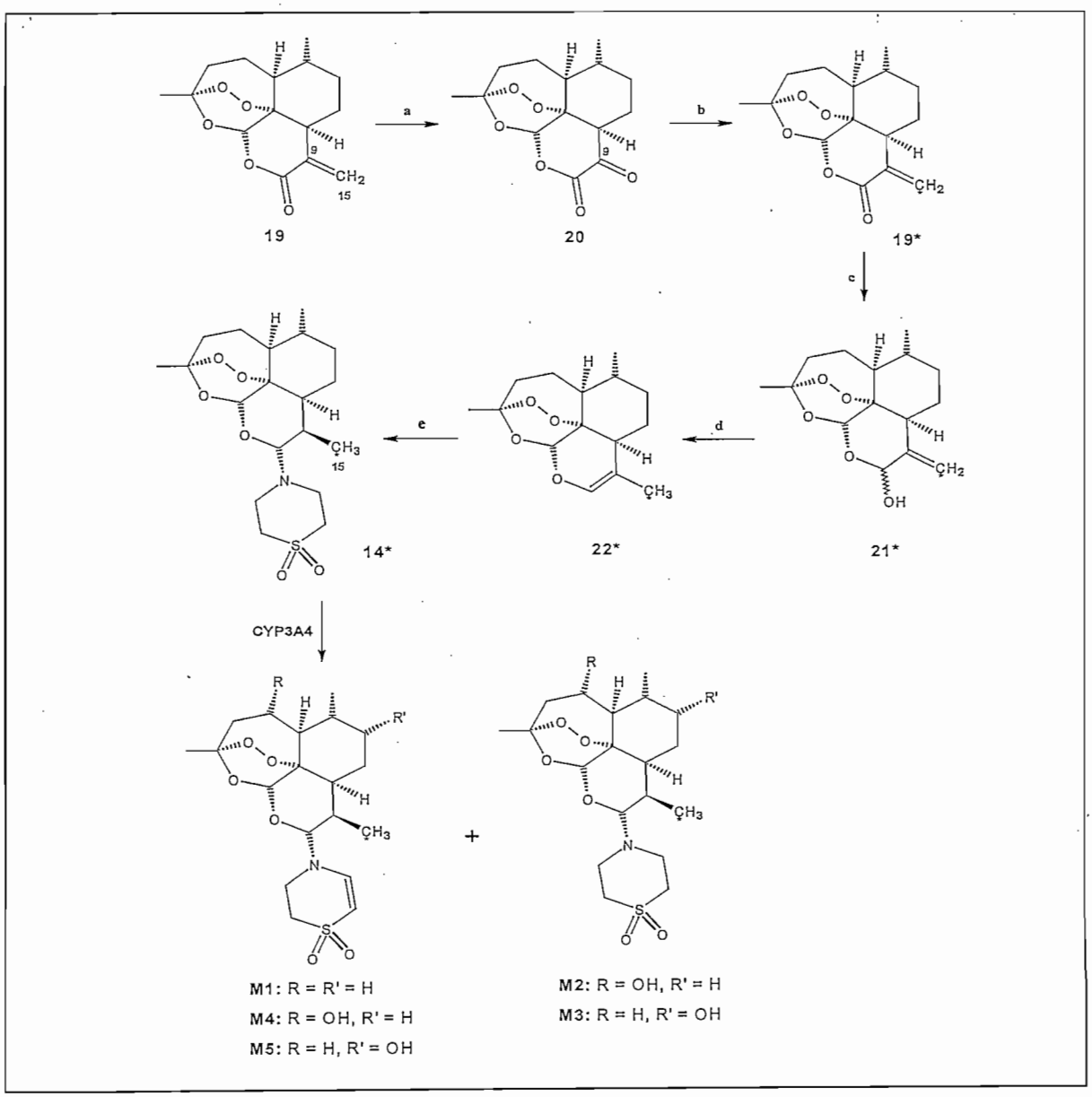

Figure 2.6: **Preparation of labeled artemisone 14* (C^*H_3 = $^{14}CH_3$ or C^2H_3) and CYP3A4-mediated formation of metabolites M1 – M5 (Ph_3P = $^{14}CH_2$, Et_3SiH for ^{14}C label; Ph_3P = C^2H_2, Et_3Si^2H for ^{2}H label; yields quoted for isolated deuteriated compounds)** (Haynes *et al.*, 2006)

Reagents and conditions:

a. $NaIO_4/RuCl_3$, CCl_4, MeCN, 20 °C, 54%;
b. C^2H_3 PPh_3 Br, n – C_4 H_9 Li, N_2, THF, -78 °C; then 20, -78 – 20 °C, 20h, 48%;
c. iBu_2 AlH, CH_2 Cl_2, -78 °C, 2h, >98%;
d. Et_3 SiH or Et_3 Si^2H, TFA, -78 °C, 4h, 40%
e. HCl, CH_2 Cl_2, 0 °C; then thiomorpholine, Et_3N, 0 °C, 4h; 50% to 12*; then mCPBA, K_2 CO_3, Et_2 O, 0 °C, to 14*, 72%. TFA = trifluoroacetic acid (Haynes *et al.*, 2006).

The carbonyl group in 19* was first reduced. The resulting allylic alcohol 21* was then reduced with Et_3 SiH for the ^{14}C-labeled compound or with Et_3 Si^2H for the ^2H-labeled compound to obtain glycol 22*. Glycol was then converted by stereo selective addition of HCl and treatment of the intermediate 10β-chloride with thiomorpholine to give labeled 12*. The labeled 12* was then converted to labeled artemisone (14*) by means of oxidation (Avery *et al.*, 1996).

In vitro experiments were conducted with the labeled artemisone (14*). It was administered to human liver microsomes and incubated for 30 minutes. Metabolites M1 – M5 were identified, M1 accounted for ≈ 34% of the total radioactivity (TRA) while M2 and M3 accounted for ≈ 19% and ≈ 16% of TRA respectively. Metabolites M4 and M5 accounted for a smaller fraction of less than 6% TRA each, leading to an approximate total conversion of 77% of labeled artemisone (14*). The respective metabolites were isolated and identified unambiguously with the aid of 1D and 2D NMR spectroscopic analysis and was coupled with high-resolution mass spectrometry. DHA could not be detected in any of the systems (Haynes *et al.*, 2006).

In vivo experiments, carried out on rats, were also conducted. Artemisone was administered to the rats via the oral route, the plasma samples contained M2 and M3 and minor amounts of M1, M4 and M5. The *in vivo* plasma profile of these metabolites correlate very closely with previous *in vitro* results obtained from studies using rat liver microsomes and hepatocytes. Based on this observation it is very likely that the metabolism of artemisone in humans will reflect the same results as was obtained in human microsomes and hepatocytes for metabolites M1 – M5 (Haynes *et al.*, 2006).

Incubation of ^{14}C-labeled artemisone (14*) with human liver microsomes and 14 recombinant CYP isoforms in conjunction with selective inhibitors rendered interesting results. Artemisone

was only metabolized, to a significant extent, by CYP3A4 while troleandomycin, azamulin and ketoconazole were identified as effective inhibitors (Haynes *et al.*, 2006).

Treatment of human hepatocytes with artemisone in ascending concentrations from 4 - 10 000 ng/L indicated only a borderline inductive effect on CYP2B6 and CYP3A4 at the highest tested concentration. No induction was observed with CYP1A2 or CYP2C19. Standard substrates were also incubated in the presence of artemisone. There was no recorded effect on substrate metabolism of substrates CYP1A2, -2A6, -2C9, -2C19, -2D6, -2E1 or -3A4. The assumption can be made that induction of either CYP3A4 or CYP2B6 with artemisone will most likely not be a problem in clinical applications, and this stands in stark contrast to other artemisinins currently in use (Haynes *et al.*, 2006).

2.8 TOXICITY

2.8.1 Toxicity of the most common artemisinin derivatives

Unlike most antimalarial drugs, no serious side-effects of artemisinin or its derivatives such as artesunate, artemether or arteether have been reported during treatment. The most common adverse effects are mild by nature and include nausea, vomiting and diarrhea. All of these effects are also characteristic of acute malaria (Wongsrichanalai *et al.*, 1997). All clinical trials which implicated the use of artemisinin drugs were subjected to a meta-analysis. The verdict of that analysis was that these compounds were safe and well tolerated and that no clinical difference was found in terms of safety or tolerance between the various derivatives (Ribeiro & Olliaro, 1998).

In contrast to the clinical trials in humans, toxicity in cell lines and neurotoxicity in laboratory animals have been observed (Singh & Lai, 2004). This discrepancy may be attributed to the fact that laboratory animals were exposed to much higher dosages than those used in humans. The dosage difference between laboratory animals and humans may vary between 20 – 30 fold. Human dosages are usually in the range of 2 – 10 mg/kg while animal studies usually include dosages as high as 250 mg/kg (Gordi & Lepist, 2004).

The route of administration may also play an important role in the presentation of toxic effects, most of the recorded toxic effects presented with the administration of large volume, oil-based, injections of up to 0.5 ml/kg. This approach leads to a depot-effect with sustained release of the drug for an extended period of time usually being the resultant effect, this

effect is thought to be the main factor responsible for toxicity in animals (Woodrow *et al.*, 2005). A combination of short treatment time, small injection volumes and reduced dosages, in comparison to animal studies, would minimize the risk of neurotoxic side-effects in humans (Gordi & Lepist, 2004).

Neurotoxicity, particularly to brainstem centres, involved in hearing and balance, have been described in 3.3% of patients receiving artemisinin related drug treatment. These effects are in addition to the toxic effects recorded in animals and contrary to the above explanation (Schmuck *et al.*, 2002). Neurodegeneration may be linked to this apparent neurotoxicity; studies also indicate hearing loss in patients treated with oral artemether-lumefantrine (Toovey & Jamieson, 2004). Schmuck *et al.* (2002) conducted *in vitro* studies to investigate the effect of artemisinin on neuronal cell cultures isolated from rats. Cortical neurons, cortical astrocytes and brain stem cells were exposed to artemisinin for up to 7 days and then compared to each other. The brain stem cells showed selective sensitivity to artemisinin, the EC_{50} value was 0.014 µM. The cortical neurons exhibited a delayed effect which became apparent only after 7 days of exposure, the artemisinin EC_{50} in that case was 1.058 µM.

Extensive studies on artemisinin and its derivatives revealed that DHA was the most neurotoxic drug, followed by artemisinin, artemether and then artesunate (Schmuck & Haynes, 2000). Toxicity to neuronal cells and astrocytes was found to be a direct result of mitochondrial function inhibition and induction of oxidative stress. Respiratory chain interference is responsible for inducing irreversible neurodegeneration. This raises the suspicion that the neurotoxic mechanism of artemisinin may be related to the production of radicals in conjunction with inhibition of the mitochondrial respiratory chain. The antioxidant defense system in neuronal cells is less specialized and also less active than in non-neuronal cells and may explain the increased sensitivity of neuronal cells to artemisinin (Schmuck *et al.*, 2002).

Parenteral administration of artemisinins has been known to cause embryo loss in mice, rats and rabbits. This confirms the possibility of artemisinin toxicity to foetuses *in utero*, although extensive monitoring of human pregnancies has not shown any definite teratogenic or embryo toxic effects (McGready *et al.*, 2001).

Clark *et al.* (2004) reasons that the number of pregnant woman exposed to artemisinins during the first trimester, the most critical stage of pregnancy, is too small to collect a sufficient amount of data to demonstrate drug safety. They investigated the effect of artesunate administered to rats on days 6 – 17 and to rabbits on days 7 – 19 of gestation.

Based on the results they concluded that artesunate is highly embryo toxic. The foetuses that survived the treatment presented various complications such as growth retardation, skeletal defects and also cardiovascular defects. Defective haematopoiesis and angiogenesis were suggested to be the cause of tissue damage and morphological defects attributed to treatment with artemisinin drugs (Longo *et al.*, 2006). Based on this evidence, the potential benefits of artemisinin treatment should be weighed against the potential risk and alternative treatment options should be considered for woman during the first trimester of pregnancy if feasible.

2.8.2 Toxicity of artemisone

Very little data is available concerning the toxicity profile of artemisone. Schmuck and Haynes (2000) assessed the neurotoxicity with an *in vitro* model, developed for screening compounds required for medicinal and other purposes. Primary neuronal brain stem cell cultures from foetal rats, with endpoints of cytotoxicity and neurofilament integrity, were employed in the study. The intracellular ATP level is a sensitive and early marker of cytotoxicity and was also measured during the study. DHA displayed the greatest neurotoxocity while artemisone did not show any signs of neurotoxicity. DHA was also found to be cytotoxic towards cortical neurons and non-neuronal cell lines. The results of that comparative neurotoxocity study between a few artemisinin derivatives, including artemisone and artemiside are presented in table 2.2.

Table 2.2: Effect of artemisinin and a few derivatives on viability and neurofilaments in rat primary neuronal brain stem cell cultures (Haynes *et al.*, 2006)

Compound	Viability [µg/mL](a)		ATP [µg/mL](b)		Neurofilaments [µg/mL](c)	
	NOEC (d)	IC_{50}	NOEC (d)	IC_{50}	NOEC (d)	IC_{50}
Artemisinin	1.0	> 10	0.01	0.08	0.001	0.05
DHA	0.1	5.0	0.01	0.08	< 0.001	0.01
Artesunate	0.1	> 10	-	-	0.1	5.0
Artemether	1.0	> 10	-	-	0.01	0.1
Artemiside	1.0	> 10	1.0	8.5	0.001	> 10
Artemisone	> 10	> 10	> 10	> 10	> 10	> 10

a) The potential cytotoxicity was measured by viability based on the activity of neuron-specific enolases.
b) ATP (adenosine triphosphate) levels measured at 1 day after initial administration.
c) Neurotoxicity was assessed by the effect on the cytoskeleton on day 7.
d) No observable effect concentration (Haynes *et al.*, 2006).

The general toxicity of artemisinins is most likely attributable to facile *in situ* hydrolysis to DHA. Artemisone in contrast, as described earlier under the heading of metabolism, is not converted to DHA and this is considered to be the main reason for its lack of neurotoxicity (Haynes *et al.*, 2006).

2.9 COMPARATIVE ANTIMALARIAL DRUG ACTIVITIES

Haynes *et al.* (2006) conducted comparative *in vitro* screens against a variety of multidrug-resistant *P. falciparum* isolates. In those screens it could be clearly seen that artemisone was significantly more active than artesunate, chloroquine and also pyrimethamine, irrespective of the resistance profile. Figure 2.7 is a bar-diagram containing the comparative results of that study.

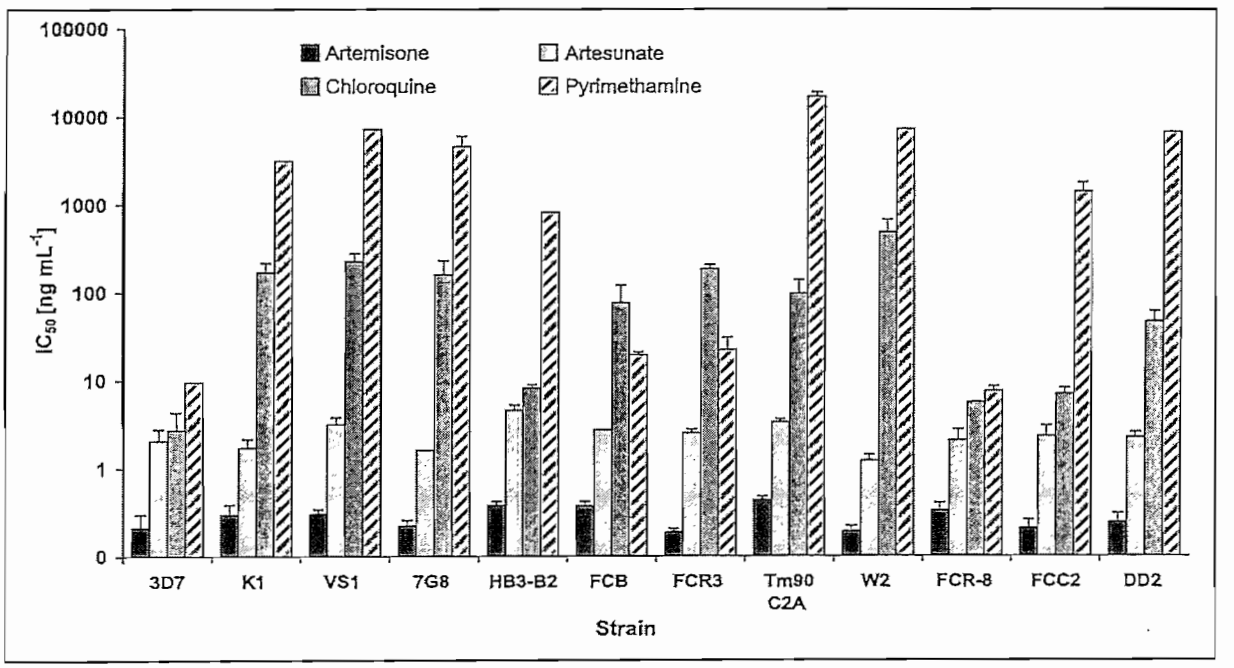

Figure 2.7: Comparative *in vitro* antimalarial activities for artemisone, artesunate, chloroquine and pyrimethamine against drug-sensitive (3D7) *P. falciparum* and also to isolates with varying degrees of drug resistance (Haynes *et al.*, 2006)

The above study was assayed by administration of the compounds in DMSO to parasites incubated with ^{3}H-hypoxanthine. Inhibition of the hypoxanthine uptake is expressed as the concentration at 50% inhibition (IC_{50}) in ng/mL. Error bars represent the standard deviation about the arithmetic mean (Haynes *et al.*, 2006).

Ex vivo bioassays are very useful and effective in assessing both the comparative bioavailability and efficacy of an orally administered drug. Healthy Saimiri monkeys were dosed with 30 mg/kg of either artemisone or artesunate. Plasma samples collected from these monkeys against *P. falciparum* K1 revealed that artemisone had a much greater bioavailability than artesunate. It was clearly reflected in the greater and substantially more sustained activity of artemisone in plasma when compared to artesunate (Haynes *et al.*, 2006).

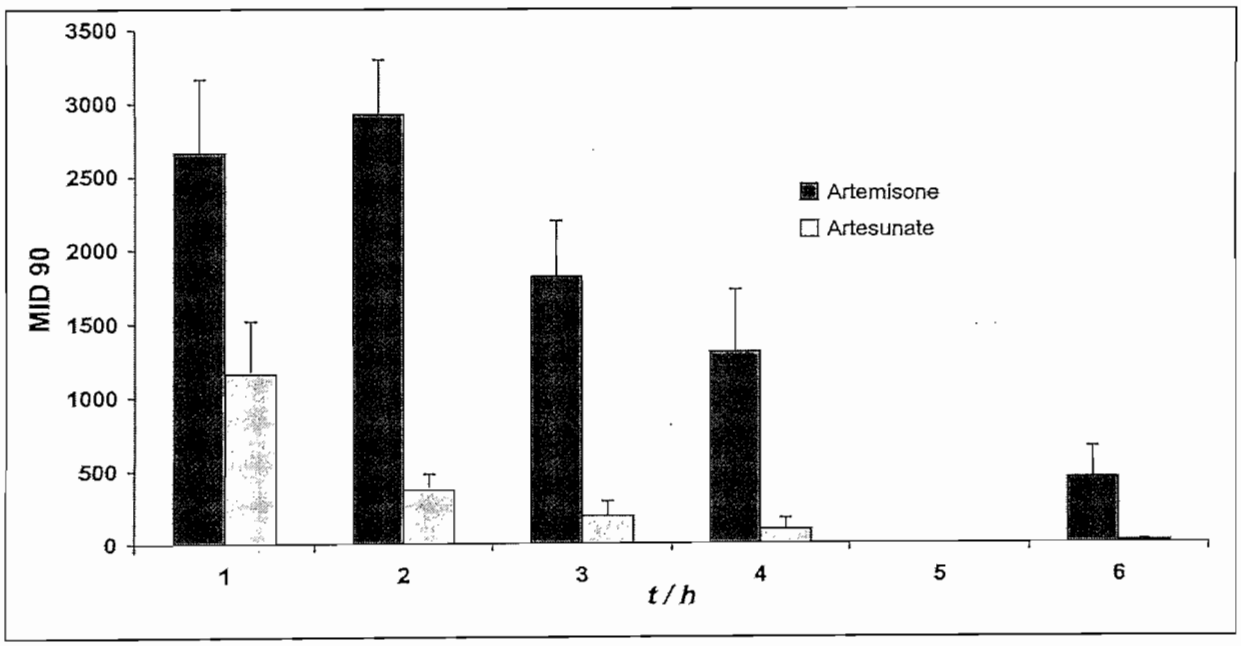

Figure 2.8: Maximum inhibitory dilutions (MID), *ex vivo*, of plasma samples obtained from Saimiri monkeys at 1 – 4 and 6 hours after a single oral dose of either artemisone or artesunate at 30 mg/kg (Haynes *et al.*, 2006)

A dose of 30 mg/kg was required to inhibit maturation of ≥ 90% *P. falciparum* K1 from the ring phase to schizonts. The standard error of the mean of the MID data, obtained for four monkeys with each drug, is represented by the error bars (Haynes *et al.*, 2006).

Aotus monkeys were next infected with a *P. falciparum* FVO isolate. This isolate is resistant to both the antifolates and chloroquine. The monkeys were treated with either artesunate or artemisone, but this time a dose of 10 mg/kg was administered every day for three

consecutive days. After 24 hours, since starting with the treatment, there was no evidence of parasites in the blood samples of the artemisone treatment group. The artesunate treatment was not as successful and parasites were still present 48 hours after initiating treatment. Artemisone treatment succeeded in curing one monkey while the others presented with recrudescence. The recrudescence occurred after approximately 16 days after artesunate treatment while recrudescence was delayed to approximately 23 days after artemisone treatment (Haynes *et al.*, 2006).

Table 2.3: ***In vivo* activity study results of oral artesunate and artemisone against *P. falciparum* FVO in an Aotus monkey model** (Haynes *et al.*, 2006)

Animal	Initial *Parasitaemia* [x10^3 µ/L]	Compound [10 mg/kg/day] days 0 – 2	*Parasitaemia* [x10^3 µ/L] on day *n*			Time to recrudescence (days)
			day 1	day 2	day 3	
1	145	Artesunate	2.1	0.2	0	9
2	515	Artesunate	15	0.6	0	20
3	340	Artesunate	40	0.4	0	18
4	490	Artemisone	0	0	0	20
5	10	Artemisone	0	0	0	20
6	150	Artemisone	0	0	0	29
7	85	Artemisone	0	0	0	-

Parasitaemia Thick blood films were examined microscopically twice a week. These were considered to be negative when no parasites were detected after 100 microscopic fields were examined (≈ 0.1 ml blood).

***Parasitaemia* (day 1, day 2, day 3)** Represent the *parasitaemia* on days 1, 2 and 3 after initial treatment on day 0.

Artemisone combination therapy was also evaluated, that approach achieved the desired results. Complete cure was achieved in infected monkeys receiving a single oral dose of artemisone at 10 mg/kg in combination with mefloquine at 5 mg/kg. Artemisone (10 mg/kg) was also effective when combined with amodiaquine at a dose of 20 mg/kg, both drugs were administered once daily for three consecutive days.

The results proved artemisone's *in vivo* efficacy. By combining artemisone with a longer acting partner drug the problem of recrudescence was also easily addressed. Combination therapy with artemisone and a longer acting partner drug will also additionally protect both drugs against the emergence of resistance (White, 1999).

2.10 CONCLUSION

Artemisone has been subjected to all aspects of preclinical screening, including pharmacokinetic studies and a detailed toxicological evaluation. Artemisone is structurally distinct to current artemisinins but measurably more effective in its antimalarial activity. This marked increase in activity could be attributed to the metabolically inert polar groups which are embedded within the alkyl amino substituents which in turn are attached directly to C10 of the artemisinin nucleus. Artemisone has a different metabolic profile and exhibits very favourable physicochemical properties with virtually zero neurotoxicity/cytotoxicity.

The most likely artemisinin target, the parasite Ca^{2+} pump PfATP6, is targeted and inhibited to a much greater extent by artemisone than by artemisinin. The respective K_i values for artemisone and artemisinin are 1.7 ± 0.6 nM and 169 ± 31 nM for *P. falciparum* and 0.072 ± 0.012 nM and 7.7 ± 4.9 nM for *P. vivax*.

The production cost of artemisone is compatible with the requirements for an antimalarial drug due to its relatively simple method of synthesis. Its enhanced activity over current artemisinins will permit the use of smaller antimalarial dosages with artemisone, thereby augmenting the supply of artemisinin. Artemiside was reported to be very active against *in vitro P. falciparum* cultures but the evidence is very limited. It is essential to conduct more extensive research on this novel compound in order to exploit its full potential.

The advent of these new artemisinin derivatives, such as artemisone and artemiside, marks the dawn of a new era in antimalarial treatment regimens.

CHAPTER 3

PHEROID™ TECHNOLOGY

3.1 INTRODUCTION

As already mentioned, the Pheroid™ delivery system is a patented system consisting of a unique submicron emulsion type formulation. The Pheroid™ delivery system, based on Pheroid™ technology, will for ease of reading be referred to as either Pheroid or Pheroids throughout the rest of this thesis.

The structure of the Pheroid can be described as a stable structure which is suspended within an aqueous system. Pheroids can be manipulated in terms of structure, size, morphology and function to suit the solubility characteristics and size of the drug molecules which need to be delivered. The first basic Pheroid formulation was used for the treatment of Psoriases. The basic ingredients of that initial formulation was also found to be present in banana peel extract and was later identified as specific essential fatty acids. The main ingredients of the Pheroid system are plant and essential fatty acids which are able to entrap, transport and deliver virtually any type of pharmacologically active compounds and other useful molecules (Schlebusch, 2002).

The novelty of Pheroid technology is irrefutable. Various patents have been registered in China, Europe, South Africa and the United States. The abovementioned patents all emphasize the ability of the Pheroid delivery system to enhance the absorption and in turn increase the efficacy of biological, oral and dermatological medicines. Several national and international clinical trials, employing Pheroid technology, have also confirmed the effectiveness of this delivery system (Saunders *et al.*, 1999). The Pheroid delivery system is definitely very unique and its potential application only limited by the human imagination. This creates a pressing need to further investigate its usefulness in context to this project.

3.2 PHEROID TYPES, CHARACTERISTICS AND FUNCTIONS

3.2.1 Pheroid types

There are mainly three different types of Pheroid:

- lipid-bilayer vesicles in nano- and micrometer sizes;
- microsponges, and
- depots containing pro-Pheroids.

The Pheroid delivery system is a colloidal system which can be manipulated in various ways to suit a specific application. The system contains unique and stable lipid-based structures which range in size from submicrons to microns and are uniformly distributed in a dispersion medium. The morphology, structure and size of these dispersed structures can be manipulated which in turn alters the specific function of the formulation (Grobler *et al.*, 2008).

The size of the lipid-bilayer vesicles range typically between 80 – 300 nanometers and that of the microsponges usually range between 0.5 and 5.0 micrometers. The size of the depots on the other hand are determined by the amount of pro-Pheroid which is contained within the depot (Schlebusch, 2002).

Pheroids consist of three phases, namely an aqueous phase, an oil phase and nitrous oxide. The aqueous phase consist mainly of sterile water while the oil phase is a unique combination of essential fatty acids. Essential fatty acids cannot be manufactured by human cells but is still very necessary to maintain various cell functions which is why the essential fatty acids have to be ingested during the normal daily diet. Research, however, have shown that western diets often lack these basic essential lipid molecules (Grobler, 2004).

The fatty acids contained in the Pheroid system are ethylated and pegylated polyunsaturated fatty acids, including both omega-3 and omega-6 fatty acids but with the exclusion of arachidonic acid. The fatty acids in the human body are mostly of the *cis*-formation and so is the fatty acids included in the Pheroid system which ensures compatibility and virtually zero side effects (Vasir *et al.*, 2003; Zeng *et al.*, 1995).

The Pheroid system has inherent therapeutic qualities, due to its high essential fatty acid content, such as the maintenance of membrane integrity of mammalian cells, modulation of the immune system and energy homeostasis. These characteristics of the Pheroid system affords it significant advantages over other similar delivery systems (Grobler, 2004).

The Confocal laser scanning microscopy (CLSM) micrographs in figure 3.1 show active compounds entrapped in several Pheroid types. Each type has a specific composition.

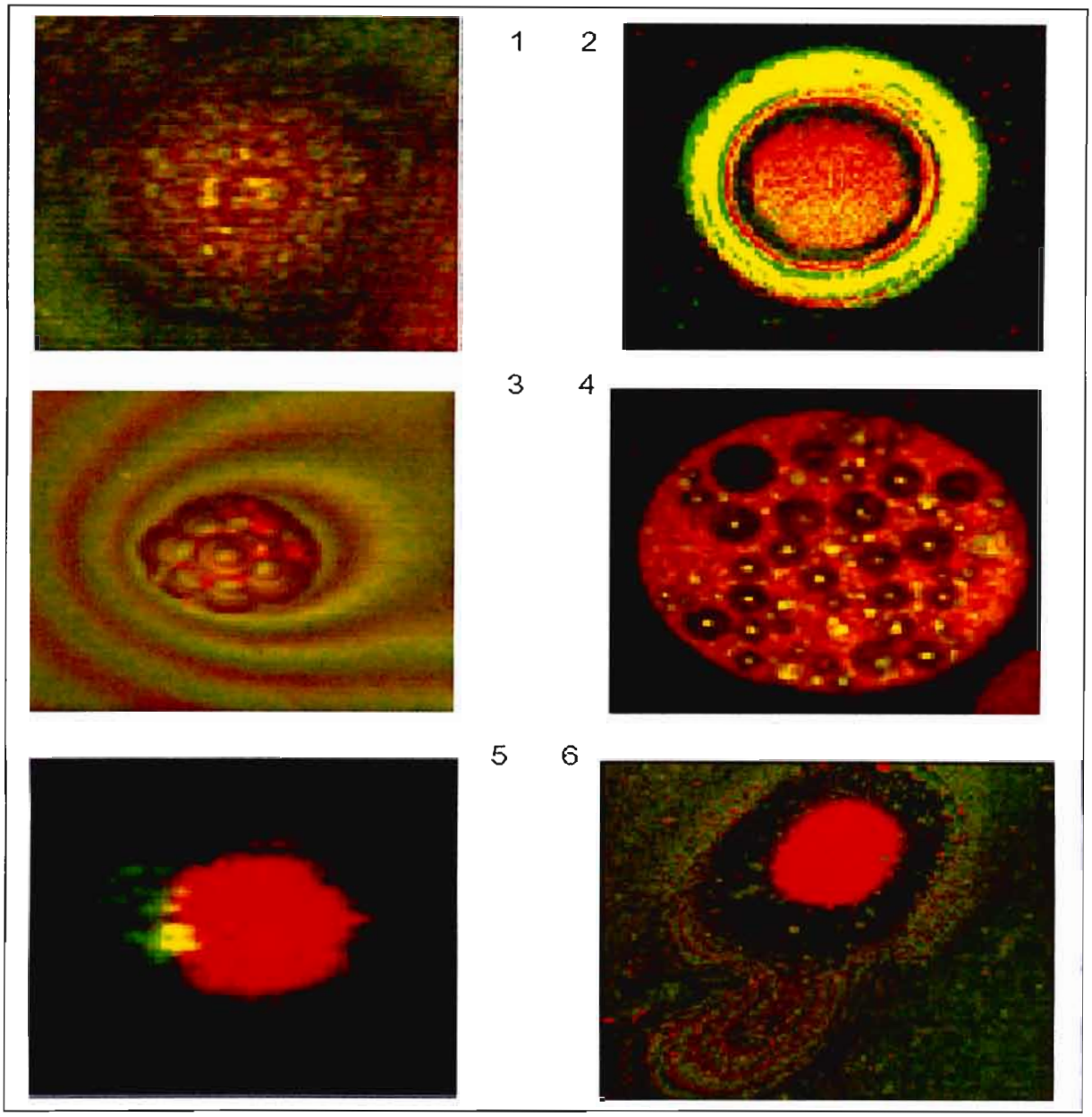

Figure 3.1: Basic Pheroid types: freshly entrapped active compounds in various Pheroid formulations (Grobler, 2004)

The micrographs in figure 3.1 illustrate some of the basic Pheroid types:

1) A bilayer membrane vesicle with a diameter of 100 nm.
2) A highly elastic or fluid bilayered vesicle with loose lipid packing.
3) The formation of small pro-Pheroids, used for some oral administrations.
4) The reservoir contains multiple particles of coal tar. Reservoirs have large loading capacity to surface area ratios and are able to entrap insoluble compounds. General size is 1 – 10 µm.

5) This Pheroid is in the process of entrapping fluorescently labeled water-soluble diclofenac. It is very small (about 30 nm) and the membrane packing is sponge-like.
6) A depot with a hydrophobic core containing pro-Pheroid formulation, a surrounding hydrophilic zone and an outer vesicle-containing zone. Selective addition of fluid results in the release of vesicles from a release zone. The depots are used for sustained release according to a concentration gradient and can range in size from 5 to 100 µm. The sizes of Pheroid reflected above are not all to scale.

3.2.2 Structural and functional features

The structural and functional features of the Pheroid can easily be manipulated by:

- adding charge-inducing agents;
- changing the active compound in terms of character and concentration;
- manipulating the composition and/or the concentrations of the fatty acids;
- alterations in the preparation method;
- adding non-fatty acids or a phospholipid e.g., cholesterol;
- cryo-protectant addition, and
- changes in the hydration medium such as pH adjustments and alterations in ionic strength (Wang & Quinn, 2002).

Manipulation of both the structural and functional characteristics can easily be accomplished by applying one or several of the abovementioned tactics. The surface charge of the Pheroid can be altered by adjusting the degree of hydrogenation of the fatty acids. The composition and ratio of the fatty acids have a great influence on the mean particle size and reproducibility of the Pheroid. The addition of hydrogenated fatty acids to the Pheroid induces an increase in the molecular radius of the fatty acids with less elasticity of the membranes. Increasing the pegylated ricinoleic acid content will in turn render smaller particles with thicker membranes and proportionally smaller interior spaces (Grobler *et al.*, 2008).

All Pheroid formulations, currently in use, contain tocopherol or a tocopherol-based derivative. The inclusion of these substances in Pheroid formulations can be attributed to their emulsion stabilizing effects and also to their anti-oxidative qualities. Tocopherol is fat

soluble by nature and forms an integral part of membranes and cells throughout the body. The prevention of oxidation of unsaturated fatty acyl residues of membrane lipids are believed to be the primary function of tocopherol. Tocopherol reacts directly with lipid peroxyl radicals within the cell membrane and is then converted to oxidation products. This stands in contrast to other anti-oxidant defense mechanisms and acts by breaking the normal radical chain reaction when tocopherol itself is oxidized (Wang & Quinn, 2002).

Due to its mechanism of action tocopherol is classed as a scavenger of lipid peroxyl radicals, of the chain carrying species, that are responsible for the propagation of lipid peroxidation. A secondary attribute of tocopherol is its ability to stabilize cell membranes by means of complexation of products liberated by membrane lipid hydrolysis such as free fatty acids and lyso-phospholipids and thus effectively eliminating their disruptive effects (Grobler *et al.*, 2008).

3.2.3 Functional characteristics

In order to deliver an active compound effectively it is important to consider the efficacy of the delivery system and the nature of the tissue and cell type to which the active compound needs to be delivered.

The effective delivery of an active compound to target cells or organs will involve at least one or more of the following processes:

- entrapment of an active compound in the Pheroid carrier system;
- penetration of an active, entrapped, compound across biological barriers;
- uptake of the entrapped active compound in the circulatory system;
- release of the entrapped active compound from the Pheroid carrier system (Grobler *et al.*, 2008).

In retrospect it is quite apparent that there are two different types of controlling entities present in the abovementioned processes, namely the control exerted by the manufacturer of the product and also the control exerted by the body in terms of physiological and biochemical control of the target cells. An intimate knowledge of these physiological and biochemical processes is absolutely critical in order for the manufacturer to control and optimize the total delivery process (Grobler *et al.*, 2008).

3.3 VERSATILITY OF THE PHEROID DELIVERY SYSTEM

The versatility of the Pheroid system enables its users to target various sites in the body for pharmaceutical and even cosmetic applications. The enhanced efficacy of a large variety of active compounds, the vast array of investigated Pheroid-based administration routes and the large variety of Pheroid-based dosage forms provide ample proof of the functional versatility of Pheroid-based technology. The pliability of the system can be attributed to the addition of pliable pegylated tails, added to the fatty acids, in conjunction with the use of a gas component which in turn forms extremely elastic structures. Pegylation serves to sterically stabilize the Pheroid system and to maintain the interior spaces. Pheroids are robust by nature and will not shatter under extravasations or moderate pressure. By adding polyethylene glycol (PEG) to the Pheroid formulation it is possible to further increase drug stability, bioavailability, circulating life and drug solubility (Torchilin, 2001).

3.3.1 Efficiency of entrapment (EE)

The efficiency of entrapment of an active compound in the Pheroid system can be expressed as the fraction of the total amount of active compound, added to the system, which is entrapped in the Pheroid. The EE is calculated as a percentage value.

$$EE\ \% = \frac{\text{Amount of entrapped active}}{\text{Total amount of active added}} \times 100 \qquad \textit{Eq. 1}$$

CLSM is the key to determining the efficiency of entrapment of Pheroid-based products and both the Pheroid and the active compounds can be visualized by means of fluorescence labeling. An entrapment efficiency of at least 90% is the benchmark set for all Pheroid-based products. The general formula, πr^3, is used to calculate the approximate interior entrapment volume of the Pheroids where "r" represent the mean radius of the Pheroids (Grobler *et al.*, 2008). Based on the above formula it is clear that an increase in diameter is directly proportional to an increase in the interior entrapment volume. The theoretical entrapment volume of Pheroids, differing in size, can be predicted with the aid of figure 3.2.

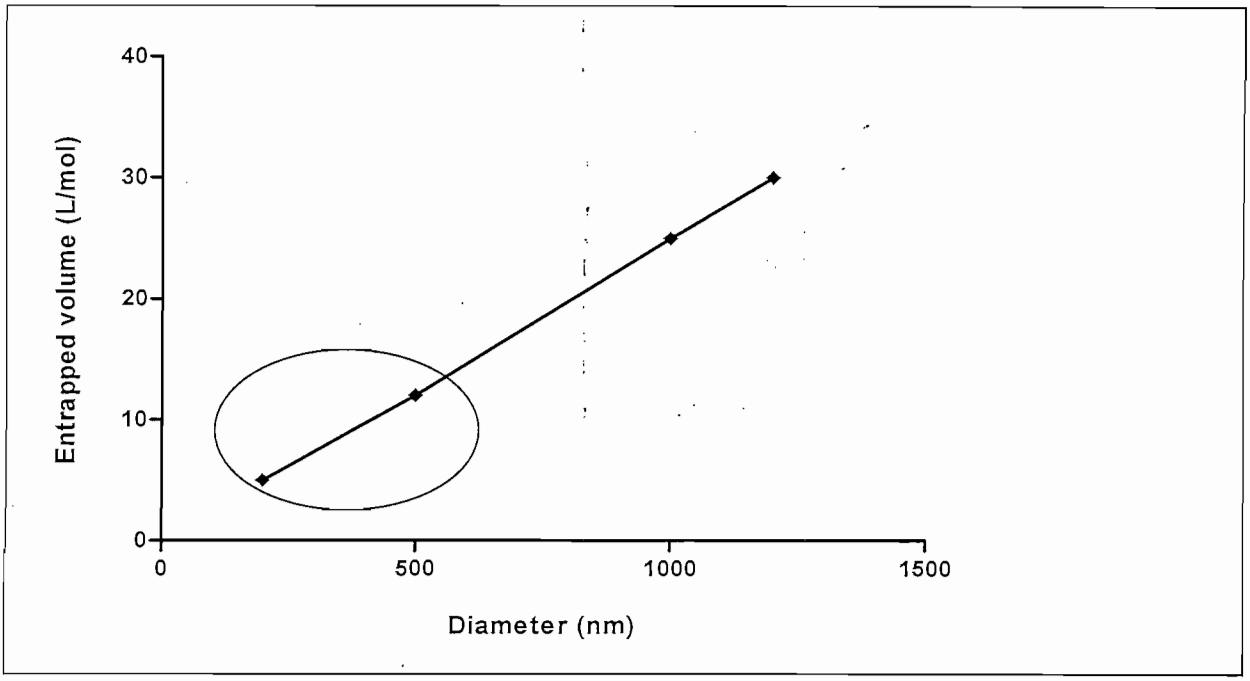

Figure 3.2: A graph to roughly predict the theoretical interior volume of Pheroids in various size ranges (adapted from Grobler *et al.*, 2008)

The circled area indicates the size range of the average Pheroid. The interior volume is influenced by various factors such as:

- the characteristics of the hydration medium, e.g. electric capacity, presence of charged molecules, pH, ionic strength, etc.;
- the characteristics and concentration of the active compound;
- the size of the specific vesicles, and
- the character and concentration of the utilized fatty acids (Grobler *et* al., 2008).

The entrapment capabilities of a single Pheroid vesicle depend almost exclusively on the size, charge and solubility of the active compound and it is possible to entrap quite a few active compound molecules per single vesicle. The accurate determination of the entrapped compounds is performed by means of a 3-D analysis of optical sections, without physical interference, with the aid of CLSM. A simple calculation is necessary to determine the number of Pheroids required per volume of active molecules, namely:

$$\text{Number of Pheroids (\%)} = \frac{\text{Total number of active molecules}}{\text{Average number of active molecules per Pheroid}} \times 200 \qquad \textit{Eq. 2}$$

For the complete entrapment of a certain amount of active compound molecules it is preferable to have a Pheroid to active compound molecule ratio of 2:1. This explains the multiplication of the ratio with 200 in the above formulation in order to calculate the percentage value of the required number of Pheroids. The most basic Pheroid formulation contains approximately 150 Pheroids/µl of formulation (Grobler *et al.*, 2008).

3.3.2 Penetration efficiency across biological barriers

The penetration efficiency of a specific compound can largely be attributed to its physicochemical properties such as molecular weight, melting point, water solubility and its oil/water partition coefficient. All of these characteristics and their specific role in penetration efficiency are described by Fick's laws of diffusion (Roy, 1997).

The easiest route to follow when determining the efficiency of penetration is to employ a comparative investigation. This method involves testing and determining the enhancement in penetration efficiency achieved by the experimental carrier and then comparing those values to that of an existing commercial product. The area under the curve of enhancement in relation to elapsed time is then determined. The total enhancement is then calculated from the respective total areas under the curves. Various studies of this kind have confirmed the phenomenal ability of the Pheroid system to rapidly traverse biological barriers and to subsequently deliver its entrapped compound to the desired target area (Grobler *et al.*, 2008).

3.3.3 Cellular uptake of Pheroid entrapped compounds

Electrochemical interactions ensure the sterical stabilization of Pheroids. This stands in contrast to conventional lipid-based delivery systems which depend on cholesterol for stabilization. The inclusion of cholesterol in conventional structure-based systems result in a rigid vesicular structure with an inability to deform in order to cross densely packed cell layers without fracturing. The electrochemical stabilization of the Pheroid system on the other hand allows for a very elastic vesicular structure and a solution to the flexibility problems encountered with other lipid-based systems (Grobler *et al.*, 2008).

The formulation and type of Pheroid as well as the mechanism of uptake by the target cells all play a role in the extent of the Pheroid uptake. The cellular permeation of the Pheroid formulation may be influenced by one or a combination of the following factors:

- the size and type of the Pheroids;
- the morphology of the Pheroids;
- the ratio and concentration of the various fatty acids;
- the molecular geometry of the specific fatty acids;
- the concentration and character of the active compound;
- the presence of molecules that influence the electrostatic milieu;
- the presence of charge-changing molecules;
- the pH of the formulation, and
- the character of the hydration medium (Grobler *et al.*, 2008).

The mechanism of Pheroid uptake by living cells is still speculative to some degree, however, preliminary evidence indicate that the fatty acid membrane-binding proteins may be instrumental in the active facilitated uptake of Pheroids with the aid of lipid rafts within the cell membrane. The mechanism of the fatty acid transport into cells is a controversial topic and can be divided into two possible categories, namely diffusion or passive transport and protein-mediated or active transport. Based on existing evidence it is more feasible that the Pheroids are transported by means of protein-mediated or active transport rather than passive transport (Hertzel & Bernlohr, 2000).

Proteins have the capability to efficiently mediate trans-bilayer movement of fatty acids. These specific intracellular proteins are aptly named fatty acid-binding proteins (FABPs) and are members of a multigene family, encoding ~ 15kDA proteins that bind to hydrophobic ligands in a non-covalent and reversible manner. The individual members of this protein family show unique, tissue-specific, expression patterns and these members are not all expressed equally in all tissues. This distribution variation may lead to the development of Pheroid formulations which will be able to target specific cell types and tissues (Hertzel & Bernlohr, 2000).

3.4 DISTRIBUTION AND METABOLISM

The distribution of Pheroids are greatly influenced by the type and extent of the fatty acid modifications. Pheroids are metabolized, depending on their composition, by either the peroxisomes of the cells or the mitochondria. Metabolization of the Pheroids result in the subsequent release of the entrapped active compound and can be confirmed by co-localization studies of Pheroids and other sub-cellular organelles (Grobler *et al.*, 2008).

3.5 THE PHEROID DELIVERY SYSTEM COMPARED TO OTHER LIPID-BASED DELIVERY SYSTEMS

Table 3.1 provides a comparison of the similarities, differences and key advantages of the Pheroid and other lipid-based or liposomal drug delivery systems currently in use today.

Table 3.1: Key advantages of the Pheroid system compared to lipid-based delivery systems (Grobler, 2004)

Pheroid	Lipid-based delivery systems
• Cytokine reactions to Pheroid were reported to be very low during a cytokine level study and cannot be regarded as being of any clinical significance.	• Some liposomal formulations have been shown to elicit immune responses.
• It is very simple to optimize the Pheroid system for a specific active compound by manipulation of the size, charge, lipid composition and membrane packing, thus compatibility and repeatability pose no problems.	• Problems concerning repeatability of liposomal systems have been encountered and some have proven to be very troublesome and difficult to overcome.
• A high affinity exist between Pheroid and cell membranes due to the fact that Pheroid is comprised mainly of fatty acids. Enhanced penetration and delivery are ensured due to the fact that the Pheroid travels through the cell membrane and follow endosomal sorting mechanisms.	• Problems are encountered with both penetration and delivery due to the lack of specific binding and uptake mechanisms in mammalian cells.
• The rate of binding to and uptake of Pheroid by cells are very high and fast. This is due to the affinity between essential fatty acids contained within the Pheroid and the micro-domains in the cell membrane. This can be compared to the rate observed for active transport across the cell membrane.	• A specific cellular binding mechanism has not yet been established for liposomes.
• By manipulating the fatty acid content and/or the pH of the Pheroid formulation it is possible to target sub cellular organelles.	• Targeting of sub cellular organelles is very complicated, if at all possible, as a result of the metabolism of phospholipids.
• No evidence of Pheroid cytotoxicity has been documented. This may be attributed to the fact that it forms part of the natural biochemical pathways, displays rapid clearance and even assists with the maintenance of cell membrane integrity.	• Impaired cell integrity and cytotoxicity are common problems encountered with foreign substances that enter the body.
• The Pheroid has a unique ability to penetrate a vast array of potential barriers such as skin, keratinized tissue, intestinal lining, the vascular system, fungi, bacteria and parasites.	• Other delivery systems do not demonstrate such versatility.
• The Pheroid system display high levels of stability in various products.	• Other delivery systems often display physical and chemical instability.

Table 3.1: Key advantages of the Pheroid system compared to lipid-based delivery systems (continued) (Grobler, 2004)

Pheroid	Lipid-based Delivery Systems
• The polyphilic nature of Pheroids enables the system to encapsulate active compounds with differing degrees of solubility and even insoluble compounds.	• Other delivery systems are limited due to having either a hydrophilic or lipophilic nature.
• The Pheroid enhances bioavailability due to its ability to inhibit the drug efflux mechanism in the intestinal lumen.	• No liposomal formulation has been documented which exhibit this ability. Cremaphor EL is a separate compound which is used to achieve a similar effect in patients.
• The interior volume of the Pheroid is very stable although it does not contain any cholesterol to assist in the maintenance of the vesicles.	• Cholesterol and phospholipids form the basis of most lipid-based delivery systems. Cholesterol is included for the maintenance of the interior of the vesicles.
• An entrapment efficiency of 85 – 100% can be achieved with virtually any active or essential compound.	• Charge and steric effects are often limiting factors in the entrapment efficiencyof these systems.
• Batch to batch reproducibility has been proven in various Pheroid containing products.	• Size control is a common problem with these systems and low batch to batch reproducibility is often encountered.
• Pheroid formulations can be sterilized with gamma radiation with no ill effects.	• Other delivery systems are sensitive to heat, radiation and chemical sterilization. Filtration is also not suitable for sterilization of formulations which contain large vesicles.
• There are currently three main types of Pheroid, namely: * single lamellar vesicles * microsponges * depots (pro-Pheroids)	• There are quite a few liposome types in use such as: * single lamellar vesicles * multi-lamellar vesicles * nanosomes * multi-vesicular vesicles
• The Pheroid is able to protect the entrapped compound from opsonization, inactivation and metabolism.	• Some liposomal formulations is also able to protect an entrapped compound from opsonization.
• Pheroids are able to entrap peptides and antibodies which enables the Pheroid to interact with specific micro-domains on cells in culture.	• Drug targeting is possible with the aid of antibody-containing liposomes.
• Pheroids can be formulated in the form of pro-Pheroids.	• Pro-liposomes can also be formulated.
• The reticulo-endothelial system (RES) is passively targeted by the Pheroids and accumulate in both the liver and spleen.	• The RES are also targeted by liposomal systems and they also accumulate in the liver and spleen.
• The Pheroid system is able to change the pharmacokinetic profile of entrapped compounds. Faster time to onset of action and greater peak plasma levels are usually achieved with the aid of Pheroid technology.	• Liposomes have also been known to change the pharmacokinetic profile of a wide variety of entrapped compounds.

3.6 KEY CHARACTERISTICS OF THE PHEROID SYSTEM AND ITS PHARMACEUTICAL APPLICABILITY

The Pheroid delivery system exhibits a few key characteristics which make it superior to most, if not all, of the other active compound carrier systems currently available on the market.

3.6.1 Increased delivery of active compounds

The Pheroid system is able to greatly increase the percentage of active compound delivered to the desired site of action. This has been proven in both *in vitro* and *in vivo* studies.

Membrane diffusion studies have rendered interesting results. In table 3.2 below are a few of the results of those studies which mimic the delivery of active compounds across specific membranes. The abbreviations in this table, namely "PHR" and "COM" represent the Pheroid-entrapped product and a comparable commercial product respectively (Grobler, 2004).

Table 3.2: Diffusion rates and percentage release per label claim for product tested (Grobler, 2004)

Active Agent	% Active/Product	Diffusion Rates ($\mu g/cm^2/h$)	% Release per label claim
Acyclovir PHR	0.5	69.1533	0.1214
Acyclovir COM	0.5	54.0942	0.0952
Miconazole Nitrate PHR	2	389.9238	6.8155
Miconazole Nitrate COM	2	111.2222	1.0466

3.6.2 Decreased time to onset of action

The Pheroid system is capable of rapidly traversing most physiological barriers, as already mentioned, and is able to transport and release the entrapped compound quickly and effectively at the desired site of action. This characteristic of the Pheroid system suggests that it would be possible to achieve potentially faster relief from target symptoms (Grobler, 2004).

Figure 3.3 illustrates the obtained average plasma levels of Rifampicin for 14 healthy volunteers after oral administration of combination anti-tuberculosis DOTS treatment, with and without the use of Pheroid technology. Pyriftol is a Pheroid-based formulation and Rifafour serves as a reference which is not a Pheroid-based formulation.

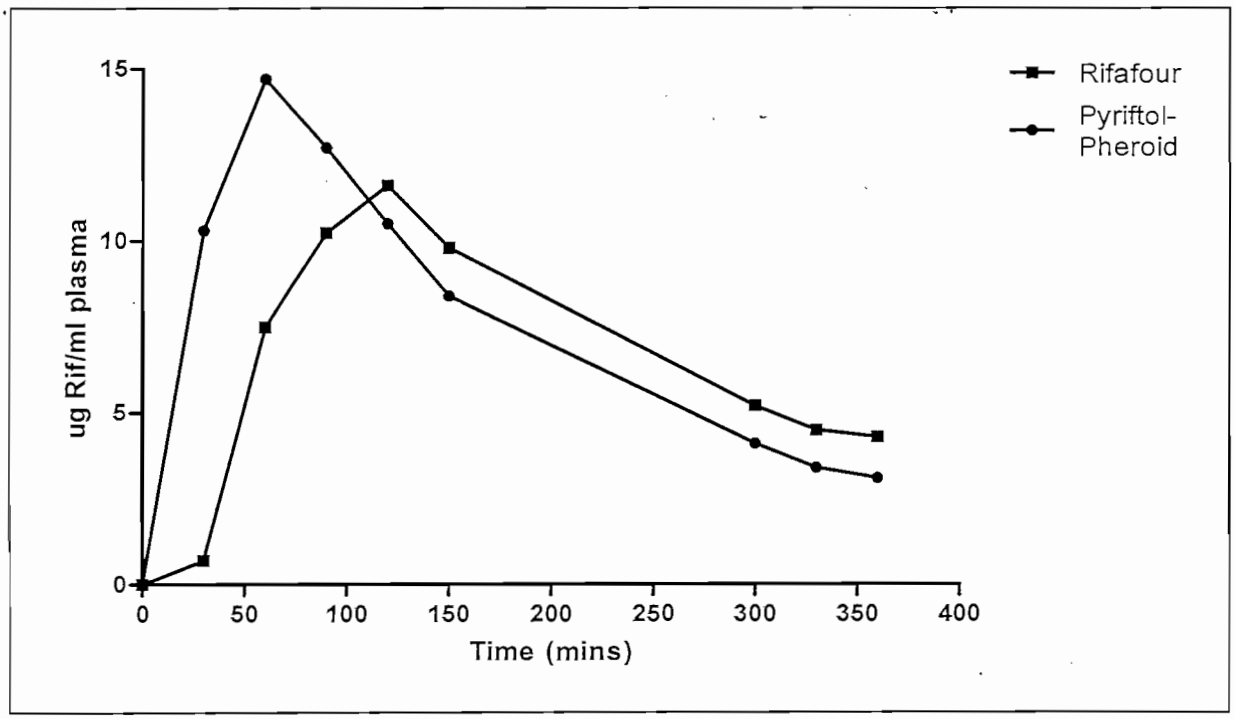

Figure 3.3: The time needed to achieve plasma C_{max} is halved by entrapment in Pheroid when compared to that of one of the preferred comparative products. Pyriftol contained only 60% (400 mg) of the amount of Rifampicin contained in the commercially available Rifafour (600 mg) (Grobler, 2004)

3.6.3 Increased therapeutic efficacy

The enhancement of action of anti-infective agents, entrapped in a Pheroid formulation, are clearly demonstrated in table 3.3. The Pheroid formulations were compared to a few commercially available products and from the results it is very clear that the Pheroid formulation is able to greatly increase the efficacy of an active compound. The results are all based on zone inhibition studies (Grobler, 2004).

Table 3.3: Zone of inhibition study: Five commercially available products (COM) versus Pheroid (PHR)-formulations of the same active compound (Grobler, 2004)

Active Agent	PHR/COM	Dose mg/ 5 ml	*S. aureus*	*P. aeruginosa*	*B. cereus*	*E. coli*	*A. niger*	*C. albicans*
Cloxacillin	PHR	125	30.74	23.96				
Cloxacillin	COM	125	29.45	19.78				
Erythromycin	PHR	250	26.7		28.89			
Erythromycin	COM	250	25.84		27.78			
Ciprofloxacin	PHR	250	33.05			35.78		
Ciprofloxacin	COM	250	30.14			33.40		
Cotrimoxazole	PHR	240	13.95			24.64		
Cotrimoxazole	COM	240	11			22.83		
Itraconazole	PHR	50					16.03	14.28
Itraconazole	COM	50					11.47	10.21
Control			9	9	9	9	9	9

All the above formulations made use of one single Pheroid type. Reformulation of ciprofloxacin and erythromycin in microsponges has since increased efficacy (Grobler, 2004).

3.6.4 Reduction in cytotoxicity

Cellular damage is quite a common occurrence attributed to the harmful side effects of active compounds. The Pheroid system has the ability to actually enhance normal cell integrity which in turn will potentially minimize cellular damage (Grobler, 2004).

3.6.5 Penetration of tissue, organisms and most known barrier cells

Research has shown that the Pheroid is capable of penetrating a vast array of barriers, commonly found in the human body such as skin, keratinized tissue, vascular walls, sub cellular organelles and intestinal epithelium (Grobler, 2004).

The Pheroid has also demonstrated its ability to effectively penetrate and deliver active compounds to bacteria, viruses, fungi and parasites. The Pheroid is also able to penetrate human skin which means that it is possible to administer active compounds via the topical

route. Topical administration focuses on the active near the desired site of action which in turn relates to a significant reduction in systemic side effects (Grobler, 2004).

3.6.6 Reduction in minimum inhibitory concentration (MIC)

A reduction in the MIC would suggest that it may be possible to use less of the active compound and still achieve comparable results with the aid of the Pheroid system. In some studies it was demonstrated that as little as 1/40th of the original dose was still able to achieve an effective drug plasma concentration when administered in the form of a Pheroid formulation. This trend would in turn lead to cost savings in product formulation and a reduction in patient side effects caused by the active compound (Grobler, 2004).

In figure 3.4 the growth of reference strain H37 RV is depicted. Growth was determined with the use of radio-active labels in a BACTEC system (Grobler, 2004).

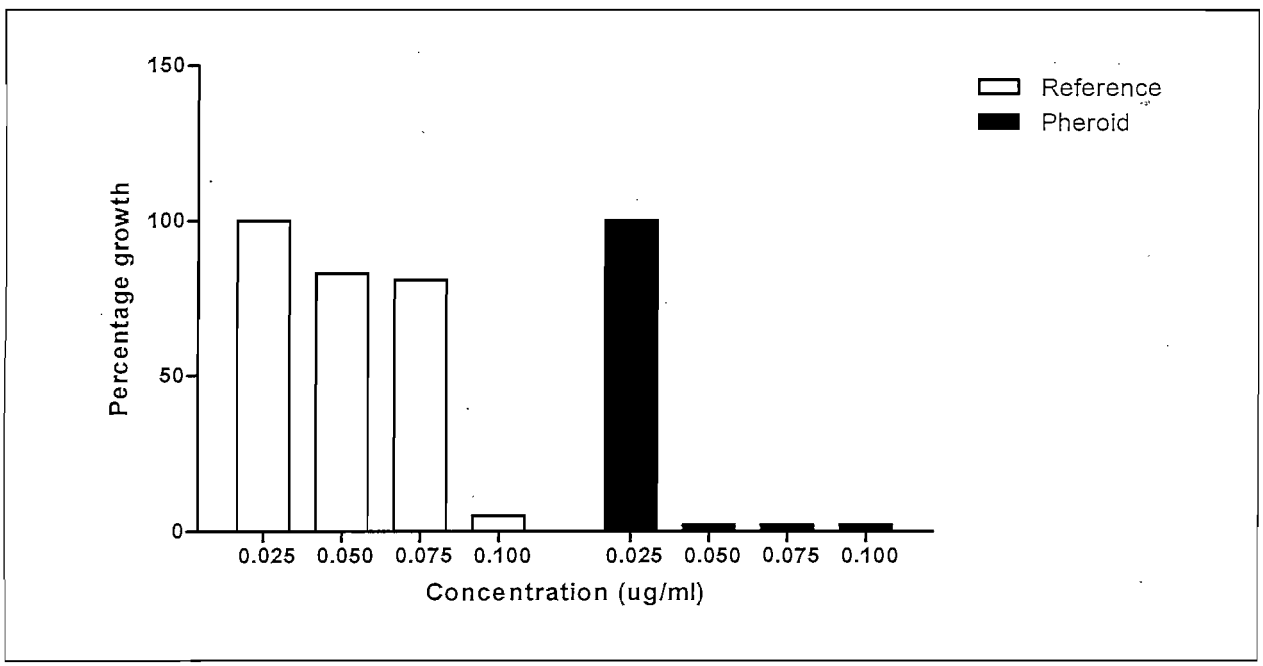

Figure 3.4: ***In vitro*** **inhibition of bacterial growth by isoniazid** (Grobler, 2004)

3.6.7 Adaptability and flexibility

The Pheroid system is capable of entrapping a wide variety of active compounds with contrasting characteristics such as hydrophilic, lipophilic and even insoluble substances. This character trait puts the Pheroid system a cut above most other delivery systems. Other

delivery systems are not as flexible and can either carry lipophilic or hydrophilic compounds but not both types and most other systems are unable to carry insoluble compounds. A further bonus of the Pheroid system is that with the deletion or addition of selected components it is possible to change the structure, size and carrying capacity which make it even more specific and versatile (Grobler, 2004).

3.6.8 Immunological responses

The Pheroid is capable of masking the active compounds, such as peptides or proteins, which in turn reduces the recognition of these compounds by the body's immune system. This ability of the Pheroid system would also reduce the occurrence of adverse intolerance and immunologic responses in the patient (Grobler, 2004).

3.6.9 Targeting of the treatment area

The Pheroid can be designed in a variety of different structures in order to target specific body systems or organs. This in effect means that it is possible to target only the affected area and avoid cellular damage and side effects to other parts of the body (Grobler, 2004).

3.6.10 Ability to entrap and transfer genes to cell nuclei

In vitro studies have demonstrated the effective entrapment of both human and viral DNA, of various lengths, in Pheroid vesicles. Various other experiments conducted on the Pheroid delivery system also demonstrated its applicability in both DNA vaccines and gene therapy. Reproducible expression of appropriate proteins was also observed after transfection of cells by Pheroid entrapped genes (Grobler, 2004).

3.6.11 Reduction and possible elimination of drug resistance

Incorporation of an active compound into a Pheroid vesicle can reduce or even eliminate existing drug resistance. This has been clearly demonstrated in various *in vitro* studies. Analysis of bacterial growth of multidrug resistant TB have shown that formulations containing the standard antimicrobial, rifampicin, are clearly less effective when compared to a Pheroid-based rifampicin formulation. The potential to revive the effectiveness of various antibiotics such as penicillin will have a huge impact in the healthcare industry. Figure 3.5 illustrates the effectiveness of rifampicin when entrapped in a Pheroid formulation. The

entrapment of rifampicin in Pheroids resulted in complete bactericidal activity against resistant Mycobacterium isolated from a MDR patient. The free rifampicin shows no growth inhibition (Grobler, 2004).

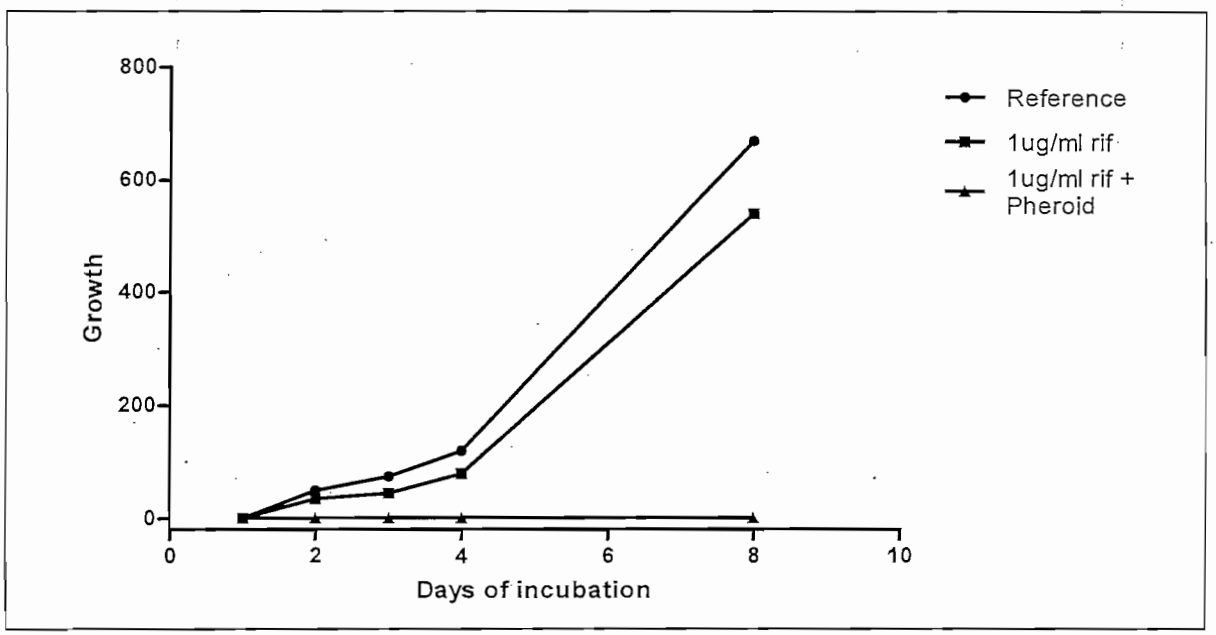

Figure 3.5: Effectiveness of rifampicin when entrapped in a Pheroid solution (Grobler, 2004)

3.7 THERAPEUTIC AND PREVENTATIVE APPLICATIONS OF PHEROID TECHNOLOGY

3.7.1 Therapy of Tuberculosis

According to the WHO "*Tuberculosis kills 2 million people each year. Overall, one third of the world's population is currently infected with the TB bacillus*".

Various *in vitro* and *in vivo* studies suggested that the Pheroid delivery system might have significant benefits when used in conjunction with the current TB treatment regimen. Based on the abovementioned evidence, a bioequivalence study was launched to determine whether the Pheroid delivery system would remain as effective when administered via the oral route as it has proven to be in topical applications (Grobler, 2004).

The goal of the aforementioned study was to prove that the Pheroid system could:

- enhance the absorption of antimicrobials from the gastrointestinal tract without an increase in toxicity;
- increase the plasma levels of the administered compound;
- increase intracellular concentration of antimicrobials in target cells which are the breeding ground of the tuberculosis bacillus;
- increase the circulatory time of active compounds;
- increase the bactericidal effect of the antimicrobials inside target cells; and
- decrease side effects caused by the antimicrobials (Grobler, 2004).

Results of the bio-equivalence study revealed that:

- the plasma levels of the antimicrobials did in fact increase after oral administration which would suggest that the Pheroid-entrapped antimicrobials was better absorbed from the gastrointestinal tract;
- a lower dosage could be used to obtain similar results;
- entrapment of the antimicrobials led to an increased cellular response due to an increased rate of absorption;
- the therapeutic concentrations of the drugs were maintained for longer and the circulatory time of the drugs was extended which would indicate that the exposure time of the bacillus to the antimicrobials was increased;
- the minimum inhibitory concentration (MIC) was decreased, this can be attributed to the fact that the delivery of the antimicrobials to the target cells was increased by the Pheroid delivery system, and
- fewer side effects occurred and in turn patient compliance increased dramatically (Grobler, 2004).

3.7.2 Preventative therapies

Historically, vaccination is the only strategy that has led to the elimination of the viral disease, smallpox. An indirect relationship has been observed between vaccine immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines, administered with standard adjuvant, tend to be poor. This highlights the urgent need for effective vaccine adjuvant to enhance the immunogenicity and immunostimulatory properties of vaccines (Grobler, 2004).

3.7.2.1 A peptide-based vaccine: *Hepatitis B*

The efficacy of a commercially available *hepatitis B* vaccine, incorporated in a Pheroid formulation, was investigated and compared. Non-recombinant *hepatitis B* vaccines are generally based on the use of the surface molecules of the virus as an antigen. For comparative animal studies, different formulations of this peptide-based vaccine were used, namely:

- the peptide only;
- peptide with alum (aluminium hydroxide) as an adjuvant, and
- a Pheroid incorporated peptide formulation.

The Pheroid-based formulation led to more than a 10-fold increase in the efficacy of the peptide-based *hepatitis B* vaccine and was measured by the antibody response. The Pheroid plays and obvious dual role in vaccinology, primarily as a delivery system for disease specific antigens but also as an immune-stimulatory adjuvant (Grobler, 2004).

3.7.2.2 A virus-based vaccine: *Rabies*

The efficacy of both a commercially available *rabies* vaccine and a Pheroid-based *rabies* vaccine was investigated and compared. An inactivated virus is used in the manufacturing of *rabies* vaccines. For the comparative animal studies, different formulations of the virus were used, namely:

- the inactivated virus only;
- the inactivated virus with alum as adjuvant, and
- the inactivated virus incorporated in a Pheroid-based formulation.

The inactivated virus incorporated in a Pheroid-based formulation showed a 9-fold increase in antibody response when compared to the other formulations (Grobler, 2004).

3.7.3 Pheroid technology for nasal vaccine delivery

The hypothesis for the nasal delivery of vaccines, by means of a Pheroid-based formulation, is based on the same principles than that of micro particulate systems such as chitosan and *N*-trimethyl chitosan chloride micro particles. The antigen is entrapped in the Pheroid

formulation and then subsequently administered via the nasal cavities. Absorption is mediated by the micro fold cells (M-cells), situated in the nasal epithelium, which are responsible for the sampling and transporting of antigens to the underlying nasal associated lymphoid tissue (NALT), germinal centers containing both B and T cells, plasma cells and antigen presenting cells (APCs). These aforementioned cells are involved in the regulation and induction of antigen-specific effectors cells, which produce the protective humeral and cellular immune responses (Grobler, 2004).

3.8 CONCLUSION

Despite the fact that there are many similarities between the Pheroid delivery system and other lipid-based delivery systems, there are a few definite advantages which count in the favour of Pheroid technology and have already been proven by previous research. Pheroid technology has infinite possibilities in the pharmaceutical industry due to its versatility as a delivery system. The most significant advantage of the Pheroid system is its ability to greatly enhance the absorption of both peptide and protein drugs. Speeding up the absorption of these drugs will in turn lead to higher plasma levels, faster cellular responses and a significant decrease in the T_{max}.

By using Pheroid technology it is possible to maintain therapeutic concentrations for longer periods of time and lower drug dosages is required to achieve the MIC. Lower drug dosages in turn relates to a much lower incidence of drug related adverse effects. These characteristics of the Pheroid system promotes itself as a great delivery system for both peptide and protein drugs as well as other poorly absorbable compounds. The relatively low manufacturing costs, biocompatibility and low toxicity suggests that this system may hold great potential for the future development of novel drug delivery.

CHAPTER 4

ACTIVITY STUDIES OF ARTEMISONE AND ARTEMISIDE

4.1 INTRODUCTION

As discussed earlier it is obvious that Pheroid technology has the potential to greatly enhance the absorption and bioavailability of most therapeutic compounds. This ability of the Pheroid system may equate to a substantial reduction in the IC_{50} values of encapsulated antimalarial compounds.

Ongoing research and development in the field of novel antimalarial compounds has led to the synthesis of two new artemisinin derivatives namely artemisone and artemiside. Previous studies, as mentioned in chapter 2, rendered very promising results with artemisone during both *in vitro* and *in vivo* antimalarial efficacy evaluations. Artemiside on the other hand has not been evaluated previously and no data is available.

The first objective of this study was to evaluate the *in vitro* antimalarial activity of both artemisone and artemiside in comparison to that of artesunate. These drugs were evaluated in the presence and absence of Pheroid vesicles. The second objective was to subsequently evaluate the *in vivo* antimalarial activity of these new derivatives, with and without Pheroid vesicles.

4.2 *IN VITRO* ANTIMALARIAL ACTIVITY EVALUATION

4.2.1 *In vitro* cultivation of *P. falciparum*

4.2.1.1 History

Trager and Jensen (1976) were the first to describe an effective method for the continuous cultivation of *P. falciparum.* This method was termed "the candle jar method" (Basco, 2007; Trager & Jensen, 1976). The original method utilized *P. falciparum* infected red blood cells of human origin, human serum and buffered RPMI 1640 medium (Basco, 2007). The original method was later refined and the use of human serum was excluded and rather substituted with human serum substitutes such as Albumax II (Ringwald *et al.*, 1999).

The synchronization of cultures and their application in efficacy studies were first reported by Lambros and Vanderberg (1979). Synchronization was achieved by treating the cultures with a specific concentration of sorbitol. This is essential to ensure that the majority of parasites in a culture are at a specific stage of their life cycle. The isolation of specific stages of the parasite life cycle in continuous cultures are necessary for conducting quantitative studies concerning immunological, biochemical and physiological differences during stage development (Lambros & Vanderberg, 1979).

4.2.1.2 Materials

RPMI 1640, HEPES, hypoxanthine, gentamycin, D-(+)-glucose powder, sodium bicarbonate, sodium chloride and sorbitol were all obtained from Sigma Aldrich® (South Africa). Scientific Group supplied the Albumax II. A special, three component, gas mixture was obtained from Afrox (Germiston, South Africa). The gas mixture consisted of 5% oxygen (O_2), 5% carbon dioxide (CO_2) and 90% nitrogen gas (N_2). Giemsa stain, sodium phosphate and potassium phosphate were also supplied by Sigma Aldrich® (South Africa). MERCK (South Africa) provided the methanol while frosted, 1.2 mm, ground edge microscope slides were purchased from either Lasec (South Africa) or Seperations (South Africa).

4.2.1.3 Method of cultivation

P. falciparum was cultivated by means of a modified version of the original Trager and Jensen (1976) method. The *in vitro* cultivation, in continuous culture, was achieved by using a specific culture medium mixture which consisted of the following reagents:

- RPMI 1640 medium (1.04 g/100 ml)
- HEPES (0.6 g/100 ml)
- D-(+)-glucose (0.4 g/100 ml)
- Hypoxanthine (0.004 g/100 ml)
- Gentamycin (0.04 g/ml conc. = 0.12 ml/100 ml)
- Sodium bicarbonate (4.2 ml/100 ml of a 5% solution)
- Albumax II (0.5 g/100 ml)

These reagents were added together and dissolved in sterile water for injection. The mixture was then filtered through a 0.22 µm filter under vacuum. It is important to conduct this exercise in a laminar flow cabinet, under sterile conditions, in order to prevent contamination

and subsequent infection of the cultures. The chloroquine resistant RSA11 strain was used during the *in vitro* drug efficacy evaluations. The cultures were kept in culture flasks with air tight caps which were purchased from Scientific Group (South Africa). The cultures were maintained at an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The culture medium (10 ml) was changed every second and fresh human erythrocytes were added once weekly to maintain a haematocrit and parasitaemia of approximately 5%. Erythrocytes were obtained from human A^+ whole blood samples donated by healthy volunteers. The samples were purged with wash medium to remove all the leukocytes. The wash medium consisted of the same ingredients as the normal culture medium but with the exclusion of Albumax II. The wash procedure was performed by first centrifuging the whole blood sample and then removing the Buffy coat by means of aspiration. This was followed by the addition of wash medium and again centrifuging with subsequent removal of the supernatant. This procedure was repeated thrice, the remaining erythrocytes were then stored in sterile culture medium at 4 °C (De Ridder, 2007).

4.2.1.4 Determination of parasitaemia

Giemsa stained thin blood smear microscope slides were prepared, three slides of each culture flask intended for experimental purposes. The slides were prepared by placing a small amount of erythrocytes from the culture flask onto a pre-labeled microscope slide. A second slide was placed on the first slide and moved backwards into the drop of erythrocytes spreading it across the width of the slide. The second slide was then moved forward, subsequently smearing the erythrocytes in a thin film over the first slide. The slides were left to air dry after which the erythrocytes were fixed with methanol. The slides were then covered with a giemsa solution consisting of one part giemsa and four parts phosphate buffer. The phosphate buffer was prepared by dissolving 0.65 g sodium phosphate and 0.41 g potassium phosphate in one litre of sterile water for injection. The giemsa was filtered to remove any small particles and unwanted crystals before it was used. The slides were left to stain to 10 minutes after which they were rinsed with running water to remove the excess stain. The slides were again left to air dry. The slides were then evaluated under a light microscope with a 100 X objective. The total amount of infected and uninfected erythrocytes was counted in 10 different microscope fields on all three slides. The percentage parasitaemia was then calculated with equation 3.

$$\% \text{ Parasitaemia} = \frac{\text{Total amount of infected erythrocytes}}{\text{Total amount of erythrocytes}} \times 100 \qquad \textit{Eq. 3}$$

During continuous cultivation the haematocrit and parasitaemia were both maintained at approximately 5%. Parasitaemia was adjusted if deemed necessary by the addition of fresh, uninfected, erythrocytes. Haematocrit was adjusted by the addition or removal of culture medium.

4.2.1.5 Synchronization

Synchronized cultures were used throughout the *in vitro* study. Cultures were synchronized to ensure that all parasites in the culture are in the same phase (De Ridder, 2007; Lambros & Vanderberg, 1979). All cultures were synchronized in the ring phase or early trophozoite phase. It was accomplished by adding 4 ml of a sterile 15% (w/v) sorbitol solution to the packed infected erythrocytes. It was left to incubate for 5 minutes at 37 °C after which 8 ml of a sterile 0.1% (w/v) glucose solution was added. It was again incubated at 37 °C for 5 minutes, the solution was then centrifuged for eight minutes at 2000 revolutions per minute (r.p.m.). The supernatant was aspirated, the erythrocytes resuspended in culture medium and transferred to culture flasks. Normal cultivation procedures, as described earlier, was then followed.

4.2.2 Experimental design: *in vitro activity evaluation*

The study and all experimental procedures were approved by the Ethics Committee of the North-West University (NWU-0008-08-55).

The general experimental design was the same for all the *in vitro* experiments. All the experimental procedures, drug solution volumes and culture medium / erythrocyte volumes were kept constant. The only differences being the type of drug and the drug concentrations used. The experiments were performed in 96 well plates and all experiments were performed in triplicate. For experimental purposes a volume of 20 µl of the individual drug solutions were added to the wells. This was followed by the addition of 180 µl of the culture medium / infected erythrocyte suspension.

The chloroquine resistant *P. falciparum* strain, RSA11, was used at a parasitaemia of 2% and the haematocrit was set at 2%. All procedures were performed in a laminar flow cabinet under sterile conditions. After all the well plates were correctly filled, they were placed in an airtight container with an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The container was incubated for 48 hours at a constant temperature of 37 °C. After 48 hours have elapsed the

well plates were removed from the container and the parasitaemia of each well was determined by means of flow cytometry. Giemsa stained thin blood smear microscope slides were also prepared from each well to serve as a reference and also as a backup. The percentage parasitaemia was determined and the 50% inhibitory concentration (IC_{50}) was then calculated and statistically evaluated.

4.2.3 Preparation of experimental formulations

4.2.3.1 Materials

Artemisone and artemiside were kindly donated by the Department of Chemistry, Open Laboratory of Chemical Biology, Institute of Molecular Technology for Drug Discovery and Synthesis, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (P.R. China). The ingredients for the Pheroid formulations were kindly provided by the Department of Pharmaceutics of the North-West University (Potchefstroom, South Africa).

4.2.3.2 Methodology

The drug containing reference formulations were all prepared by completely dissolving the appropriate drug amounts in 100% analytical grade DMSO. The solutions were then made up to volume with sterile water for injection (SWI) and used as stock solutions. The stock solutions were serially diluted with SWI to achieve the required experimental drug concentrations.

The Pheroid vesicle formulations were prepared by first dissolving the appropriate drug amounts in the Pheroid oil-phase. The drug/oil-phase solutions were then adjusted to volume with nitrous oxide water (NW) to achieve a 2% oil in NW solution. These stock solutions were then also serially diluted to achieve the required experimental drug concentrations. The dilutions were performed with a Pheroid vesicle and NW solution in a ratio of 1:250. The final Pheroid vesicle to NW ratio, after dilution in the 96 well plates, was 1:2 500.

The drug free reference formulations were SWI and a Pheroid vesicle formulation with a Pheroid vesicle to NW ratio of 1:250. A volume of 20 µl of either the reference formulation or the Pheroid vesicle formulation were added to corresponding the wells.

The final drug concentrations after dilution in the 96 well plates are presented in table 4.1.

Table 4.1: Final drug concentrations in the 96 well plates

Compound	Concentration range (nM).										
Artemisone	0	0.05	0.10	0.20	0.40	0.50	0.65	0.80	1	5	10
Artemiside	0	0.05	0.10	0.20	0.40	0.50	0.65	0.80	1	5	10
Artesunate	0	10	15	20	25	30					

Artesunate was included in the study based on the fact that it is currently the most widely used artemisinin derivative against *P. falciparum* malaria. The antimalarial activity and IC_{50} value of artesunate were included to give an indication of the relative antimalarial activity of both artemisone and artemiside.

4.2.4 Preparation of culture medium / erythrocyte suspension

4.2.4.1 Materials

Culture medium, as described under 4.2.1.3 was used to prepare the experimental suspensions. Uninfected erythrocytes were obtained from human A^+ whole blood samples donated by healthy volunteers. Erythrocytes infected with *P. falciparum* strain RSA11 were maintained in continuous culture.

4.2.4.2 Methodology

The parasitaemia of the cultures intended for experimental use was determined by microscopic evaluation as described under 4.2.1.4. The parasite cultures were then transferred from the culture flasks to 15 ml falcon tubes. The tubes were then centrifuged at 2 000 r.p.m. for 3 minutes to separate the infected erythrocytes from the used culture medium. The supernatant was removed by means of aspiration and the packed erythrocyte volume was determined. The necessary calculations were performed and the parasitaemia adjusted accordingly to 2% by the addition of freshly prepared erythrocytes, as described under 4.2.1.3. Culture medium was added to the packed erythrocytes until a haematocrit of 2% was achieved and the erythrocytes were then resuspended in the medium. This culture medium / infected erythrocyte suspension was then added to each well at a volume of 180 µl.

4.2.5 Flow cytometric determination of parasitaemia

4.2.5.1 Materials

The flow cytometric analysis were performed with a BD FACSCalibur™. Reagents and consumables were purchased from Scientific Group (South Africa). Gluteraldehyde solution and Triton® X–100 were purchased from Sigma Aldrich® (South Africa).

4.2.5.2 Methodology

Eppendorf tubes were labeled and 100 µl of a 0.04% gluteraldehyde solution was added to each tube. The samples were fixed by transferring 100 µl of each sample to the corresponding gluteraldehyde containing tube. The samples were left to incubate for one hour at 4 °C. After the incubation period the samples were centrifuged and the supernatant removed. The remainder of the samples were then resuspended in 100 µl of phosphate buffered saline (PBS) containing 0.25% Triton® X – 100. The samples were then left for a further 10 minutes, at room temperature, and again centrifuged. The supernatant was discarded and the samples resuspended in 100 µl of PBS. YOYO-1 was subsequently added to each sample to reach a final concentration of 0.50 µM. The samples were incubated in the dark for one hour and then analyzed with a FACSCalibur™ flow cytometer. The parasite area, on which the parasitized erythrocytes were plotted, was defined on a two dimensional scattergram of green and red fluorescence. The obtained results were compared to parasite-negative populations for accuracy purposes.

4.2.6 Validation of Fascalibur™ results

4.2.6.1 Materials

Giemsa stain, sodium phosphate and potassium phosphate were purchased from Sigma Aldrich® (South Africa). Methanol was purchased from MERCK (South Africa). First grade, frosted, 1.2 mm, ground edge microscope slides were obtained from either Lasec or Seperations (South Africa). A Nikon DXM1200 digital camera on a Nikon ECLIPSE TE300 confocal microscope with ACT-1 software were used to manually count the slides.

4.2.6.2 Methodology

The FACSCalibur™ results were validated by preparing giemsa stained thin blood smear microscope slides from each experimental well. The same procedure was followed as described under 4.2.1.4. The slides were evaluated using a Nikon DXM1200 digital camera on a Nikon ECLIPSE TE300 confocal microscope with ACT-1 software. The total amount of infected and uninfected erythrocytes were manually counted and the data was then used to determine the percentage parasitaemia with the aid of equation 3.

The calculated % parasitaemia was then compared to the results obtained with the FACSCalibur™ to confirm the accuracy of the results.

4.2.7 Calculation of IC_{50} values and statistical analysis

The IC_{50} is a quantitative measurement which represent the specific concentration of a drug which is required to inhibit parasite growth by 50%. Prism version 4 (GraphPad Software Inc., California USA) was used to calculate the IC_{50} values. The percentage parasitaemia values were first normalized. This was accomplished by calculating the mean of the triplicate percentage parasitaemia values of the zero drug concentration wells. The calculated mean was then set as a nominal value representing a percentage parasitaemia of 100%. The mean of all the other triplicate parasitaemia values were then set against the representative 100% value and a corresponding percentage value was then calculated for the mean of each triplicate set. Normalization of the parasitaemia values were necessary to ensure that the curves originate at 0% and plateau at 100%. The drug concentrations were transformed and expressed on a logarithmic scale on the X-axis. A sigmoidal dose-response curve was then constructed by means of non-linear regression. The corresponding IC_{50} value of each dose-response curve was then determined.

The statistical significance of the difference between the IC_{50} values of the reference formulation and that of the Pheroid vesicle formulation was determined by calculating a t-value with the student's t-test of independent samples (equation 4).

$$t = \frac{X_1 - X_2}{\sqrt{S_1^{\,2} + S_2^{\,2}}} \qquad Eq.4$$

where X_1 represent the mean IC_{50} of the reference formulation and X_2 represent the mean IC_{50} of the Pheroid vesicle formulation. S_1 and S_2 represent the standard error of the mean (SEM) of the reference and the Pheroid vesicle formulations respectively. StatSoft Inc. (2009) STATISTICA (data analysis software system), version 9.0, was then used to convert the calculated t-values to corresponding p-values.

4.2.8 Results and discussion

4.2.8.1 *In vitro* activity of artesunate

The initial parasitaemia for all the wells were the same. The results are presented in table 4.2 and graphically depicted in figure 4.1. The results are presented as the % parasitaemia versus the specific drug concentration. The effects of the reference and the Pheroid vesicle formulations, at each drug concentration, are presented alongside each other for easy comparison.

Table 4.2: Artesunate *in vitro* activity results

Conc.	Reference			Mean	SEM	Pheroid vesicles			MEAN	SEM
0	8.28	8.43	8.04	8.25	0.11	7.15	8.29	7.3	7.58	0.36
10	7.28	6.53	9.77	7.86	0.98	3.47	6.75	4.14	4.79	1.0
15	6.57	7.4	6.66	6.88	0.26	2.84	2.76	3.75	3.12	0.32
20	7.18	6.93	6.78	6.96	0.12	2.24	2.97	2.13	2.45	0.26
25	3.99	6.85	8.68	6.51	1.36	1.32	2.0	1.56	1.63	0.2
30	3.48	4.31	4.01	3.93	0.24	1.8	1.32	1.38	1.5	0.15

The slight variance in parasite density may be attributed to the release of merezoites during the course of the experiment which infected more surrounding erythrocytes.

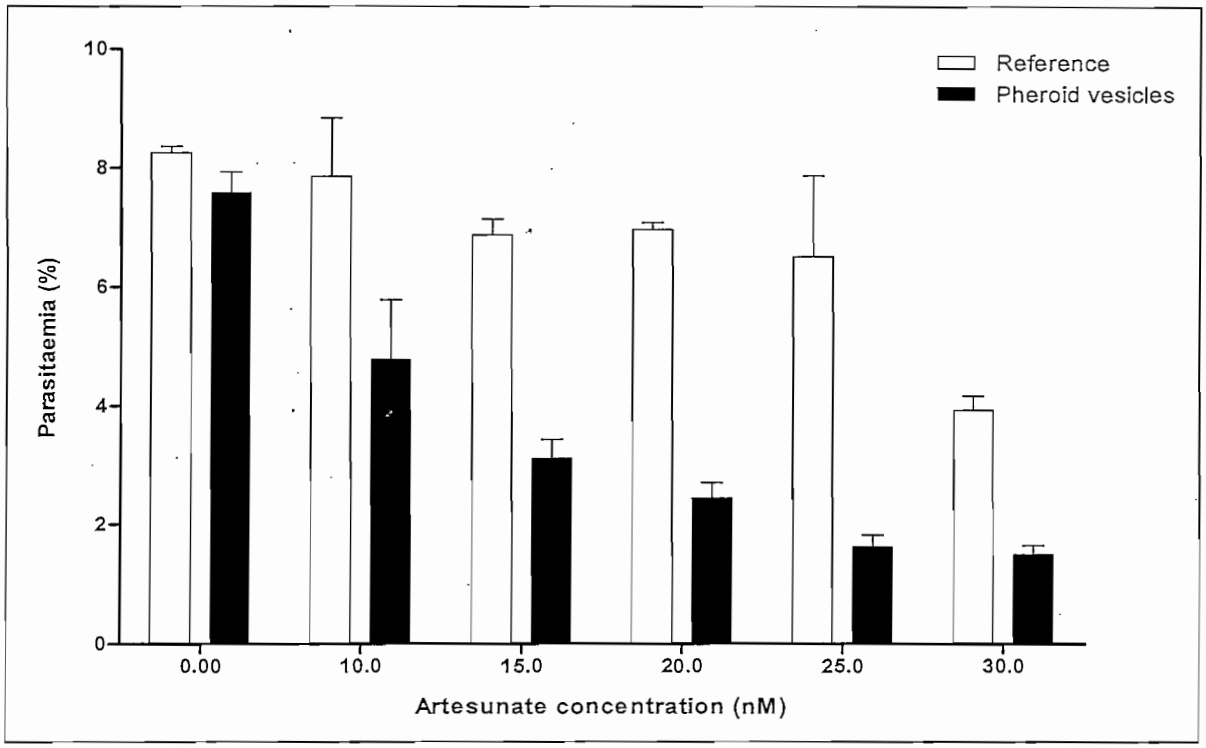

Figure 4.1: ***In vitro*** **activity of artesunate**

At the 0 nM concentration the parasitaemia was virtually the same for both the reference and Pheroid formulations, parasitaemia values were 8.3% and 7.6% respectively. The 10 nM artesunate reference formulation achieved virtually no reduction in parasitaemia while the same drug concentration in the Pheroid formulation reduced the parasitaemia to 4.8%. The 15 nM reference formulation reduced the parasitaemia to 6.9%. This remained constant for the 20 nM and 25 nM reference formulations with respective parasitaemias of 7.0% and 6.5%. The Pheroid formulation showed better antimalarial activity. The 15 nM artesunate concentration reduced the parasitaemia to 3.1%. This trend in parasite reduction repeated itself at the 20 nM concentration where the parasitaemia was reduced to 2.5%. At 25 nM the Pheroid formulation reduced the parasitaemia even further to 1.6%. At 30 nM the reference formulation succeeded in reducing the parasitaemia to 3.9% but not to the same extent as the Pheroid formulation which succeeded in reducing the parasitaemia to 1.5% at the same artesunate concentration.

The IC_{50} values of the two formulations were also calculated. The standard error of mean (SEM) was also determined but the values were so small (0.05 nM and 0.04 nM respectively) that they are not included in figure 4.2.

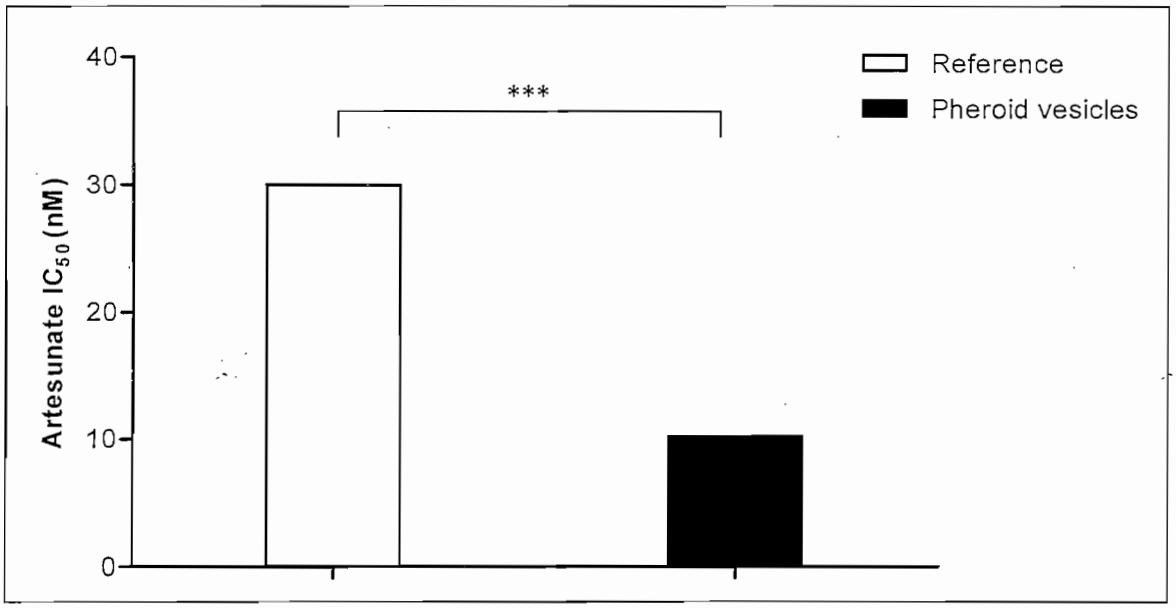

Figure 4.2: Calculated IC_{50} values of the artesunate reference and artesunate Pheroid vesicle formulations (*, $p < 0.0001$)**

The reference formulation had an IC_{50} value of 29.65 ± 0.05 nM while the Pheroid formulation had an IC_{50} of 10.20 ± 0.04 nM. The difference between the calculated IC_{50} values were statistically significant ($p < 0.0001$). This amounts to a difference of 65.6%, which suggest that the Pheroid formulation achieved the same reduction in parasitaemia at a drug concentration of 65.6% less than that required by the reference formulation. From this results it was very clear that the Pheroid formulations' performance was far superior in comparison to the reference formulation and that the same results could be achieved with a much lower drug concentration when using the Pheroid formulation.

4.2.8.2 *In vitro* activity of artemisone

The initial parasitaemia for all the wells were the same. The results are presented in table 4.3 and graphically depicted in figure 4.3. The results are presented as the % parasitaemia versus the specific drug concentration. The effects of the reference and the Pheroid vesicle formulations, at each drug concentration, are presented alongside each other for easy comparison.

Table 4.3: Artemisone *in vitro* activity results

Conc.	Reference			Mean	SEM	Pheroid vesicles			MEAN	SEM
0	8.29	8.19	8.5	8.33	0.09	8.76	8.01	7.89	8.22	0.27
0.05	8.7	8.7	8.1	8.5	0.2	6.01	5.79	5.12	5.64	0.27
0.1	8.02	8.16	8.1	8.09	0.04	5.89	5.42	4.76	5.36	0.33
0.2	7.76	7.6	7.5	7.62	0.08	4.76	4.87	4.15	4.59	0.22
0.4	7.17	7.81	7.1	7.36	0.23	4.02	4.12	3.97	4.04	0.04
0.5	7.87	6.22	6.01	6.7	0.59	3.78	3.79	3.14	3.57	0.23
0.65	4.5	4.88	4.89	4.76	0.13	2.98	3.34	2.98	3.1	0.12
0.8	4.0	4.39	4.13	4.17	0.11	2.54	3.01	2.13	2.56	0.25
1	3.32	2.93	3.48	3.24	0.16	2.01	2.34	1.98	2.11	0.12
5	2.11	2.4	2.8	2.44	0.2	1.7	1.89	1.56	1.72	0.1
10	0.3	1.51	1.98	1.26	0.5	0.87	0.8	0.76	0.81	0.03

The slight variance in parasite density may be attributed to the release of merezoites during the course of the experiment which infected more surrounding erythrocytes.

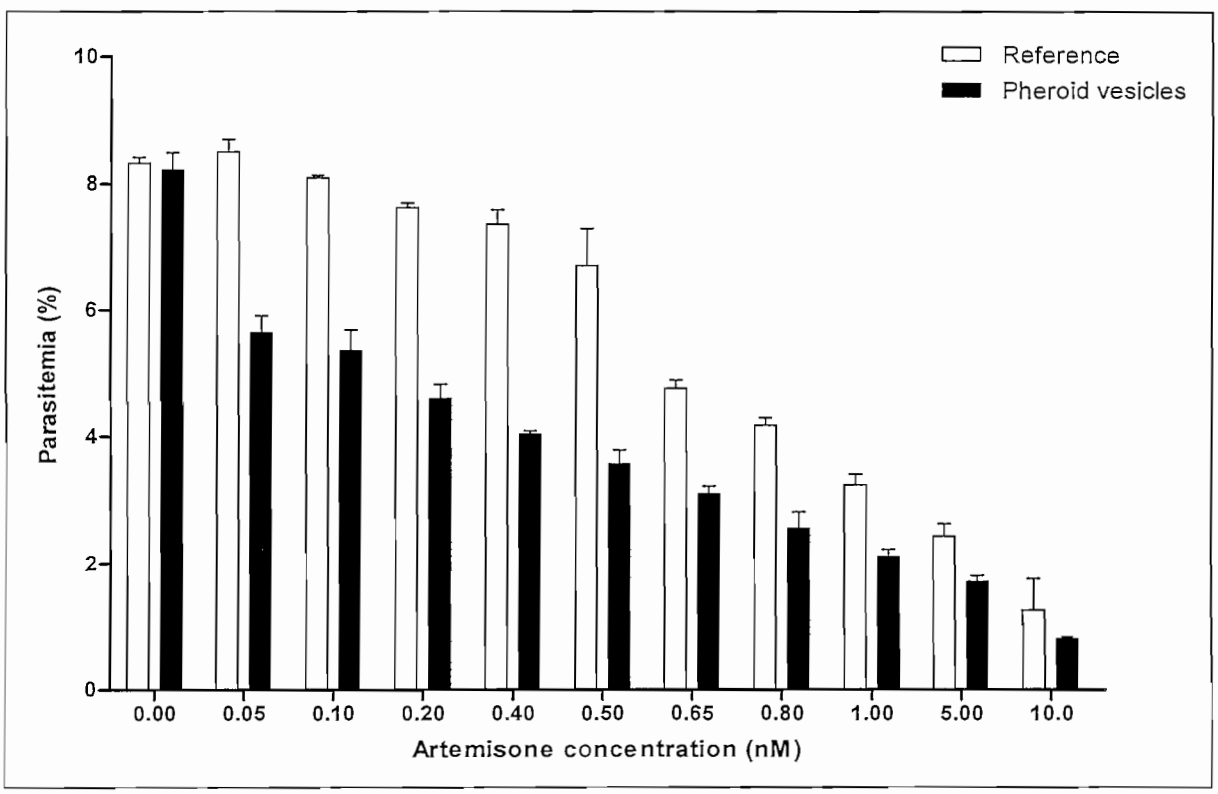

Figure 4.3: *In vitro* activity of artemisone

At the 0 nM concentration the parasitaemia was virtually the same for both the reference and the Pheroid formulations. The parasitaemia values were 8.3% and 8.2% respectively. At the first artemisone reference concentration of 0.05 nM there was no reduction in parasitaemia while the same concentration in the Pheroid formulation reduced the parasitaemia to 5.6%. The reference formulation showed a very gradual reduction in parasitaemia between concentrations of 0.10 nM and 0.50 nM. At 0.50 nM the parasitaemia was 6.7%. The Pheroid formulation reduced the parasitaemia from 5.4% to 3.6% between concentrations of 0.10 nM to 0.50 nM. The first marked reduction in parasitaemia with the reference formulation was at 0.65 nM where the parasitaemia was reduced to 4.8%. The Pheroid formulation, at the same concentration, reduced the parasitaemia to 3.1%. A trend of gradual reduction in parasitaemia was observed between concentrations of 0.80 nM and 10 nM for both formulations. The reference formulation reduced the parasitaemia from 4.2% at 0.80 nM to 1.3% at 10 nM. The Pheroid formulation was even more effective in reducing the parasitaemia, at 0.80 nM the parasitaemia was 2.6% and at 10 nM the parasitaemia was 0.8%.

The IC_{50} values for both formulations were calculated and are graphically depicted in figure 4.4.

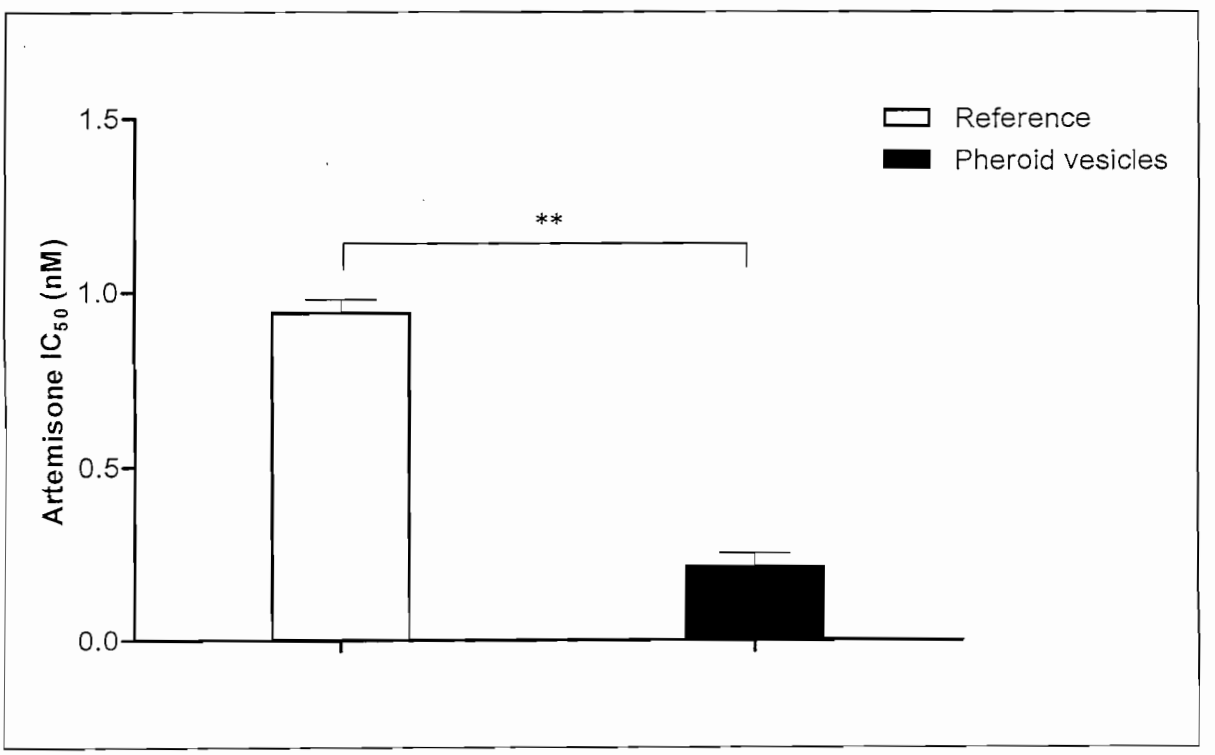

Figure 4.4: Calculated IC_{50} values of the artemisone reference and artemisone Pheroid vesicle formulations (, p = 0.0001)**

The reference formulation had an IC_{50} of 0.94 ± 0.04 nM while the Pheroid formulation had an IC_{50} value of 0.21 ± 0.04 nM. The difference between the calculated IC_{50} values were statistically significant ($p = 0.0001$). This amounts to a difference of 77.7% and suggest that the Pheroid vesicle formulation achieved the same reduction in parasitaemia than the reference formulation at a drug concentration of 77.7% less. This data suggest that the Pheroid formulation was far superior to the reference formulation and that the same results could be achieved at a much lower drug concentration when using the Pheroid formulation.

When comparing the *in vitro* results of artesunate with that of artemisone it was clear that artemisone was much more effective in reducing the parasitaemia than artesunate. The IC_{50} value of the artesunate reference formulation was 29.65 ± 0.05 nM while the IC_{50} value of the artemisone reference formulation was only 0.94 ± 0.04 nM. This amounts to a difference in reference IC_{50} values of 96.8% ($p < 0.0001$). When comparing the IC_{50} values of the Pheroid formulations the same trend was observed. The calculated difference between the two formulations was 97.9% ($p < 0.0001$). The biggest difference obtained was with comparison of the artesunate reference IC_{50} value to that of the artemisone Pheroid formulations' IC_{50}. The difference was 99.3% ($p < 0.0001$) in favour of the artemisone Pheroid vesicle formulation.

The results strongly suggest that artemisone is a much more potent antimalarial compound than artesunate and that Pheroid technology was able to enhance artemisone's antimalarial activity even further.

4.2.8.3 *In vitro* activity of artemiside

The initial parasitaemia for all the wells were the same. Results are presented in table 4.4 and graphically depicted in figure 4.5. Variance in parasite densities may be attributed to the release of merezoites during the course of the experiment which infected more surrounding erythrocytes.

Table 4.4: Artemiside *in vitro* activity results

Conc.	Reference			Mean	SEM	Pheroid vesicles			MEAN	SEM
0	15.83	14.22	17.29	15.78	0.89	7.60	8.13	9.94	8.56	0.71
0.05	9.74	11.93	11.56	11.10	0.68	7.81	11.6	7.01	8.80	1.42
0.1	11.71	14.14	11.75	12.53	0.80	3.3	6.06	6.06	5.14	0.92
0.2	8.64	8.30	7.46	8.13	0.35	3.04	2.61	2.33	2.66	0.21
0.4	8.02	6.28	6.5	6.93	0.55	2.96	2.68	2.41	2.68	0.16
0.5	5.98	5.81	5.17	5.65	0.25	1.91	2.05	2.7	2.22	0.24
0.65	5.37	3.86	5.82	5.02	0.6	2.36	2.24	2.25	2.28	0.04
0.8	3.17	3.31	3.1	3.20	0.06	1.77	2.04	1.36	1.72	0.20
1	4.11	2.92	2.38	3.14	0.51	2.02	1.0	0.92	1.31	0.35
5	2.16	1.17	2.38	1.9	0.37	1.19	0.79	0.69	0.89	0.15
10	1.29	1.26	-	1.28	0.02	0.77	0.66	-	0.7	0.06

The % parasitaemia was plotted against the specific artemiside concentration. The effects of the reference and the Pheroid vesicle formulations, at each drug concentration, are presented alongside each other for easy comparison.

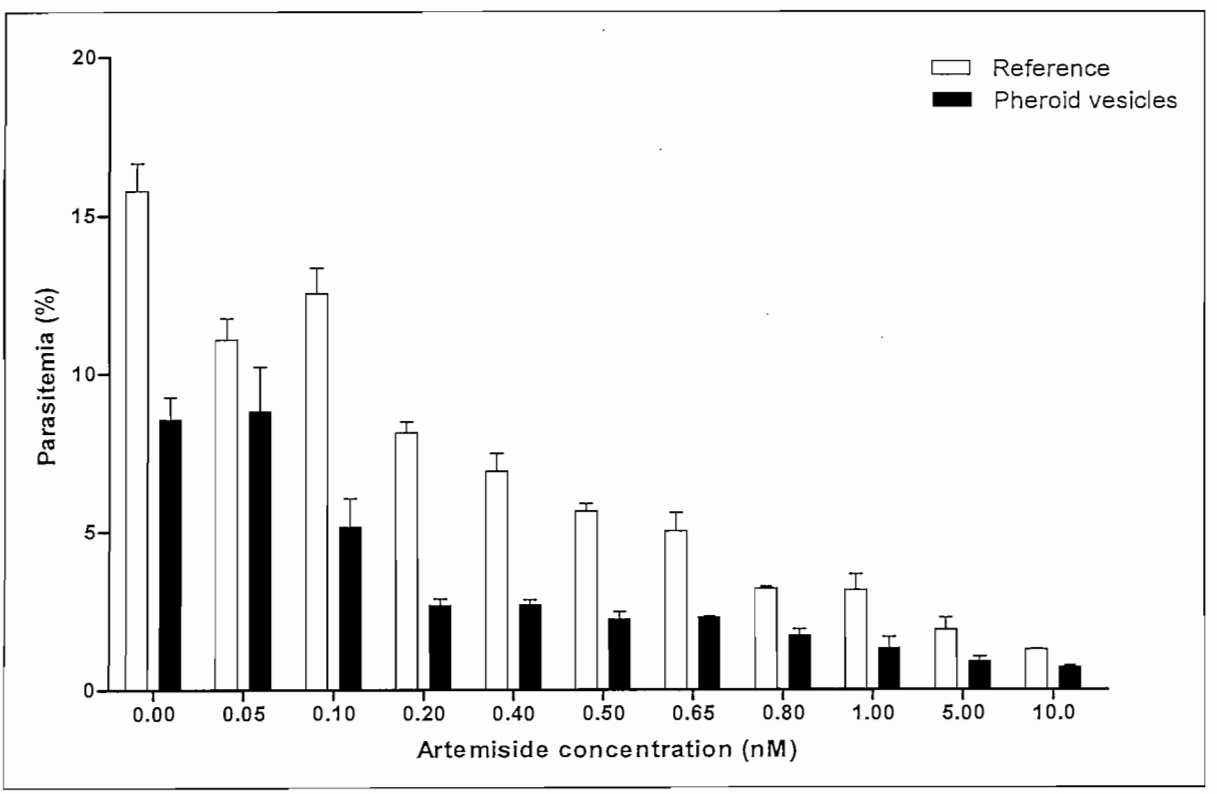

Figure 4.5: *In vitro* activity of artemiside

At the 0 nM concentration there was a marked difference in the starting parasitaemia. The occurrence was scrutinized by re-evaluating the microscope slides and the FACSCalibur™ settings and results. No discrepancies were found. At 0 nM the reference formulation presented with a parasitaemia of 15.8% while the Pheroid formulation had a parasitaemia of 8.6%. At 0.05 nM the parasitaemia of the reference was 11.1% and that of the Pheroid formulation was 8.8%. At 0.10 nM there was still no significant reduction in parasitaemia with the reference formulation while the Pheroid formulation reduced the parasitaemia to 5.1%. The first marked reduction in parasitaemia with the reference formulation was observed at 0.20 nM with a parasitaemia of 8.1%. The Pheroid formulation achieved an even greater reduction and presented with a parasitaemia of 2.7%. There was a gradual reduction in parasitaemia from 0.40 nM to 1 nM. The reference formulation reduced the parasitaemia from 6.9% to 3.1% while the Pheroid formulation succeeded in reducing the parasitaemia from 2.7% to 1.3%. At 5 nM the reference formulation reduced the parasitaemia to 1.9% while a concentration of 10 nM rendered a parasitaemia of 1.3%. The results of the Pheroid formulation were even more impressive. The 5 nM concentration rendered a parasitaemia of 0.9% and at 10 nM a parasitaemia of 0.7% was recorded.

The IC_{50} values for both formulations were calculated and are presented in figure 4.6.

Figure 4.6: Calculated IC_{50} values of the artemiside reference and artemiside Pheroid vesicle formulations (, p = 0.009)**

The reference formulation had an IC_{50} of 0.54 ± 0.03 nM while the Pheroid formulation had an IC_{50} value of 0.11 ± 0.05 nM. The difference between the calculated IC_{50} values were statistically significant (p = 0.009). This amounts to a difference of 79.6% and suggest that the Pheroid formulation achieved the same reduction in parasitaemia than the reference formulation at a drug concentration of 79.6% less than that of the reference formulation. This data suggest that the Pheroid formulation was far superior to the reference formulation and that the same results could be achieved with a much lower drug concentration when using the Pheroid formulation.

When comparing the *in vitro* results of artesunate to that of artemiside it was very apparent that artemiside posessed much higher antimalarial activity. The IC_{50} value of the artesunate reference was 29.65 ± 0.05 nM while the IC_{50} value of the artemiside reference was 0.54 ± 0.03 nM, p < 0.0001. The difference amounted to a 98.2% smaller IC_{50} value for artemiside. When comparing the IC_{50} values of the Pheroid vesicle formulations the trend still held true. The calculated difference between the two formulations was 98.9% (p < 0.0001) in favour of artemiside. The artesunate reference IC_{50} was also compared to the IC_{50} of the artemiside Pheroid vesicle formulation and it was determined that the Pheroid formulation had an IC_{50} of 99.6% less than that of the artesunate reference (p < 0.0001).

It was deemed necessary to also compare artemisone to artemiside. The IC_{50} of the artemiside reference formulation was 42.6% less than that of the artemisone reference formulation. The difference in the IC_{50} values of the two Pheroid formulations amounted to 47.6% in favour of artemiside.

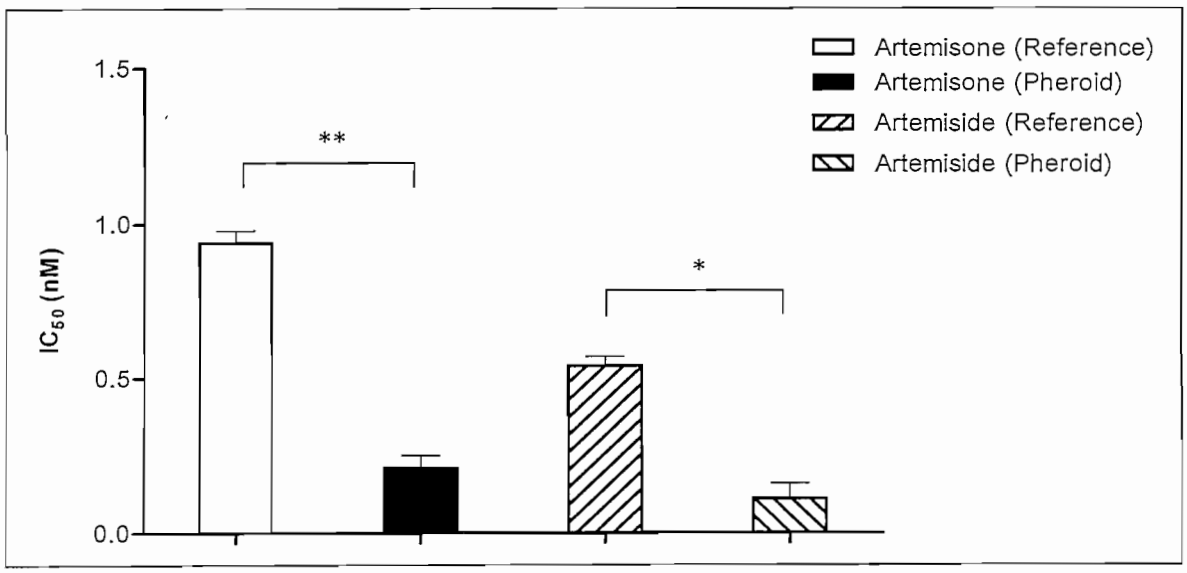

Figure 4.7: Comparison between IC_{50} values of artemisone and artemiside (, p = 0.0001 and *, p = 0.009)**

Table 4.5: Comparison of the IC_{50} values of the drugs in the reference and Pheroid vesicle formulations

Drug formulation	Artesunate (nM)		Artemisone (nM)		Artemiside (nM)	
	Mean	SEM	Mean	SEM	Mean	SEM
Reference	29.65	0.05	0.94	0.04	0.54	0.03
Pheroid vesicles	10.20	0.04	0.21	0.04	0.11	0.05

4.2.9 Summary

The *in vitro* results indicated very clearly that artemisone and artemiside is much more active against *P. falciparum* than artesunate. When comparing the respective IC_{50} values it was found that artemiside was the most potent antimalarial compound. Artemisone was also very potent. The experiments obviated the fact that it was possible to greatly enhance the *in vitro* antimalarial activity of all three test compounds by incorporating them in a Pheroid vesicle formulation. The results achieved with both artemisone and artemiside warrents additional *in vivo* and pharmacokinetic studies to further investigate the antimalarial activity of these novel compounds.

4.3 *IN VIVO* ANTIMALARIAL ACTIVITY EVALUATION

4.3.1 Introduction

After careful evaluation of the *in vitro* results it was decided to conduct *in vivo* antimalarial activity evaluations on artemisone and artemiside. The test compounds were evaluated in a C57 BL6 mouse model of malaria. Two formulations were prepared for each compound at two different doses for each. The drug formulations were administered via the oral route with the aid of a gavage tube. The Peters' 4-day suppressive test model was used as a basis to conduct the study.

4.3.2 *In vivo* model

4.3.2.1 Experimental animals

The study was conducted at the University of Cape Town (UCT) under the supervision of Dr. L. Wiesner from Cape Town Bioanalytical Services. An application for the use of C57 BL6

mice in this study was compiled and approved (Ethics number: 008/030). Male C57 BL6 mice were used as a test model to evaluate the *in vivo* antimalarial activity of artemisone and artemiside against *Plasmodium berghei* infections. C57 BL6 mice are readily available, breed very successfully and quickly in captivity and are easy to handle.

4.3.2.2 Breeding conditions

The mice were bred and kept in a closed, controlled environment at the Animal Research Centre at the UCT. The closed and controlled conditions under which the animals were kept ensured an ideal growth environment with an absolute minimum exposure to pathogens. All possible variables in the Animal Research Centre were kept as constant as possible. The specific conditions are listed in table 4.6. Mice were fed with Epol® mice cubes (Epol Pty (Ltd), Pretoria, RSA).

Table 4.6: Conditions under which mice were kept in the closed environment

Condition	Recommended value*	Value in Animal Research Centre
Temperature	19 ± 2 C°	21 ± 2 C°
Relative humidity	55 ± 15%	55 ± 10%
Rate of ventilation/air movement	15 – 20 changes per minute	18 changes per minute
Light intensity	350 – 400 lux one meter above floor level	350 – 400 lux one meter above floor level
Light period	12 hours light and 12 hours dark	12 hours light and 12 hours dark

*Values recommended according to international standards

4.3.2.3 Route of administration

The oral route was chosen since it is the most common route through which antimalarial drugs are administered to patients with uncomplicated malaria. The oral route is easily accessible, non-invasive and improves patient compliance. Based on these considerations the oral route was chosen to evaluate antimalarial activity of the test compounds, both in the presence and absence of a Pheroid vesicle formulation. The test formulations were administered via the oral route with the aid of a 1 ml syringe and an oral gavage tube.

4.3.3 Experimental design

4.3.3.1 General design

The Peters' 4-day suppressive test was used as a basis model for the study. C57 BL6 mice were randomly divided into twelve groups. Six groups were used for artemisone (A – F) and six for artemiside (A – F) (see tables 4.7 and 4.8). Five mice were included in each group to ensure that any significant statistical differences would be detected between the reference and Pheroid vesicle formulation groups (Statistical Consultation Services, UCT).

The mice were subsequently infected with *P. berghei* by injecting 2 x 10^6 parasitized erythrocytes into the peritoneum of each test animal. Directly after infection the mice in each group were treated with the pre-determined regimen for that specific group. Treatments were repeated at 24, 48 and 72 hours post infection. The treatment regimens consisted of two DMSO/water (1:9, v/v) formulations and two Pheroid vesicle formulations for each drug. Both drugs were tested at a high and a low concentration. Groups E and F (artemisone and artemiside evaluation) received only the vehicle solutions, DMSO/water (1:9, v/v) or Pheroid vesicles, with no drug added. Fresh stock solutions were prepared for each formulation immediately before each treatment. The parasitaemia was monitored frequently by studying thin blood film slides of each test animal stained with a giemsa solution. The slides were prepared from blood obtained by means of tail-tip bleeding. The slides were examined with the aid of a light microscope with the objective set at 100 X magnification. The weight of all the test animals were also frequently monitored during the study.

4.3.3.2 Infection of test animals

Mice were randomly assigned to twelve experimental groups of five mice each (see tables 4.7 and 4.8). The mice were all infected with the chloroquine sensitive isolate of *P. berghei.* It is known to be a highly virulent and lethal strain of malaria and presented a suitable model system for the evaluation. Parasites were maintained in two host animals until the parasitaemia reached a level of between 20 – 30 percent. At that stage the host mice were sacrificed and the parasites were harvested via cardiac puncture. The two parasite stocks were pooled and the average parasitaemia of the pooled stock was determined microscopically. The total number of erythrocytes per milliliter of the pooled stock was also determined to ensure a uniform infection of all test animals. The harvested parasitized erythrocytes (pRBC) were transferred to microfuge tubes and maintained in phosphate-

buffered saline at a level of 5 x 10^7 pRBC/ml as a secondary stock. The mice of the various groups (A – F, artemisone and A – F, artemiside) were all infected. A volume of 200 µl of the secondary stock was injected into the peritoneum of the test animals in order to introduce 2 x 10^6 pRBC to each. Calculations are included in annexure 1.

4.3.3.3 Preparation and administration of experimental formulations

Tables 4.7 and 4.8 gives a summary of the different treatment groups. The same batch of artemisone and artemiside, used for the *in vitro* evaluation, was also used for the *in vivo* evaluation. The materials for the Pheroid formulations were kindly provided by the Department of Pharmaceutics of the North-West University (Potchefstroom, South Africa).

Fresh stock solutions of each test formulation were prepared immediately before each treatment. The correct calculated amounts of each drug were weighed and placed in seperate containers labeled A – D. Containers E and F were reserved for the vehicles only and no drugs were added.

The reference formulations were prepared by dissolving the appropriate amount of each drug in analytical grade DMSO. Purified water was then added until the desired volume was reached. The final DMSO to water ratio was 1:9 v/v. All of the reference formulations formed a micro-suspension after the addition of water to the DMSO-drug solution.

The Pheroid vesicle formulations were prepared by first dissolving the drugs in the Pheroid oil-phase. A measured volume of NW was then added to the oil-phase to achieve the desired drug concentration. The Pheroid/drug formulations were then mixed by shaking the formulations for 30 minutes in an IKA VIBRAX VXR basic at 700 MOT/min.

A total volume of 200 µl were orally administered to each test animal. Treatments were given on the day of infection and then again at 24, 48 and 72 hours post infection.

Table 4.7: **Treatment regimen for the artemisone experimental groups (Groups E and F = untreated reference/placebo)**

Group	A	B	C	D	E	F
Drug	Artemisone	Artemisone	Artemisone	Artemisone	Vehicle	Vehicle
Formulation	DMSO/H_2O (1:9, v/v)	Pheroid vesicles	DMSO/H_2O (1:9, v/v)	Pheroid vesicles	DMSO/H_2O (1:9, v/v)	Pheroid vesicles
Volume (µl)	200	200	200	200	200	200
Dose (mg/kg)	20	20	2.5	2.5	-	-

Table 4.8: **Treatment regimen for the artemiside experimental groups (Groups E and F = untreated reference/placebo)**

Group	A	B	C	D	E	F
Drug	Artemiside	Artemiside	Artemiside	Artemiside	Vehicle	Vehicle
Formulation	DMSO/H_2O (1:9, v/v)	Pheroid vesicles	DMSO/H_2O (1:9, v/v)	Pheroid vesicles	DMSO/H_2O (1:9, v/v)	Pheroid vesicles
Volume (µl)	200	200	200	200	200	200
Dose (mg/kg)	10	10	2.5	2.5	-	-

4.3.4 Results and discussion

4.3.4.1 *In vivo* activity of artemisone

The results of the drug formulations (A – D) and the drug free reference formulations (E and F) are given in table 4.9 and graphically depicted in figure 4.8. The variations in weight of the test animals during the course of the study are presented in table 4.10 and graphically depicted in figures 4.9 and 4.10.

Three mice (B2, C1 and D1) died during or shortly after the final treatment between days 1 – 5. They are believed to have sustained internal injuries, either during the infection procedure or during the treatment procedure since the % parasitaemia was not sufficiently high to be lethal. All the test animals presented with a marked decrease in weight during the first three days, this phenomenon may be attributed to stress which was induced by the infection and

treatment procedures. The initial decrease in weight was soon recovered and most of the test animals had returned to their initial recorded weight by day six.

Table 4.9: Average parasitaemia values in mice

T (days)	A			B			C		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
0	0	0	5	0	0	5	0	0	5
1	0.44	0.05	5	0.11	0.05	5	0.22	0.05	5
4	0.9	0.12	5*	0.37	0.07	5	0.53	0.08	4
5	0.29	9.1	5	0.07	0.04	4	1.09	0.35	4
9	1.56	1.02	5	1.83	0.59	4	6.39	1.93	4
12	2.27	1.04	5	3.99	1.4	4	8.83	1.09	3
16	2.12	1.96	4	5.32	1.95	4	22.45	1.45	3
18°	6.74	6.16	3	13.86	5.98	4	-	-	-
T (days)	D			E			F		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
0	0	0	5	0	0	5	0	0	5
1	0.44	0.1	5	0.12	0.03	5	0.3	0.1	5
4	0.76	0.2	4	1.76	0.15	4	2.06	0.46	5
5	0.32	0.02	4	11.78	2.13	4*	9.99	2.01	5
9	5.16	1.63	3	18.62	2.83	4	24.21	4.82	5
12	8.25	2.32	3*	24.71	2.07	4	30.63	2.28	4*
16	15.88	4.7	3	49.72	0.65	4	44.05	3.08	4
18°	-	-	-	-	-	-	-	-	-

n - Indicate the number of surviving animals at that point (T, days).

* - At least one slide was too thick to accurately quantify parasitaemia.

° - Parasitaemia values were determined only in the high-dose group to determine whether recrudescence occurred after treatment ceased.

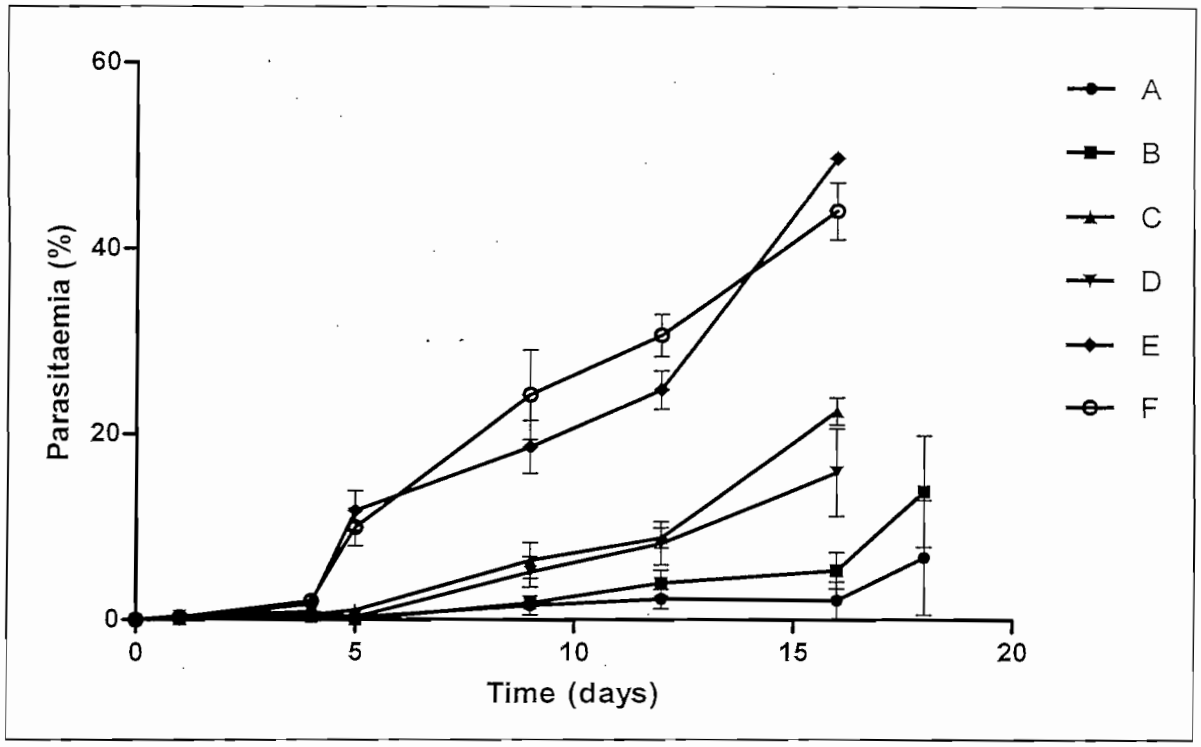

Figure 4.8: Results of the artemisone *in vivo* activity evaluation

Artemisone was effective in both the DMSO/water and Pheroid vesicle formulations. Formulations A and B (20 mg/kg) were both successful in reducing the parasitaemia. Both formulations were successful in keeping the parasitaemia at bay until day 16. The parasitaemia varied between 0.0% and 2.1% for A and between 0.0% - 5.3% for B. The parasitaemia increased notably after day 16 and the last parasitaemias were recorded on day 18. Group A presented with a mean parasitaemia of 6.7% and group B with 13.9%.

Formulations C and D (2.5 mg/kg) were less successful in reducing the parasitaemia. Similar results were obtained with C and D up until day 12. The recorded parasitaemia ranged between 0.0% and 8.8% for C and between 0.0% - 8.3% for D. Between day 12 and day 16 there was a notable increase in parasitaemia. Group C presented with a mean parasitaemia of 22.5% and group D with a parasitaemia of 15.9% on day 16.

As expected, formulations E (DMSO/water) and F (Pheroid vesicles) were not at all successful in reducing the parasitaemia. Between days 4 and 5 there was a significant increase in parasitaemia. In group E the parasitaemia increased from 1.8% on day 4 to 11.8% on day 5. The same was true for F, the parasitaemia was recorded as 2.1% on day 4

and then as 10.0% on day 5. The parasitaemia escalated at a steady pace from day 5 onwards, on day 16 group E had a parasitaemia of 49.7% and group F a parasitaemia of 44.1%.

The parasitaemia was only monitored up to day 18 for groups A and B (figure 4.8). The occurrence of recrudescence became very apparent on day 18 and it was clear that artemisone was no longer having any effect. The parasitaemia of groups C and D was only monitored until day 16. Recrudescence became very apparent at that point and it was not deemed necessary to monitor the parasitaemia any further. No animals in groups E and F survived beyond day 19 while virtually all the animals in groups A – D survived as far as day 26. The average weight of each group was also monitored and recorded for the duration of the study.

Table 4.10: Average weights (grams) during the artemisone *in vivo* activity study

T (days)	A		B		C	
	Mean	SEM	Mean	SEM	Mean	SEM
0	21.85	1.18	20.84	0.26	20.43	0.69
1	21.12	1.2	19.94	0.32	19.93	0.61
2	20.67	1.18	19.61	0.39	19.11	0.62
3	21.24	1.17	20.17	0.41	19.14	0.78
4	21.83	1.16	20.55	0.4	19.91	0.88
5	22.2	1.09	21.02	0.43	20.14	1.0
6	22.49	1.1	21.34	0.39	20.18	1.0
7	22.44	1.09	21.52	0.46	19.78	0.85
8	21.94	1.06	21.12	0.39	18.82	0.76
9	21.94	1.04	20.94	0.42	17.82	0.81
10	21.8	0.91	20.22	0.25	17.22	0.62
11	21.27	1.03	19.65	0.58	17.34	0.73
12	21.23	1.23	18.65	0.86	18.16	1.02
13	21.81	1.17	18.84	0.93	18.9	1.12
14	22.37	1.16	19.52	0.86	19.04	0.96
15	22.51	1.13	20.07	0.7	19.02	0.93
16	22.8	1.09	20.3	0.62	18.83	0.91
17	23.12	1.17	20.54	0.65	18.4	0.94
18	23.0	1.11	20.58	0.72	17.67	0.94
19	22.84	1.38	19.78	1.01	16.26	1.04

Table 4.10: Average weights (grams) during the artemisone *in vivo* activity study (continued)

T (days)	D		E		F	
	Mean	SEM	Mean	SEM	Mean	SEM
0	22.66	1.26	22.24	1.3	24.35	0.97
1	22.13	1.2	21.6	1.22	24.12	0.72
2	21.03	1.33	20.98	1.08	23.55	0.65
3	21.46	1.49	20.87	0.85	23.68	0.57
4	21.48	1.3	22.0	1.11	24.14	0.54
5	21.79	1.23	21.75	0.91	23.76	0.53
6	22.1	1.13	20.96	0.82	23.24	0.63
7	21.83	1.11	19.83	0.47	21.79	0.71
8	20.79	1.04	18.97	0.57	20.32	0.58
9	18.44	0.96	19.04	0.47	20.01	0.31
10	17.86	0.9	19.13	0.56	20.01	0.2
11	18.12	1.16	19.39	0.68	19.95	0.31
12	18.44	1.04	19.43	0.87	20.03	0.56
13	18.8	1.03	18.89	0.79	19.64	0.72
14	18.71	0.85	18.5	0.73	20.17	0.29
15	18.86	0.79	17.99	0.73	19.92	0.29
16	18.91	0.7	17.85	0.7	19.82	0.27
17	18.17	0.72	18.03	0.81	19.29	0.44
18	17.27	0.61	16.25	0.71	18.04	0.43
19	16.48	0.69	-	-	-	-

The average weights are presented in two separate graphs (figures 4.9 and 4.10) for clarity purposes. Both graphs contain the data derived from groups E and F for comparative purposes.

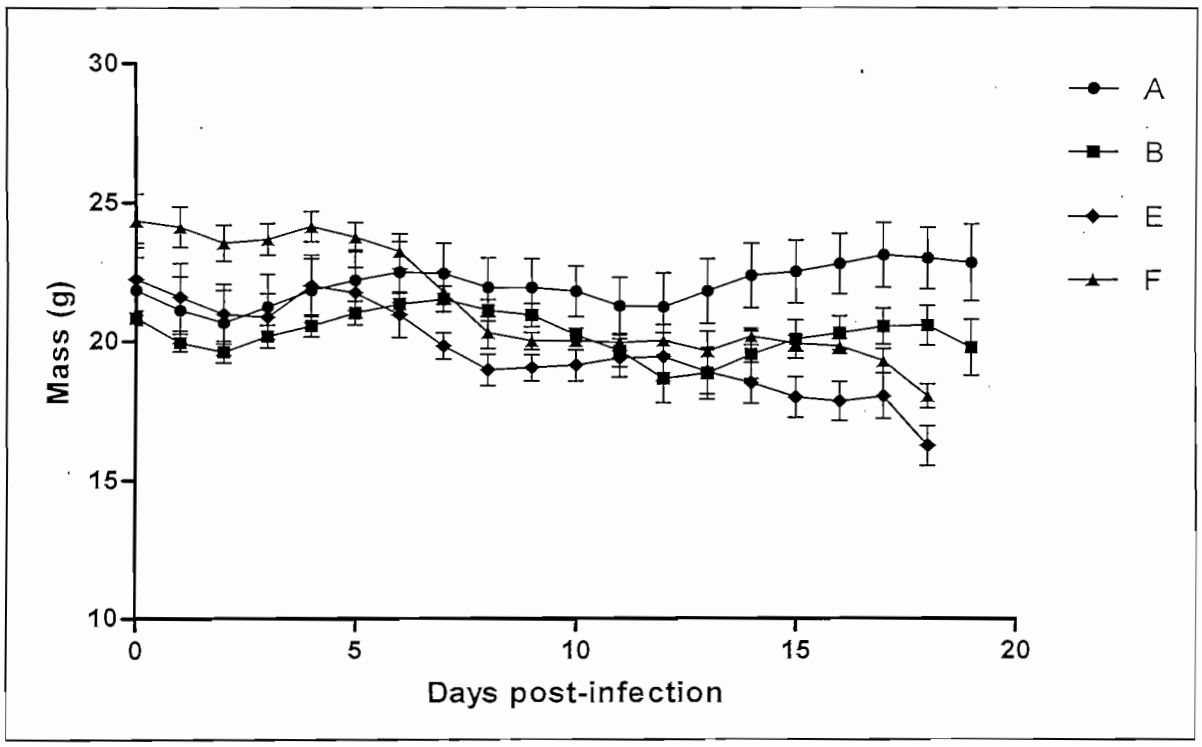

Figure 4.9: Average weights of groups A and B compared to that of groups E and F

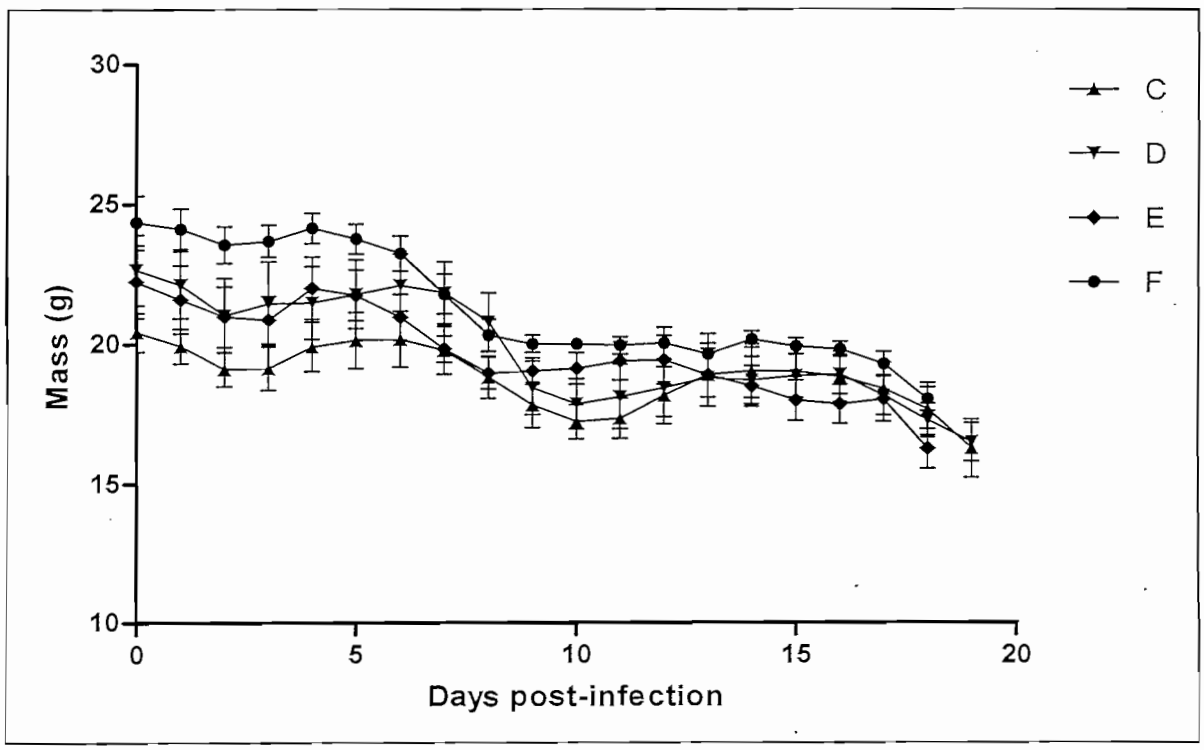

Figure 4.10: Average weights of groups C and D compared to that of groups E and F

From figure 4.9 it was obvious that the mean weight of all the groups remained fairly constant with only slight fluctuations up until about day 8. After day 8 it was apparent that the average weights of groups E and F were starting on a downward curve while the weights of groups A and B still remained relatively constant. On day 18 there was a definite difference in the mean weight of groups A and B in comparison to that of groups E and F.

In figure 4.10 the curves of groups C, D, E and F hardly diverge. All remained relatively constant up until day 8 after which there was a marked decrease in animal weight. The drop in animal weight was correlated to the increase in parasitaemia. As the parasitaemia increased the animals became more lethargic with a subsequent loss of appetite. As with the parasitaemia, the rate of change in weight increased once treatment was stopped. As anticipated, lower parasite loads (groups A and B) resulted in a smaller change in weight than in the groups with greater parasite loads (groups C, D, E and F).

4.3.4.2 Statistical analysis of artemisone results

A two-way analysis of variance (ANOVA) was performed on each treatment. Formulations A – F and the duration of the experiment (days) were the considered factors. As missing values occurred throughout the data these ANOVA's were performed by implementing mixed models. For these models the MIXED procedure of SAS (SAS Institute, Inc. 2003) was used. The significance of the treatment outcome with formulations A – F, together with their respective interactions, were determined from these ANOVA's, while estimates of the mean values of the percentages for each combination of formulation and day were also calculated from the fitted models.

When comparing the antimalarial activity of formulation A to that of formulation B, there was no significant difference (according to the Tukey-Kramer test $p > 0.05$). There was a significant difference when comparing formulations A and C ($p < 0.05$) while there was no significant difference when comparing formulations A and D ($p > 0.05$).

Formulations E (DMSO/water) and F (Pheroid vesicles) were the least effective against the *P. berghei* infection and there was a very significant difference between the activity of formulation A in comparison to formulations E and F (according to the Tukey-Kramer test $p < 0.0001$ in both instances). There was also a significant difference between the antimalarial activity of formulations B and C ($p < 0.05$). When comparing formulation B to formulations E and F there was also a significant difference ($p < 0.0001$ in both instances). Formulation C performed on par with formulation D ($p > 0.05$). There was a very significant difference

between the antimalarial activity of formulation C and that of formulations E and F ($p < 0.0001$ in both instances). Formulation D also performed much better than formulations E and F with $p < 0.0001$ for both comparisons. There was no significant difference in the antimalarial activity of formulation E in comparison to that of formulation F ($p > 0.05$).

4.3.4.3 *In vivo* activity of artemiside

The same procedures were followed during the artemiside *in vivo* activity study as with the artemisone activity study. The main difference was that artemiside was administered at 10.0 mg/kg and 2.5 mg/kg in contrast to artemisone which was administered at 20.0 mg/kg and 2.5 mg/kg. The reasoning behind this difference in drug dosage was based on the findings of Haynes *et al.* (2006) and on the *in vitro* results described earlier in this chapter. All the test animals survived the infection procedure and all the treatment procedures. On day 5 (one day after the final treatment) all test animals were still alive.

All the test animals presented with a marked decrease in weight during the first few days. The phenomenon may be attributed to stress which was induced by the infection and treatment procedures. This initial decrease in weight was soon recovered, most of the test animals had more or less returned to their initial recorded weight by day five. The results of the drug formulations (A – D) and the drug free reference formulations (E and F) are presented in table 4.11 and graphically depicted in figures 4.11 and 4.12. The variations in weight of the test animals during the course of the study are presented in table 4.12 and graphically depicted in figures 4.13 and 4.14.

Table 4.11: Average parasitaemia values in mice

T (days)	A			B			C		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
0	0	0	5	0	0	5	0	0	5
1	0.16	0.05	5	1.01	0.08	5	0.22	0.06	5
5	1.21	0.06	5	-	-	5°	1.59	0.38	5
8	1.97	0.38	4	1.60	0.44	5*	2.71	0.79	5
11	11.57	0.85	4	10.63	1.29	5	18.36	3.69	5
18	32.29	6.81	4	32.78	4.31	5	33.61	6.15	5
T (days)	D			E			F		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
0	0	0	5	0	0	5	0	0	5
1	0.42	0.07	5	0.4	0.16	5	0.28	0.07	5
5	0.69	0.15	5	1.82	0.53	5	3.01	0.28	5
8	2.39	0.38	5	4.27	0.58	4*	4.81	0.46	5
11	11.3	3.36	4	17.91	1.22	4*	17	4.39	5
18	29.1	3.02	3	39.61	1.22	4	49.99	3.93	3*

n - Indicate the number of surviving animals at that point (T, days).
* - At least one slide was too thick to accurately quantify parasitaemia.
° - Slides stained unevenly and could not be quantified accurately.

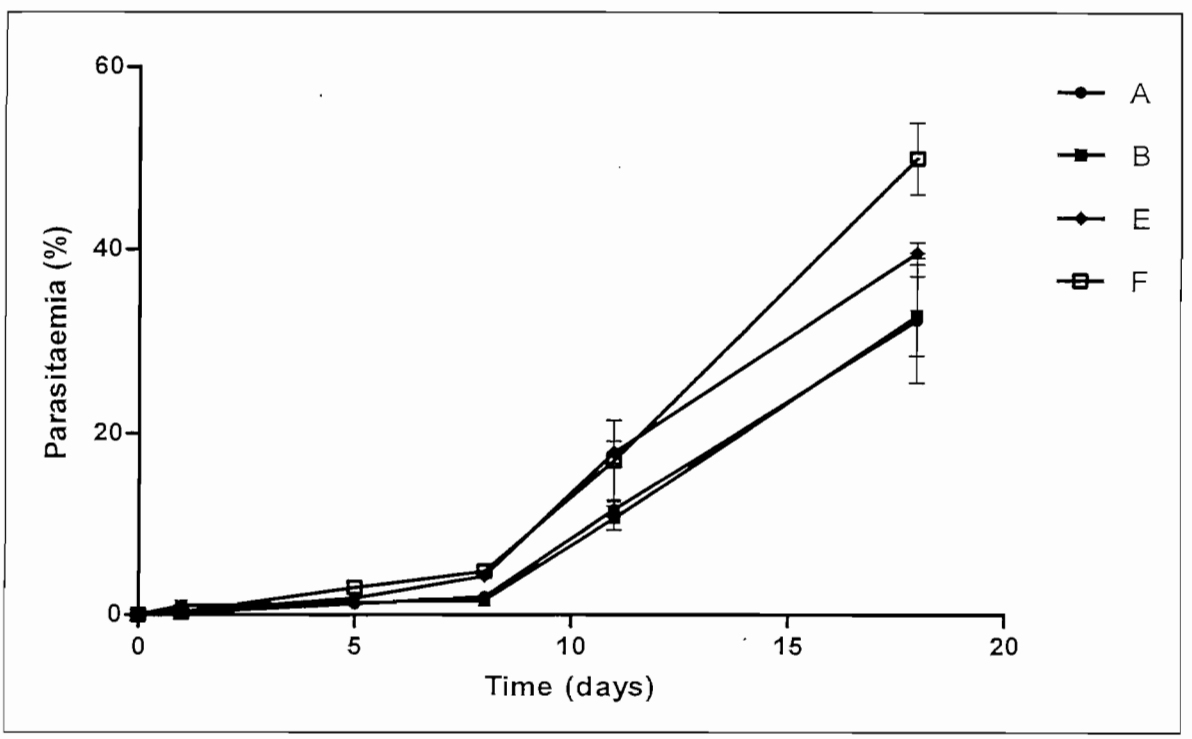

Figure 4.11: Results of the artemiside *in vivo* study (high dose groups A and B and the untreated reference groups E and F)

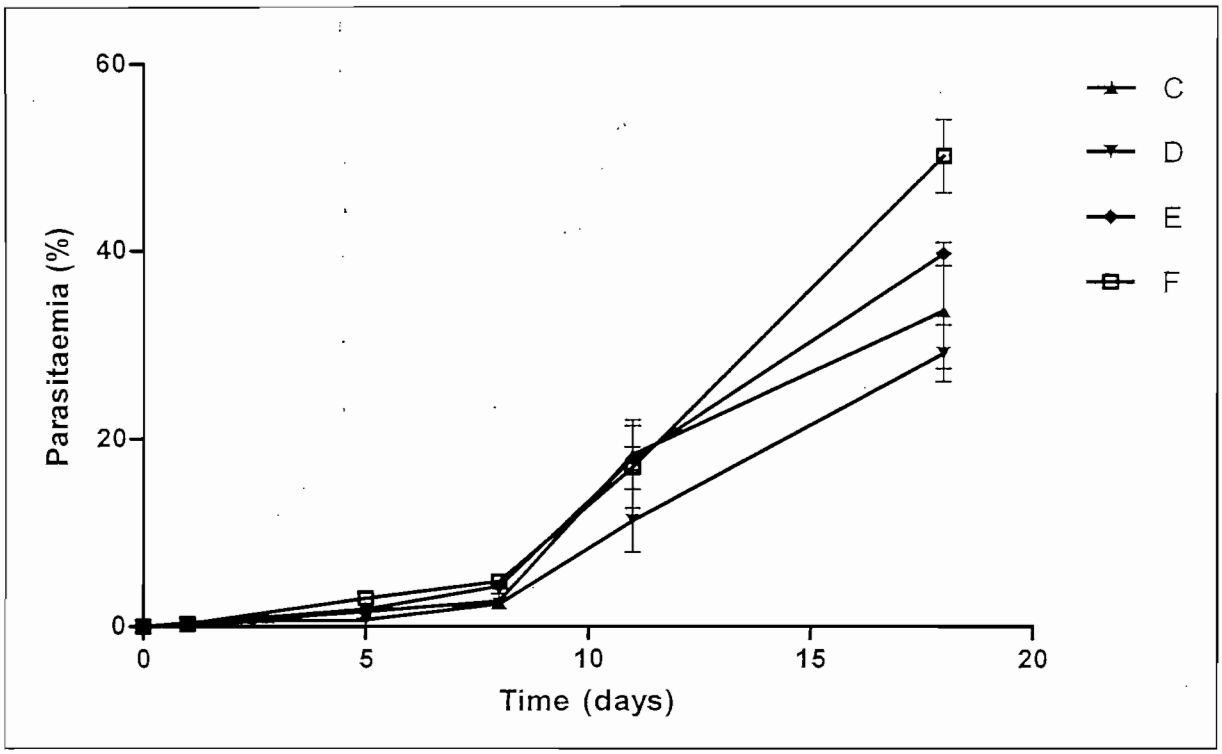

Figure 4.12: Results of the artemiside *in vivo* study (low dose groups C and D and the untreated reference groups E and F)

Artemiside was effective in both the DMSO/water and Pheroid vesicle formulations. Formulations A – D were all effective in keeping the parasitaemia at bay. There was virtually no difference between the high and low dose groups. Graphs of groups A, B, C and D hardly diverged during the course of the evaluation. The parasitaemia remained between 0.0% - 3.0% for groups A – D during the first 8 days. After day eight there was a gradual increase in parasitaemia.

After eleven days post infection the average parasitaemia was approximately 13.0% and the last recorded parasitaemia was approximately 32.0% at 18 days post infection. Parasitaemia was only monitored as far as 18 days post infection. At that time the parasitaemia was deemed to be increasing to such an extent where any remnant of the drug was clearly no longer having any effect. The untreated reference groups E and F had a parasitaemia of approximately 4.5% on day 8 and 17.5% on day 11. The last recorded parasitaemia for these groups were approximately 45.0% at 18 days post infection.

There seemed to be little difference between the DMSO/water and the Pheroid formulations in terms of mouse survival. There was also no significant difference between the high and the low dose groups in terms of parasite reduction or survival rates. It was interesting though

to note that in the low dose groups the parasitaemia was significantly higher on day 11 in the DMSO/water group than in the Pheroid group. In general the lower dose formulations (C and D) appeared to offer an increased chance of survival over the higher dose groups and the drug free reference groups. Three animals, one from the DMSO/water group (C) and two from the Pheroid vesicle group (D), survived up to three days longer than the animals included in the other groups (A, B, E, F). The average weight of each group was also monitored and recorded for the duration of the evaluation.

Table 4.12: Average weights (grams) during the artemiside *in vivo* activity study

T (days)	A			B			C		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
1	22.58	0.64	5	21.96	0.65	4	22.18	0.39	5
2	22.01	0.64	5	21.12	0.74	4	22.04	0.47	5
3	21.64	0.75	5	21.31	0.99	4	21.54	0.36	5
4	21.8	0.77	5	20.88	0.62	4	21.61	0.42	5
5	22.66	0.81	5	20.49	1.0	4	21.75	0.54	5
7	21.15	0.79	5	22.34	0.87	4	21.84	0.19	5
10	22.4	1.23	4	18.07	1.13	2	22.68	0.3	5
12	22.55	1.24	4	17.94	0	1	21.96	0.5	5
14	22.99	1.12	4	19.74	0	1	21.59	0.31	5
17	20.37	0.86	4	20.4	0.	1	19.82	0.25	5
22	17.78	0.54	4	18.54	0	1	17.23	0.48	3
26	-	-	-	-	-	-	16.13	0	1
T (days)	D			E			F		
	Mean	SEM	n	Mean	SEM	N	Mean	SEM	N
1	21.31	0.56	5	25.04	0.55	5	22.3	0.66	5
2	20.94	0.48	5	24.18	0.56	5	21.49	0.68	5
3	20.6	0.52	5	23.84	0.54	5	21.14	0.53	5
4	20.52	0.68	5	24.12	0.61	5	21.26	0.39	5
5	21.15	0.62	5	23.69	0.58	5	21.06	0.46	5
7	20.9	0.71	5	23.07	0.47	5	19.96	0.47	5
10	20.13	1.32	5	22.75	0.92	5	21.07	0.37	5
12	20.92	1.06	4	23.45	0.63	4	20.75	0.51	5
14	21.42	0.7	4	23.32	0.33	4	20.42	0.64	5
17	19.79	0.54	4	20.48	0.17	4	19.09	0.47	5
22	17.3	0.33	4	18.78	0.39	4	16.15	0.41	5
26	15.4	0.26	2	-	-	-	-	-	-

The average weights are presented on two separate graphs (figures 4.13 and 4.14) for clarity purposes. Both graphs contain the data derived from groups E and F for comparative purposes.

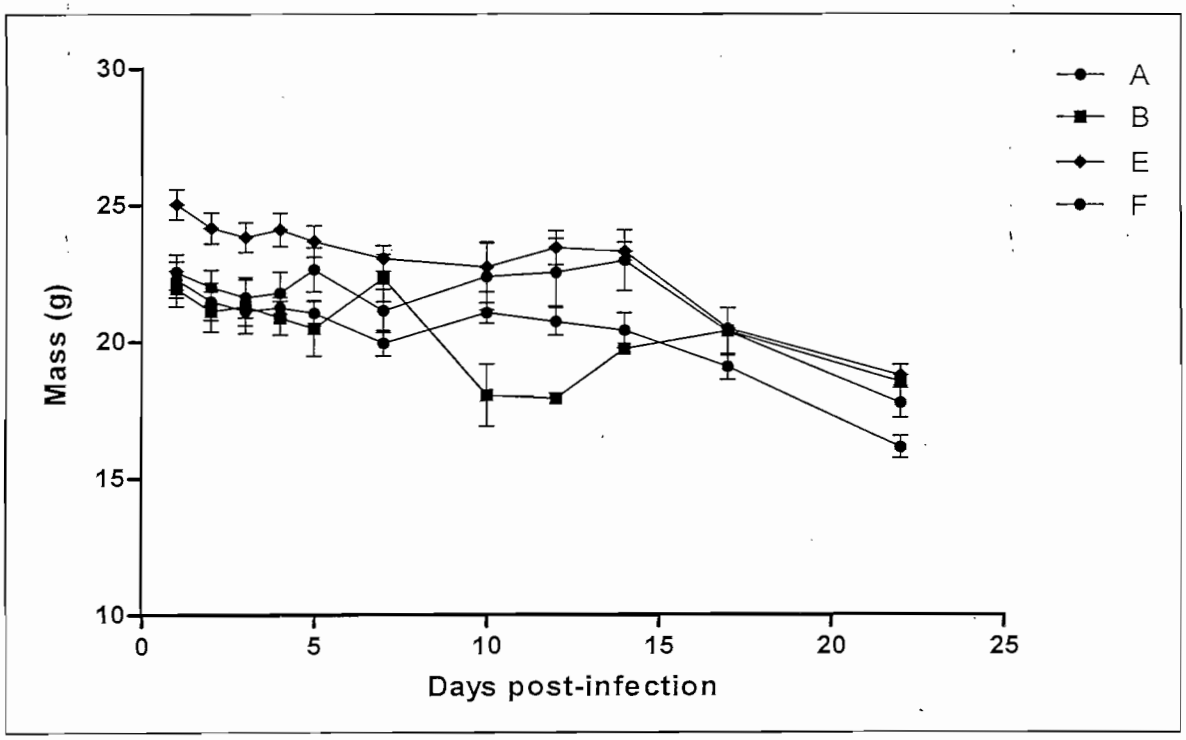

Figure 4.13: Average weights of groups A and B compared to that of groups E and F

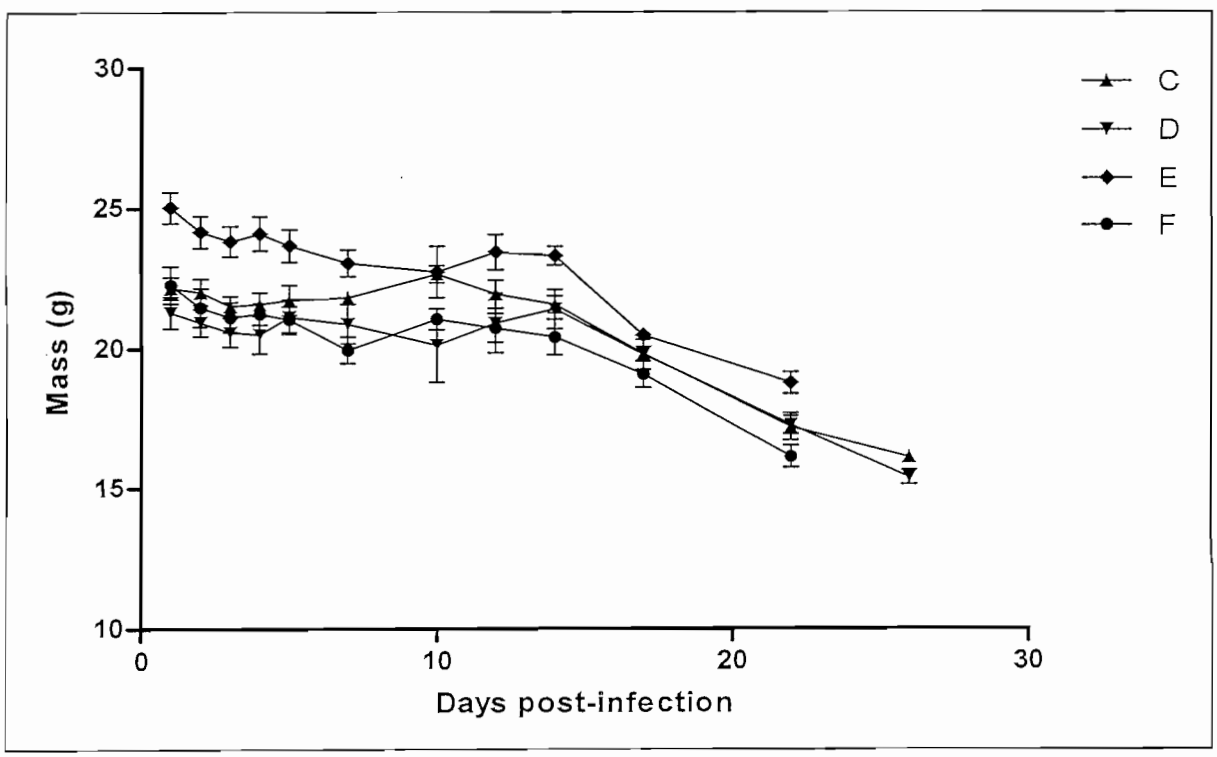

Figure 4.14: Average weights of groups C and D compared to that of groups E and F

Figures 4.13 and 4.14 clearly show that there was no significant difference in the basic configuration of curves of groups A – F. The average weight of group B showed an inexplainable drop in weight between days 7 and 14 but recovered towards day 22 and was at approximately the same average weight as the other groups at the end of the evaluation. Group E had a higher average starting weight than the other groups which apparently made it seem different from the other curves but in reality it did follow the same trend in weight reduction as the other groups. This drop in weight was correlated to the increase in parasitaemia. As the parasitaemia increased the animals became more lethargic with a subsequent loss of appetite. As with the parasitaemia, the rate of change in weight increased once treatment was stopped.

4.3.4.4 Statistical analysis of artemiside results

The same method of statistical analysis, as described for artemisone, was used to evaluate the *in vivo* antimalarial activity of artemiside. When comparing formulation A to formulation B there was no significant difference, the Tukey-Kramer test revealed that $p > 0.05$. The same trend held true when formulation A was compared to formulations C and D. The representative value for both comparisons were $p > 0.05$. Formulation A performed at least on par with formulation E and better than F. When comparing formulation A to E the Tukey-Kramer test revealed that $p > 0.05$ (0.055) while formulation A rendered a significant improvement in comparison to F with $p < 0.0001$. The results of formulation B were very similar to that obtained with formulation A. There was no statistical difference in the antimalarial activity of formulation B in comparison to that of formulation C ($p > 0.05$). The comparison of formulation B to D also rendered a $p > 0.05$. The same trend held true when formulation B was compared to E, the Tukey-Kramer test produced a p-value of $p > 0.05$. Formulation B did produce a significant improvement in antimalarial activity when compared to F ($p < 0.0001$). Formulation C did not produce a significant difference in activity when compared to D ($p > 0.05$). There was also no improvement when formulation C was compared to E ($p > 0.05$). Formulation C did, however, perform significantly better than formulation F and had a representative value of $p < 0.05$. Formulation D was more effective than formulations E and F. When comparing D to E the representative value was $p < 0.05$ and when comparing D to F the value was $p < 0.0001$. There was no significant difference when comparing formulation E to formulation F ($p > 0.05$).

4.3.4.5 Comparative discussion of *in vivo* antimalarial activity results

The *in vivo* antimalarial activity evaluation revealed that artemisone was more successful in reducing the parasite burden than artemiside. The high dose (20.0 mg/kg) artemisone formulations (A and B) were very successful in keeping the percentage parasitaemia at bay. Recrudescence became apparent at approximately 16 days post infection, and there was no significant difference between the reference (A) and Pheroid (B) formulations in terms of efficacy. Formulations C and D (2.5 mg/kg artemisone) were less effective than A and B but were still able to suppress parasite multiplication up until 12 days post infection. Recrudescence became very apparent between days 12 and 16. There was no significant difference between the low dose reference formulation (C) and the Pheroid formulation (D) in terms of efficacy. Formulations E and F were proven to have no significant effect on the parasite burden and the parasitaemia increased dramatically between 4 and 5 days post infection.

Artemiside was effective in suppressing parasite multiplication, but to a lesser extent than artemisone. Formulations A and B were both administered at a dose of 10.0 mg/kg. Both formulations were able to keep the parasite burden at bay until 8 days post infection. Recrudescence became apparent between 8 and 12 days post infection. There was no significant difference between the high dose reference formulation (A) and the Pheroid formulation (B) in terms of efficacy. The low dose formulations (2.5 mg/kg artemiside) performed equally well. Formulation C kept the parasitaemia at bay up until 8 days post infection, thereafter a very apparent increase in parasitaemia suggested the advent of recrudescence. Formulation D was also able to suppress parasite multiplication up until 8 days post infection, however, the advent of recrudescence was a bit less apparent than with formulation C. There was no apparent difference between the low dose reference formulation (C) and the low dose Pheroid formulation (D) in terms of efficacy. Formulations E and F were again proven to have no significant effect on the parasite burden. The parasite burden increased at a steady pace between 1 and 8 days post infection. A dramatic increase in parasitaemia was observed beyond 8 days post infection.

The occurrence of recrudescence was not surprising and had previously been described in the literature (Haynes *et al.*, 2006). This occurrence, following administration of artemisinin monotherapy, has previously been attributed to the rapid but incomplete absorption of these drugs. The relatively short plasma elimination half-life of this drug class was also considered to be an important factor in the occurrence of recrudescence (Duc *et al.*, 1994). In previous cases where monotherapy was deemed necessary it was recommended that a 7-day

regimen should be followed (WHO, 1998). Another study conducted by Giao *et al.* (2001) concluded that recrudescence was not reduced by extending the duration of artemisinin monotherapy to 7 days. Their findings suggested that other influences were responsible for the occurrence of recrudescence following artemisinin-based monotherapy.

The most likely explanation for the observed recrudescence during both the artemisone and the artemiside antimalarial activity studies may be linked to the occurrence of auto-induction of drug-metabolizing enzymes (Ashton *et al.*, 1997). The occurrence of auto-induction of drug-metabolizing enzymes together with the time-dependent pharmacokinetics of the artemisinin drug class may provide an explanation for the observed recrudescence during both evaluations. The Peters' 4-day suppressive test, which was used as a basis for the activity studies, may not have been sufficient to fully evaluate the potential antimalarial activity of the drugs. Auto-induction may have led to lower, subtherapeutic, levels of artemisone and artemiside towards the end of the 4-day treatment period in some test animals. The reduced drug levels may explain why radical parasite clearance was not achieved and why recrudescence occurred during both studies.

A decrease in drug levels may also translate to the emergence of drug resistance toward artemisinin-based monotherapy and highlights the importance of employing artemisinin-based combination therapy to achieve radical cure. Putative induction may also lead to increased metabolism of other drugs administered in combination regimens. Previous studies reported a decrease in mefloquine plasma levels after concomitant intake of either artesunate or artemether (Na-Banchang *et al.*, 1995; Karbwang *et al.*, 1994).

4.3.4.6 Summary

The evidence derived from this *in vivo* activity evaluation suggests that artemisone was more active against *Plasmodium* infections than artemiside in an *in vivo* model for malaria. Artemisone was more active at the high dose formulations than at the lower dose formulations while there was little to choose between the efficacy of the high and low dose formulations of artemiside. The addition of the compounds to a Pheroid vesicle formulation did not render a significant improvement in the *in vivo* antimalarial efficacy of either of the compounds. It also became evident that neither artemisone nor artemiside should be considered as monotherapy against *falciparum* malaria.

Combination therapy with a longer acting antimalarial drug such as mefloquine or piperaquine is the best approach to follow when treating uncomplicated *falciparum* malaria.

In the light of possible auto-induction of drug-metabolizing enzymes it may also be essential to critically review current recommended dosages and treatment intervals of artemisinin derivative-based combination therapies. Artemisone emerged as the most promising novel antimalarial compound during this *in vivo* efficacy evaluation and further efficacy studies is warrented to explore its full potential, not only as an antimalarial agent but also for other applications.

4.4 CONCLUSION

The *in vitro* activity results showed that artemisone and artemiside are very potent antimalarial compounds in comparison to artesunate. The incorporation of these novel artemisinin derivatives in Pheroid vesicles showed a further increase in their antimalarial activity. The *in vivo* activity results also showed that artemisone and artemiside are very active antimalarial compounds. In contrast to the *in vitro* activity results, the addition of the compounds to a Pheroid vesicle formulation did not render a significant improvement in the *in vivo* antimalarial activity of either of the compounds. This occurrence should be further investigated in future studies.

CHAPTER 5

BIOAVAILABILITY EVALUATION OF ARTEMISONE AND ARTEMISIDE

5.1 INTRODUCTION

Artemisone and artemiside were evaluated for their pharmacokinetic properties in a mouse model. The drugs were tested in both a reference formulation and a Pheroid vesicle formulation. The animals utilized were male C57 BL6 mice, weighing approximately 25 g each. The study and all procedures were evaluated and approved by the Ethics Committee of UCT, approval number 009/034.

5.2 EXPERIMENTAL DESIGN

The drugs were administered via the oral and intravenous routes and the exact procedures are described below:

- ***Artemisone***

Reference group (p.o.), (N = 10): Artemisone was administered orally at 50.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 5, 10, 30, 60 and 120 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at –20 °C.

Pheroid group (p.o.), (N = 10): Artemisone in Pheroid vesicles was also administered orally at 50.0 mg/kg. The same protocol was used as described for the oral reference group.

Reference group (IV), (N = 10): Artemisone was administered intravenously at 5.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 5, 10, 30, 60 and 120 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at –20 °C.

Pheroid group (IV), (N = 10): Artemisone, formulated in sterilized Pheroid vesicles, was also administered intravenously at 5.0 mg/kg. The same protocol was used as described for the IV reference group.

➢ *Artemiside*

Reference group (N = 10): Artemiside was administered orally at 50.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 10, 30, 60, 120 and 180 minutes after drug administration. The samples were centrifuged and 15 µl plasma was recovered and stored at –20 °C.

Pheroid group (N = 10): Artemiside in Pheroid vesicles was also administered orally at 50.0 mg/kg. The same protocol was used as described for the oral reference group.

Reference group (IV), (N = 5): Artemiside was administered intravenously at 5.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 10, 30, 60, 120 and 180 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at –20 °C.

Pheroid group (IV), (N = 10): Artemiside, formulated in sterilized Pheroid vesicles, was also administered intravenously at 5.0 mg/kg. The same protocol was used as described for the IV reference group.

5.3 INSTRUMENTATION

An Agilent 1200 series HPLC system and an API 3200 triple quadrupole mass spectrometer (Applied Biosystems) were used to perform the analysis on all the samples.

5.4 SAMPLE STORAGE

The reference standard, calibration standards and study samples (artemisone and artemiside) were all stored at -20 °C before analysis. No samples were stored for more than 7 days before performing the analysis.

5.5 METHODOLOGY: ARTEMISONE

A sensitive and selective LC/MS/MS method was developed to analyze the artemisone concentration in plasma samples collected from C57 BL6 mice.

5.5.1 Mass spectrometry

The detection of artemisone and artemisinin, internal standard (ISTD), was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode, MRM). The settings on the apparatus are summarized in tables 5.1 and 5.2. The Q1 (MS) spectrum of artemisone is presented in figure 5.1 and the Q3 (MS/MS) spectrum is presented in figure 5.2. The Q1 (MS) spectrum of the ISTD is presented in figure 5.3 and the Q3 (MS/MS) spectrum is presented in figure 5.4.

Table 5.1: ESI settings

Curtain gas	20
Collision gas	5
Ionspray voltage (V)	5000
Source temperature (°C)	500
Gas 1 (psi)	50
Gas 2 (psi)	60

Table 5.2: MS/MS settings

	Artemisone	ISTD
Q1 mass $[M+H]^+$	402.2	283.2
Q3 mass	163.2	151.1
Dwell time (ms)	150	150
Declustering potential (V)	46	26
Entrance potential (V)	2	9.5
Collision energy (V)	25	21
Collision cell exit potential (V)	4	4
Scan type	MRM	MRM
Polarity	positive	positive
Pause time (ms)	5	5

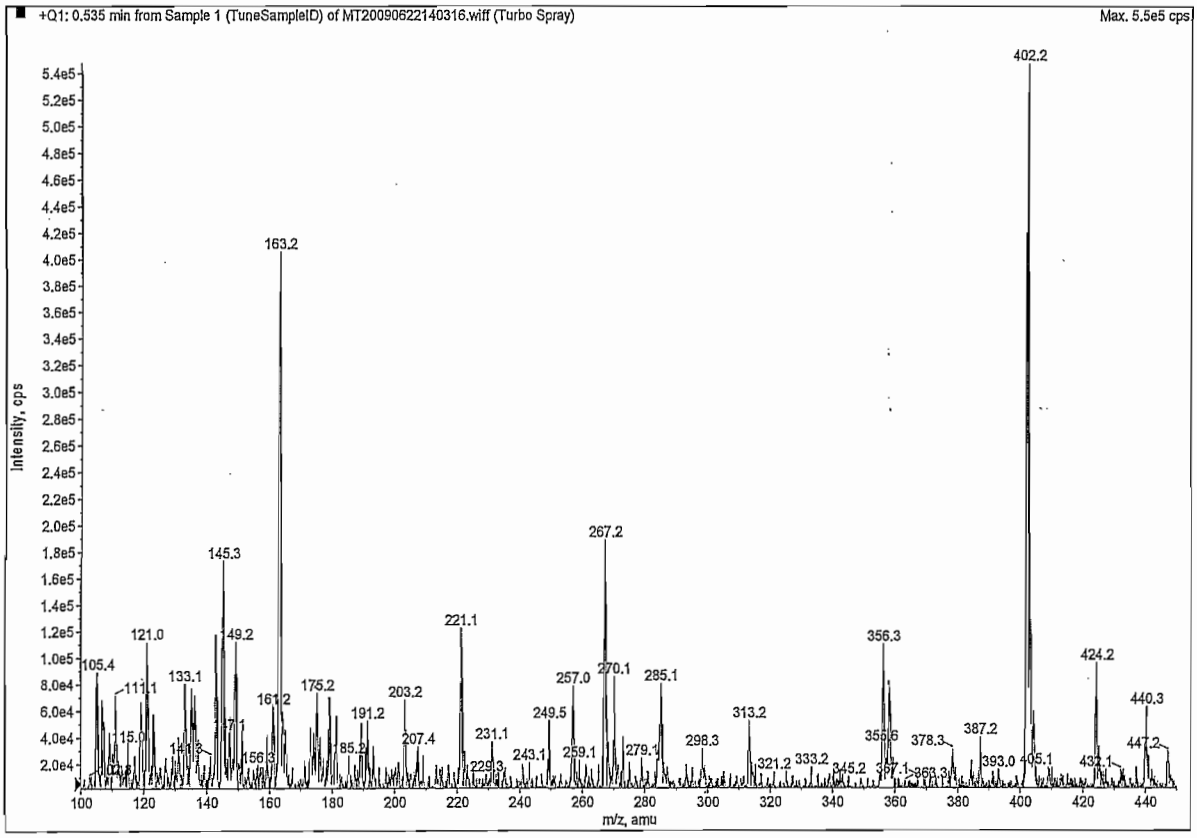

Figure 5.1: MS spectrum of artemisone

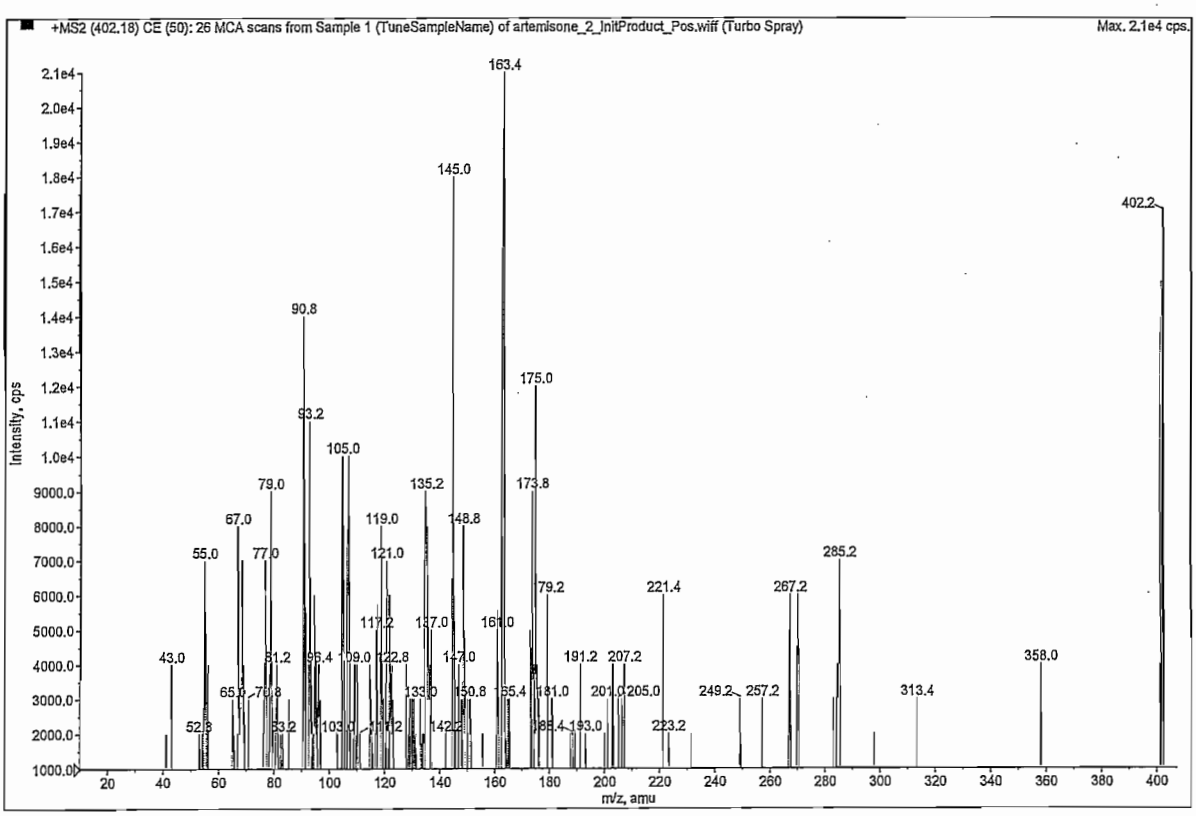

Figure 5.2: MS/MS spectrum of artemisone

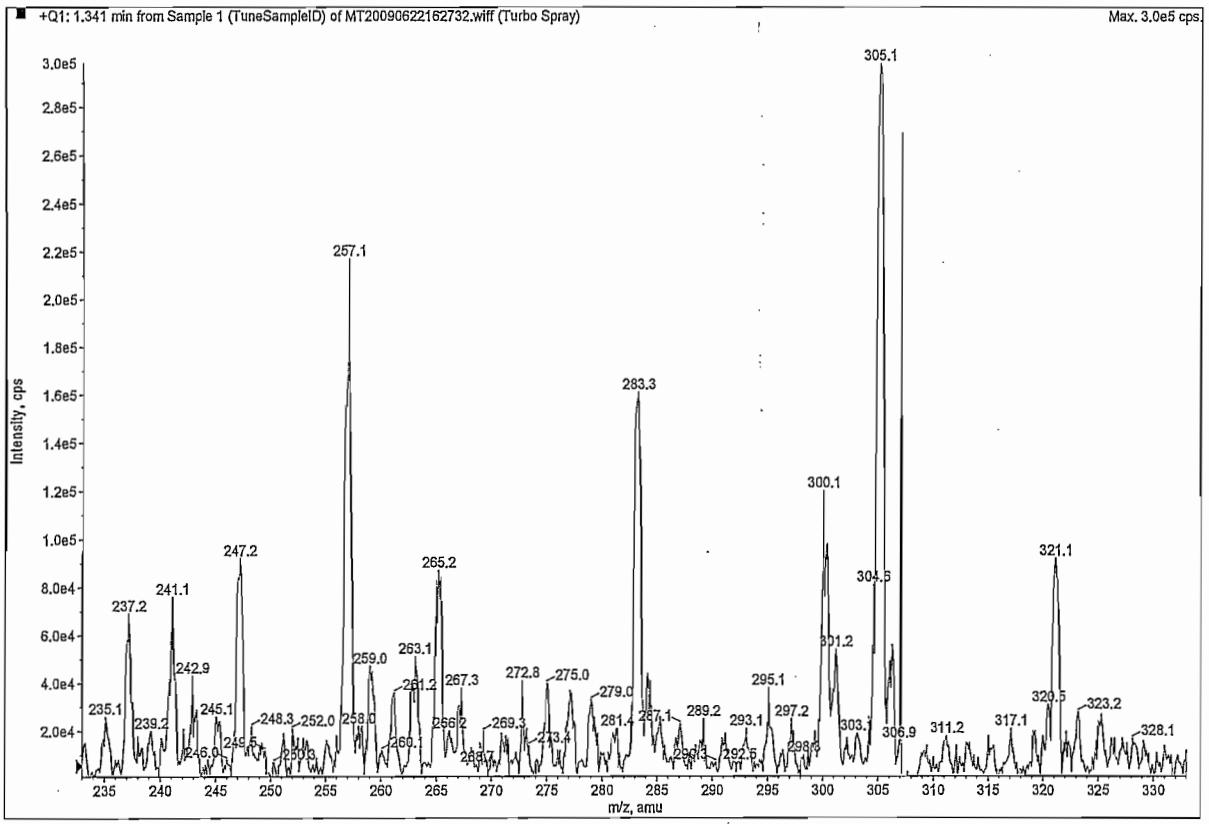

Figure 5.3: MS spectrum of the ISTD

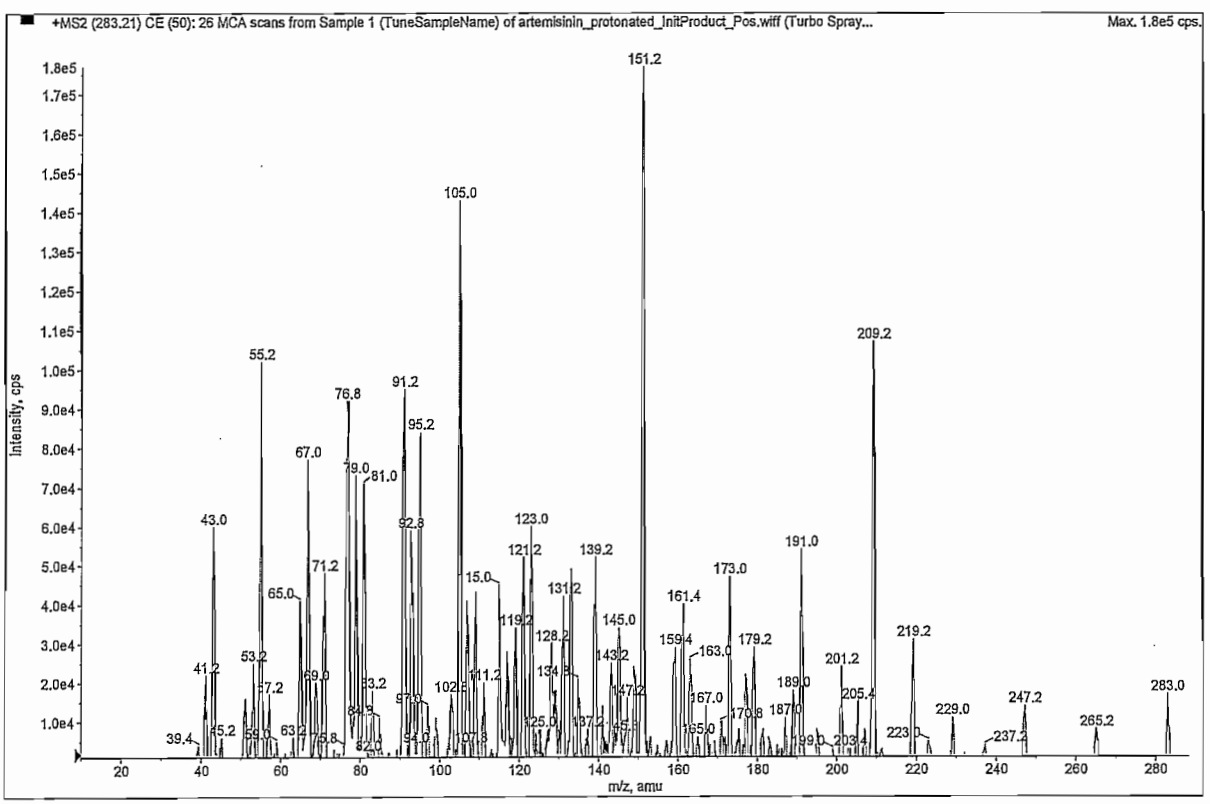

Figure 5.4: MS/MS spectrum of the ISTD

5.5.2 Chromatography

Chromatography was performed on a Phenomenex Gemini-NX (5µ, C18, 110A, 50x2 mm) analytical column using an Agilent 1200 series HPLC. The mobile phase consisted of methanol and ammonium acetate (10 mM with 0.1% acetic acid) (60:40) and was delivered at 0.5 ml/min for 3 minutes. The organic phase was increased after 3 minutes to 95% for another 2 minutes to clean the column and brought back to 60% organic for 3 minutes to equilibrate the column again. The column was kept in a column compartment at 35 °C. An autosampler injected 10 µl onto 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 cooled to 5 °C whilst awaiting injection. Representative chromatograms are presented in figures 5.5 (STD 5), 5.6 (Mouse 2, reference (p.o.), 120 minutes) and 5.7 (blank).

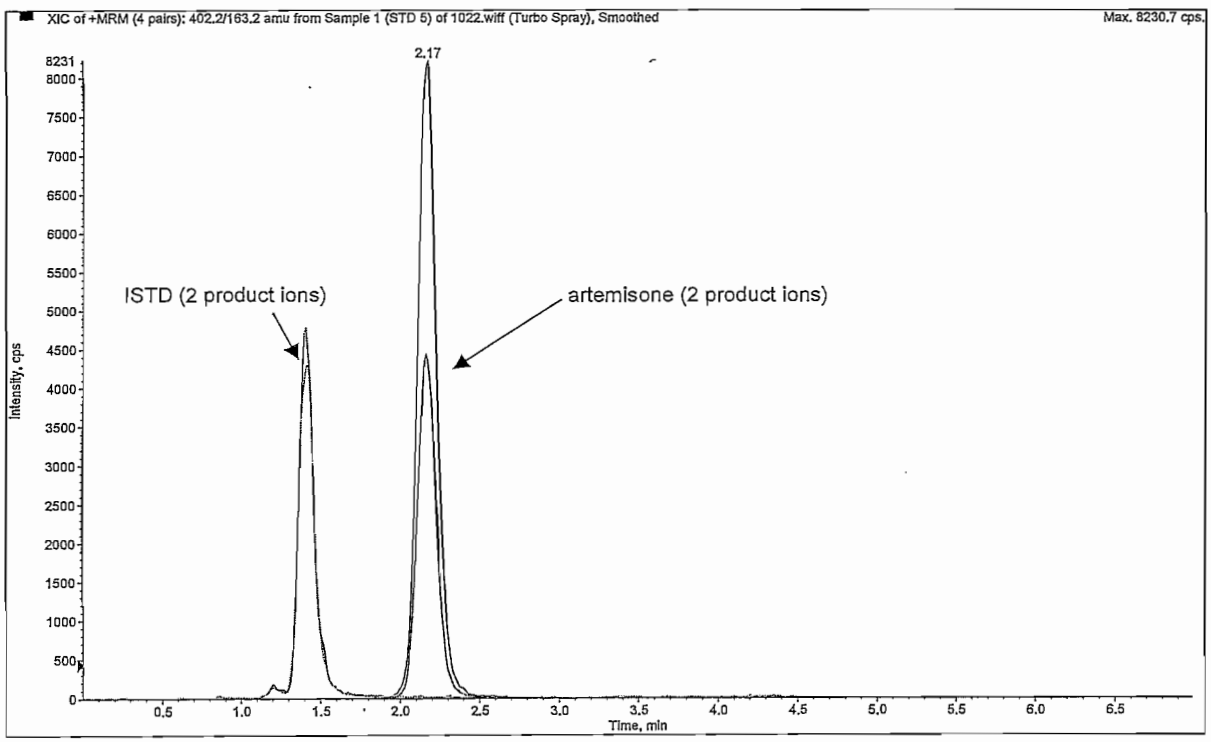

Figure 5.5: Representative chromatogram of STD 5 (500 ng/ml)

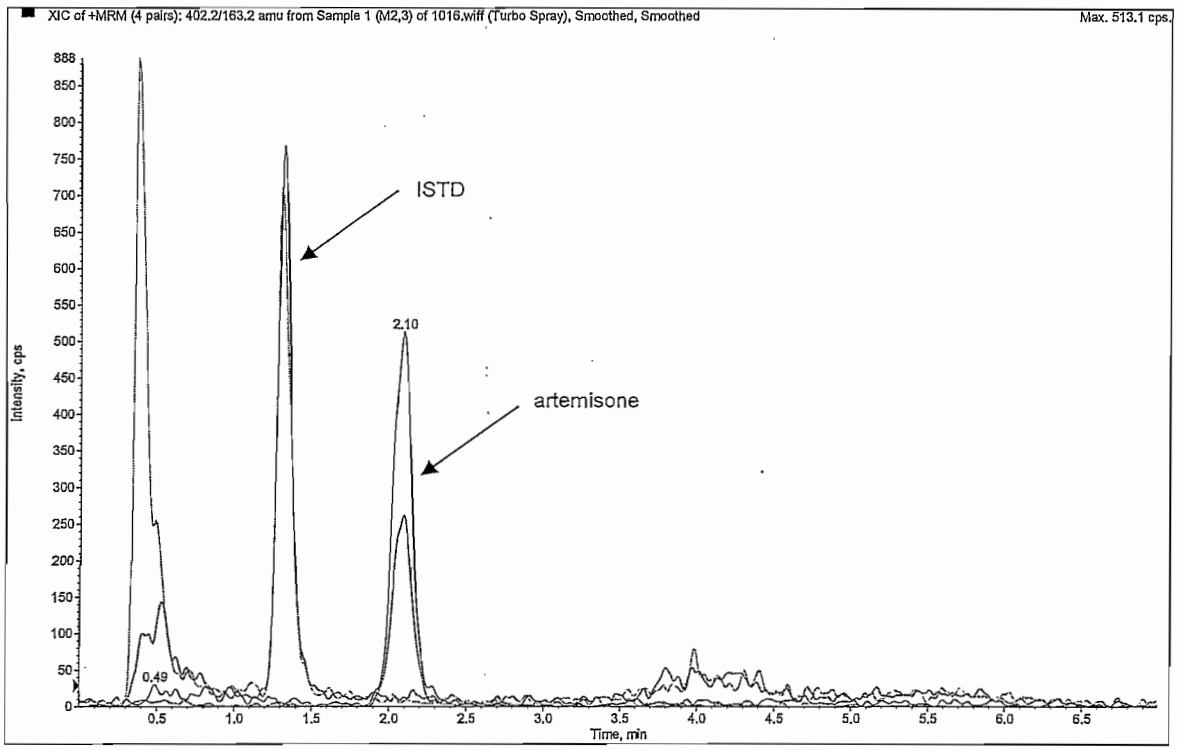

Figure 5.6: Representative chromatogram of Mouse 2 (reference [p.o.], 120 minute sample)

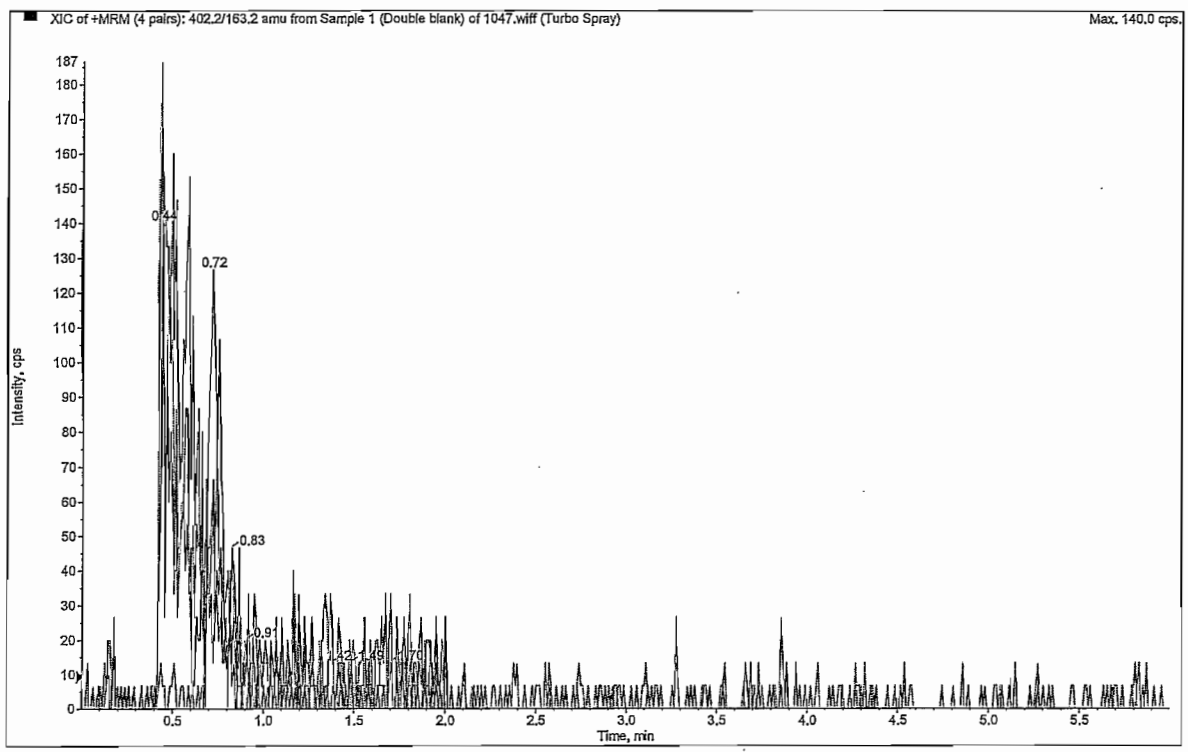

Figure 5.7: Representative chromatogram of a blank sample

5.5.3 Liquid-liquid extraction procedure

The extraction procedure was performed on ice using polypropylene test tubes. The plasma samples were thawed on ice and briefly vortexed. Twenty five microlitres of a universal Britton Robinson buffer (pH 9) was aliquotted into clean polypropylene tubes and 15 µl of the plasma sample was added. The ISTD was spiked at an appropriate concentration into the universal buffer and 25 µl was subsequently added to the extraction tubes. 1-Chlorobutane (350 µl) was also added to each tube to function as an organic solvent, the samples were vortexed for 1 minute and centrifuged for 5 minutes at high speed. The organic phase (300 µl) was transferred to clean polypropylene tubes and 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 (50:50, v/v), was added to the dry samples. The samples were then vortexed for 30 seconds and transferred to 96 well polypropylene plates. Ten microlitres of each sample was then injected onto the HPLC column and analyzed.

5.5.4 Preparation of calibration standards

A stock solution of artemisone was prepared in ethanol at a concentration of 1 mg/ml. Blank mouse plasma (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 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). The calibration standards were briefly vortexed, aliquotted into labelled polypropylene tubes, and stored at approximately -20 °C.

5.5.5 Pharmacokinetic parameters

The recovered experimental data was evaluated in terms of drug plasma concentration versus time. The following pharmacokinetic (*PK*) parameters were calculated:

- the peak drug plasma concentration (C_{max}) in ng/ml;
- time to peak plasma concentration (T_{max}), only for the oral dose experiments;
- apparent elimination half-life ($T_{1/2}$);
- area under the plasma concentration-time curve between time zero and the time of last sample collection ($AUC_{0\text{-}last}$) in ng.h/ml and

- area under the plasma concentration-time curve from time zero to infinity ($AUC_{0\text{-}inf}$); in ng.h/ml.

5.6 RESULTS: ARTEMISONE

5.6.1 Calibration curve

The calibration standards were analyzed in conjunction with the study samples. A representative calibration curve is presented in figure 5.8. The accuracy and precision statistics of this calibration curve are presented in table 5.3.

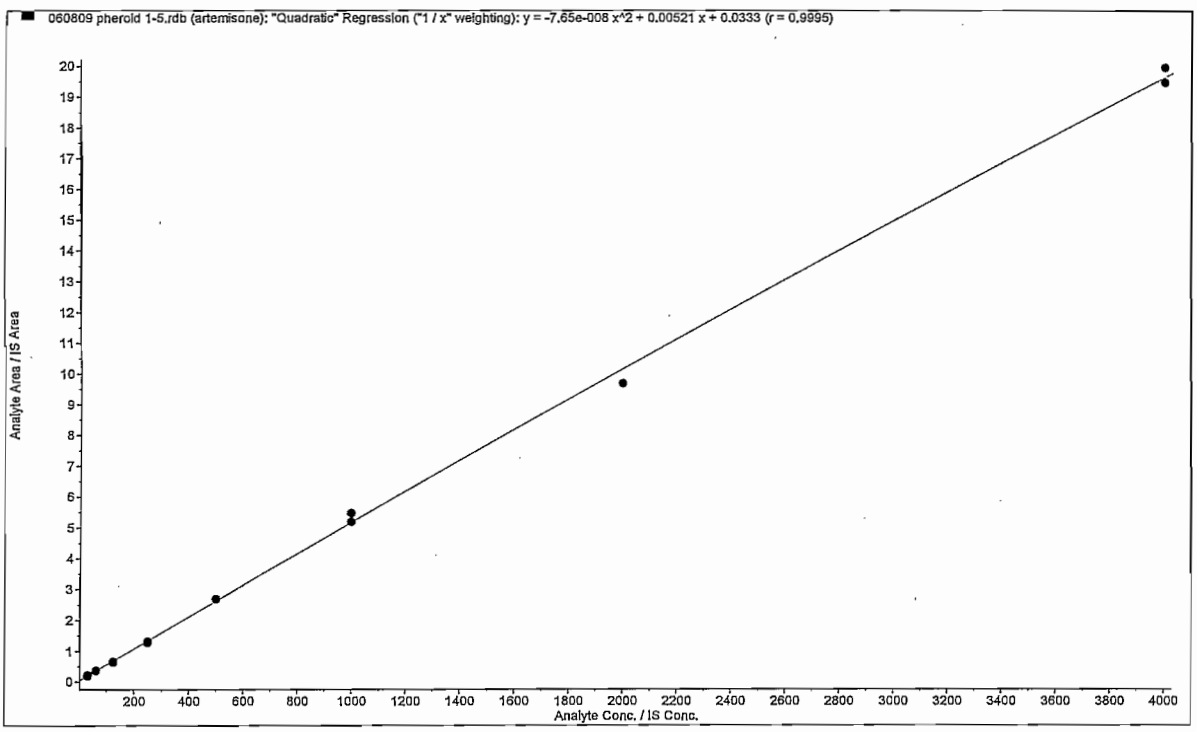

Figure 5.8: Representative calibration curve of artemisone

Table 5.3: Accuracy data of a representative calibration curve

Expected concentration (ng/ml)	STD	Mean (ng/ml)	% CV	% Accuracy
31.3	STD 9 (N=2)	32.3	15.1	103.3
62.5	STD 8 (N=2)	63.6	1.8	101.8
125	STD 7 (N=2)	118	3.3	94.1
250	STD 6 (N=2)	242	2.5	97.0
500	STD 5 (N=2)	513	0.1	102.5
1000	STD 4 (N=2)	1032	3.9	103.2
2000	STD 3 (N=1)	1906	N/A	95.3
4000	STD 2 (N=2)	4015	1.9	100.4

5.6.2 Artemisone reference (p.o.)

The samples were analyzed in two batches, the results are presented in table 5.4 and graphically depicted in figure 5.9 (N = 9).

Table 5.4: Plasma concentrations (ng/ml) of the artemisone reference group (p.o.)

Time (minutes)	0	5	10	30	60	120
Mouse 1	0	1300	802	63.6	64.9	BLQ
Mouse 2	0	680	829	479	122	BLQ
Mouse 3	0	664	1010	941	527	74
Mouse 4	0	708	961	876	498	BLQ
Mouse 5	0	473	466	124	77.1	BLQ
Mouse 6	0	530	607	468	73.6	BLQ
Mouse 7	0	1170	1240	1240	385	BLQ
Mouse 8	0	616	576	147	BLQ	BLQ
Mouse 9	0	649	609	363	129	BLQ
Mouse 10	0	410	333	98.6	BLQ	BLQ
Mean	0	720	743	480	235	74
SD	N/A	289	276	411	200	N/A

BLQ = Below the limit of quantitation.

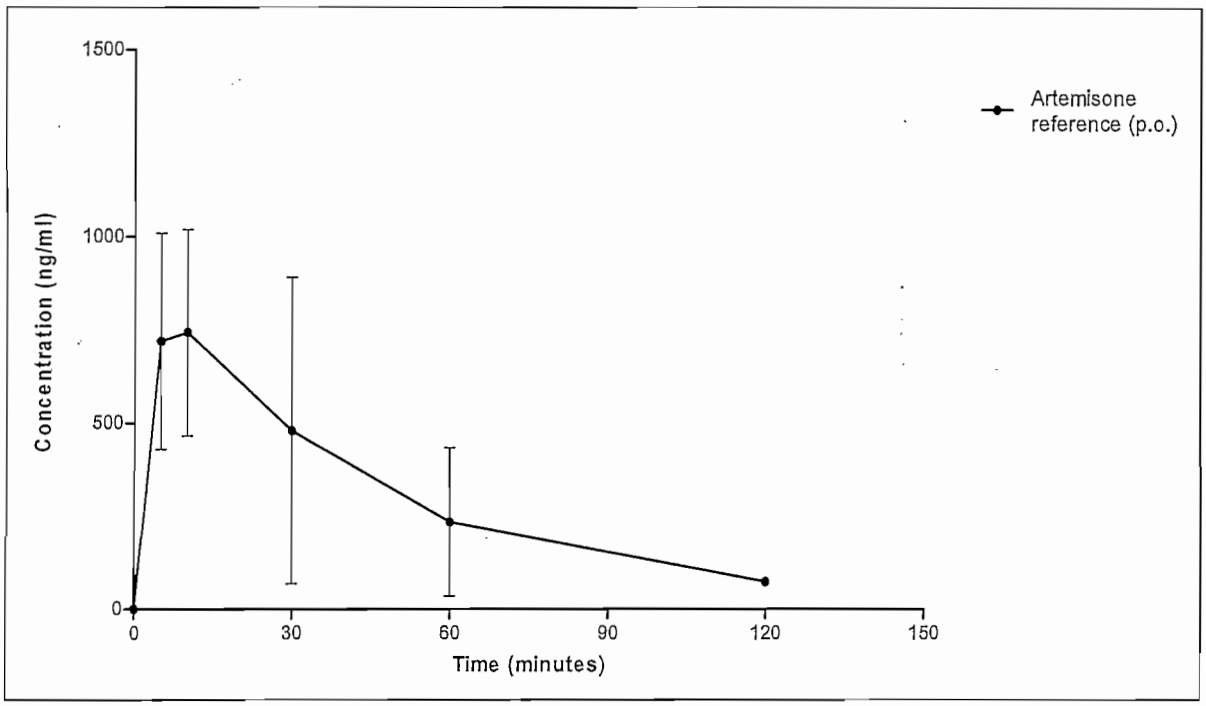

Figure 5.9: Mean concentration vs. time graph of the artemisone reference group

The first samples were collected just before administration, all of those tested negative for artemisone. Five minutes after administration the mean plasma concentration of artemisone was 720.0 ng/ml. At 10 minutes the recorded plasma concentration was 743.0 ng/ml. There was a gradual decrease in plasma concentration beyond 10 minutes, at 30 minutes the concentration was 480.0 ng/ml and 235.0 ng/ml at 60 minutes post administration. The last samples were collected at 120 minutes post administration with a mean drug concentration of 74.0 ng/ml.

5.6.3 Artemisone in Pheroid vesicles (p.o.)

The samples were analyzed in two batches, the results are presented in table 5.5 and graphically depicted in figure 5.10 (N = 10).

Table 5.5: Plasma concentrations (ng/ml) of artemisone in a Pheroid vesicle formulation

Time (Minutes)	0	5	10	30	60	120
Mouse 1	0	992	1200	1400	1160	498
Mouse 2	0	1500	1280	1640	1780	758
Mouse 3	0	*	1500	1520	733	55.3
Mouse 4	0	1880	1690	2150	985	267
Mouse 5	0	1310	1520	2020	1730	436
Mouse 6	0	839	978	1250	1110	851
Mouse 7	0	1220	1300	1570	1190	752
Mouse 8	0	826	931	1180	1150	525
Mouse 9	0	803	1040	1270	1240	501
Mouse 10	0	956	987	1360	*	1280
Mean	0	1147	1243	1536	1231	592
SD	N/A	366	263	325	333	339

* Extraction error

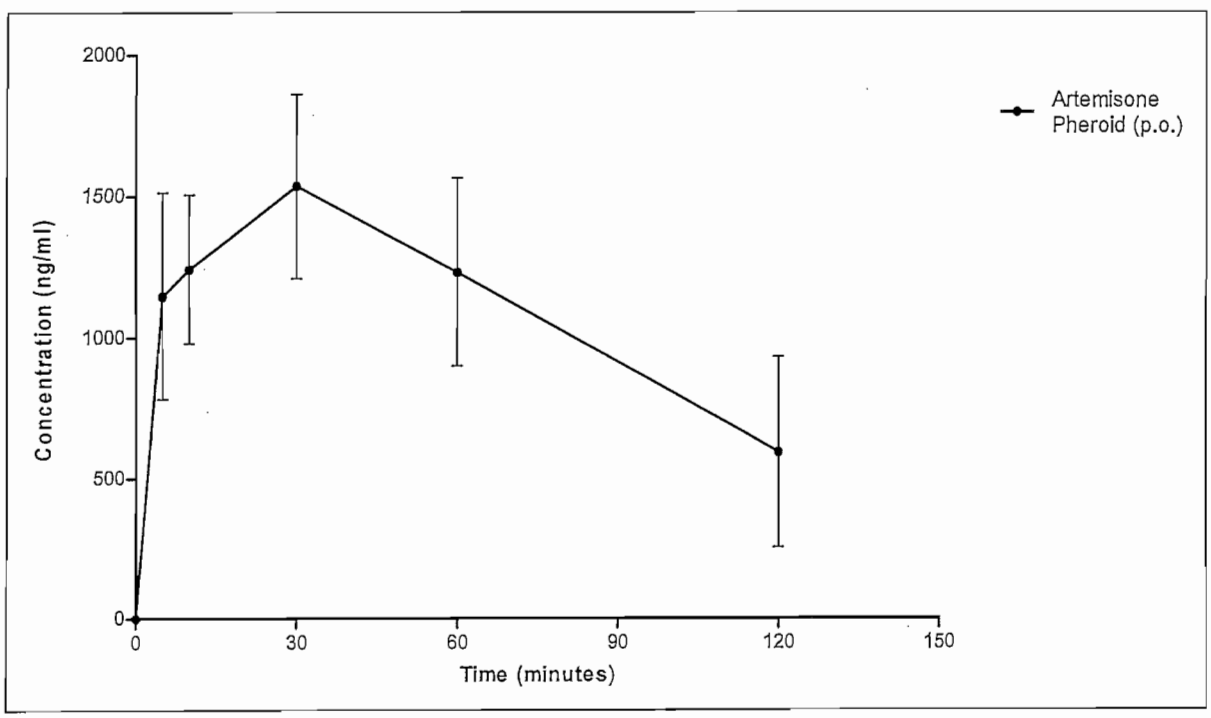

Figure 5.10: Mean concentration vs. time graph of artemisone in Pheroid vesicles

The oral artemisone in Pheroid vesicles rendered much improved results. The first samples were collected just before administering the vesicles, all those samples tested negative for artemisone. Five minutes after administration the recorded plasma concentration was 1147.0 ng/ml, almost twice the amount of the reference at the exact same time. At 10 minutes the recorded mean plasma concentration was 1243.0 ng/ml and was still on the increase. At 30 minutes after administration the peak concentration of 1536.0 ng/ml was reached, the concentration was approximately 3 times higher than that achieved by the reference at the same time point. A gradual decrease was observed after 30 minutes, a concentration of 1231.0 ng/ml was reached at 60 minutes post administration and at 120 minutes it had decreased further to 592.0 ng/ml

5.6.4 Artemisone reference formulation (IV)

The samples were analyzed in two batches, the results are presented in table 5.6 and graphically depicted in figure 5.11 (N = 10).

Table 5.6: Plasma concentrations (ng/ml) of the artemisone reference group

Time (minutes)	0	5	10	30	60	120
Mouse 1	0	1810	1270	306	271	49.9
Mouse 2	0	1400	1070	511	110	BLQ
Mouse 3	0	1440	1370	449	115	59.9
Mouse 4	0	1030	764	188	46.5	37.1
Mouse 5	0	1090	994	270	61.4	38.3
Mouse 6	0	563	371	104	61.7	BLQ
Mouse 7	0	651	448	239	77.6	31.7
Mouse 8	0	589	316	147	63.2	36.5
Mouse 9	0	439	233	134	53.0	35.6
Mouse 10	0	613	344	103	74.7	36.2
Mean	0	963	718	245	93.4	40.7
SD	N/A	466	430	142	66.4	9.39

BLQ = Below the limit of quantitation.

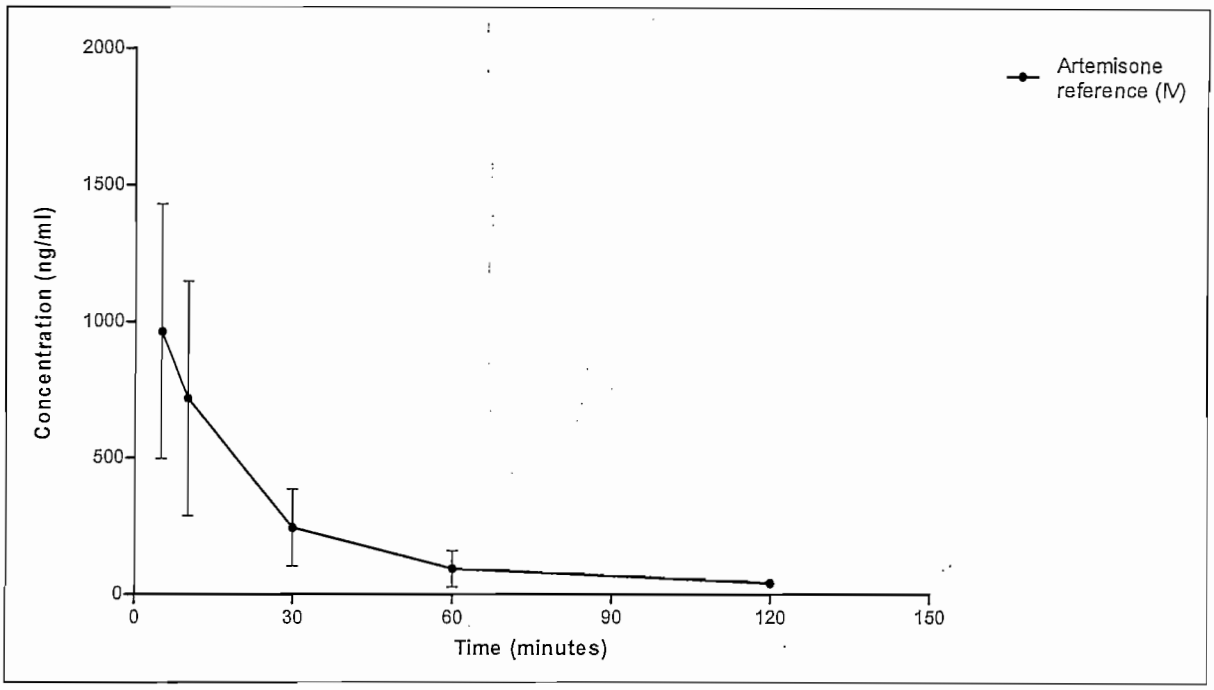

Figure 5.11: Mean concentration vs. time graph of the intravenous artemisone reference group

The intravenous artemisone reference formulation was injected at a dose of 5.0 mg/kg. The first samples were collected just before administration, all of those tested negative for artemisone. The 5 minute samples had a mean artemisone concentration of 963.0 ng/ml while the 10 minute samples had a mean concentration of 718.0 ng/ml. A further decrease was observed at 30 minutes with a concentration of 245.0 ng/ml. Beyond 30 minutes a more gradual decrease was observed, at 60 minutes the concentration was 93.4 ng/ml and 40.7 ng/ml at 120 minutes.

5.6.5 Artemisone in Pheroid vesicles (IV)

The intravenous reference formulation did not produce questionable results but it was suspected that the plasma levels could be increased by improving the solubility of the drug with the addition of a more favourable vehicle. Artemisone is a relatively lipophilic compound by nature and it was observed that both of the reference formulations formed a micro-suspension upon the addition of water to the DMSO-drug solutions. In order to address the solubility issues, artemisone was encapsulated in a sterilized Pheroid vesicle formulation and also administered intravenously. The samples were analyzed in two batches, the results are presented in table 5.7 and are graphically depicted in figure 5.12 (N = 10).

Table 5.7: Plasma concentrations (ng/ml) of artemisone in a Pheroid vesicle formulation

Time (minutes	0	5	10	30	60	120
Mouse 1	0	2180	1810	1460	927	651
Mouse 2	0	2030	1660	1140	861	415
Mouse 3	0	2130	1780	1660	1210	1090
Mouse 4	0	2280	1900	1780	1060	723
Mouse 5	0	2840	1970	1890	1220	732
Mouse 6	0	2120	1890	1680	1240	450
Mouse 7	0	5050	3280	2730	1990	1180
Mouse 8	0	3080	2230	1440	1150	122
Mouse 9	0	2850	2480	1840	1230	935
Mouse 10	0	5500	3870	2560	2230	1200
Mean	0	3006	2287	1818	1312	750
SD	N/A	1255	732	490	444	357

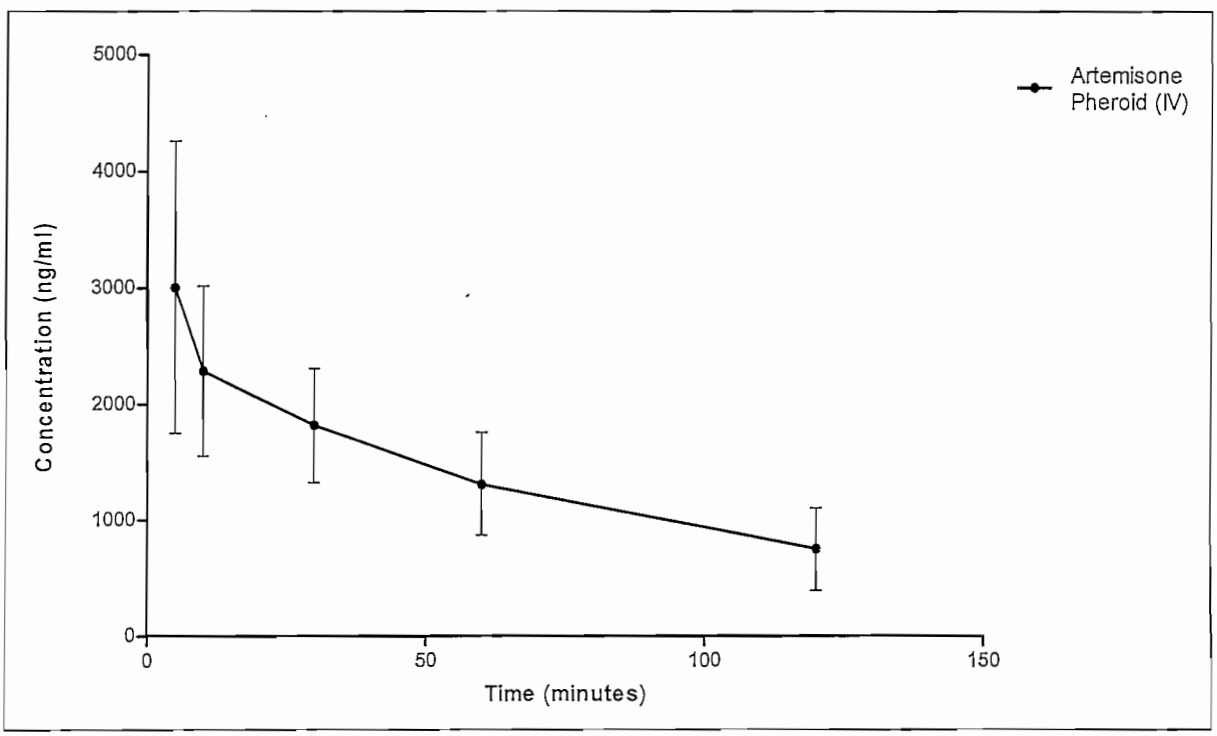

Figure 5.12: Mean concentration vs. time graph of artemisone in Pheroid vesicles

The intravenous artemisone Pheroid vesicle formulation was injected at a dose of 5.0 mg/kg. The first samples were collected just before administration, all of those tested negative for artemisone. The 5 minute samples had a mean artemisone concentration of 3006.0 ng/ml while the 10 minute samples had a mean concentration of 2287.0 ng/ml. A further decrease was observed at 30 minutes with a concentration of 1818.0 ng/ml. Beyond 30 minutes a more gradual decrease was observed, at 60 minutes the concentration was 1312.0 ng/ml and 750.0 ng/ml at 120 minutes.

5.7 STATISTICAL EVALUATION: ARTEMISONE

Noncompartmental analysis was used to calculate the *PK* parameters for artemisone in mice (WinNonlin version 5.2, Pharsight Corporation, California, USA). Linear interpolation was used to determine the area under the concentration time curve. $AUC_{0\text{-last}}$ is defined as the AUC computed from time zero to the time of the last Y-value above the lower limit of quantitation of the assay. All values below this limit were treated as "missing". $AUC_{0\text{-Inf}}$ 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 (λz). 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 4 (GraphPad Software Inc., California, USA).

5.7.1 Evaluation of oral formulations

The *PK* parameters of the oral reference and the oral Pheroid vesicle group were calculated, the results are presented in the form of an overlay of the mean concentration versus time graphs in figure 5.13. A summary of the pharmacokinetic data is presented in tables 5.8 and 5.9.

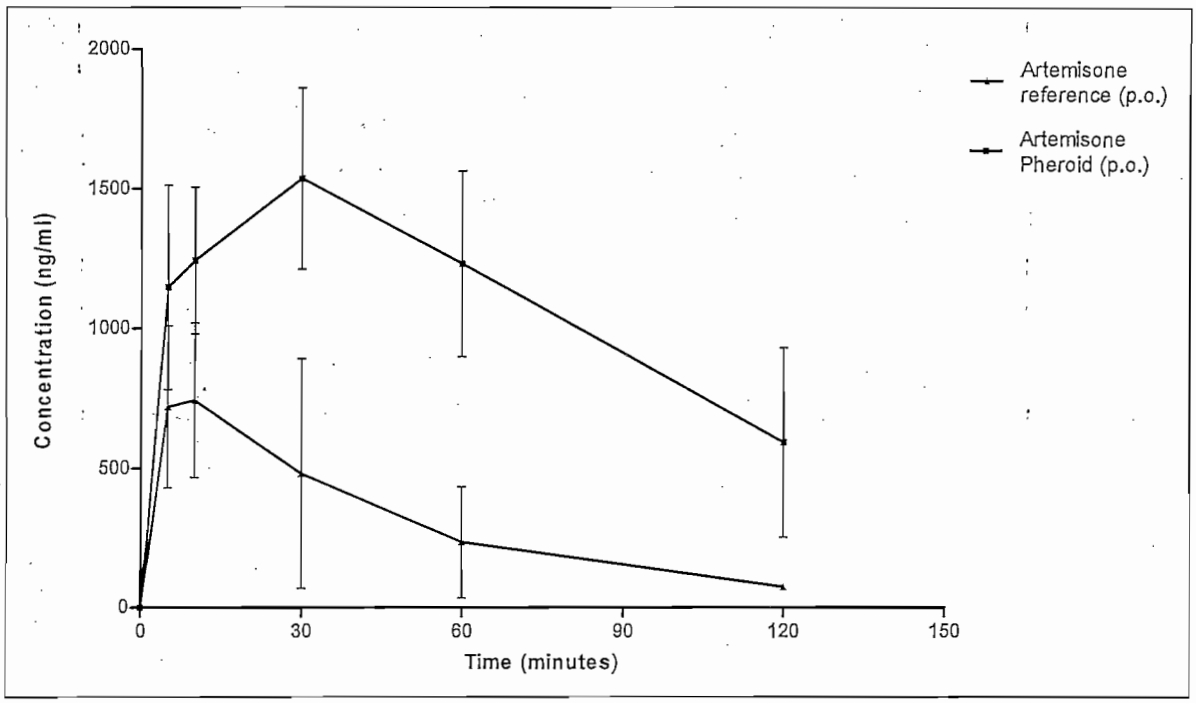

Figure 5:13: Mean concentration vs. time graph of the oral artemisone reference and Pheroid vesicle data

The incorporation of artemisone in a Pheroid vesicle formulation produced outstanding results. The differences in all the following values were scrutinized and subjected to the Mann-Whitney non-parametric test. A time delay in the T_{max} of artemisone, when incorporated in Pheroid vesicles, was obseved. When evaluating and comparing the T_{max} of the two data sets, the reference rendered a T_{max} of 7 minutes (0.12 hours) while the Pheroid vesicles increased the T_{max} to approximately 30 minutes (0.55 hours) ($p < 0.0001$). The T_{max} of artemisone, when incorporated in Pheroid vesicles, was extended by a factor of 4 when compared to that of the reference. The C_{max} was greatly increased by the Pheroid vesicles, the reference produced a C_{max} of 809.5 ng/ml while the Pheroid vesicles produced a C_{max} of 1550.0 ng/ml ($p < 0.005$). The increase in artemisone's C_{max} was calculated at approximately 90% with the aid of the Pheroid delivery system. The incorporation of artemisone in Pheroid vesicles also had a major impact on the $T_{1/2}$ of the drug, the reference had a $T_{1/2}$ of approximately 20 minutes ($T_{1/2}$ = 0.36 hours) while the Pheroid vesicles extended the $T_{1/2}$ to more than 60 minutes ($T_{1/2}$ = 1.10 hours) ($p < 0.005$). The increase in artemisone's $T_{1/2}$ was calculated at approximately 200% with the aid of the Pheroid delivery system. The difference amounts to an increase of more than 3-times compared to that of the reference.

The bioavailability of artemisone was also determined. The data obtained from the intravenously administered artemisone reference was required to determine the absolute bioavailability, the data is presented in table 5.6 and graphically depicted in figure 5.11. The relative bioavailability was determined by using the calculated arithmetic mean of the area under the curve ($AUC_{0\text{-last}}$) values of both the oral reference and the oral Pheroid vesicle data. There was a very significant difference between the two values (reference, 458.7 ng.h/ml and Pheroid, 2219.0 ng.h/ml) ($p < 0.0001$). The $AUC_{0\text{-inf}}$ for the reference was calculated as 604.6 ng.h/ml and 3094.0 ng.h/ml for the Pheroid vesicle formulation ($p < 0.0001$). The following equations were used to calculate a) the relative bioavailability and b) the absolute bioavailability:

$$\text{a)} \quad \text{Relative availability (RA)} = \frac{[AUC]_A}{[AUC]_B} \qquad \textit{Eq. 5}$$

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

$$\text{b)} \quad \text{Absolute availability (F)} = \frac{[AUC]_{po} * dose_{IV}}{[AUC]_{IV} * dose_{po}} \qquad \textit{Eq. 6}$$

where $[AUC]_{po}$ is represented by the arithmetic mean of the $AUC_{0\text{-last}}$ values of either the oral reference or the oral Pheroid vesicle formulation. The arithmetic mean of the $AUC_{0\text{-last}}$ values of the intravenous reference is represented by $[AUC]_{IV}$. The dose, IV and po, are respectively represented by the intravenous reference dose of 5.0 mg/kg and the oral dose of 50.0 mg/kg.

The relative bioavailability of the Pheroid vesicle incorporated artemisone was RA = 4.57 in comparison to the reference which was represented by RA = 1.00. These calculated values imply that the Pheroid vesicle entrapped artemisone was 4.57 times more bioavailable than the reference, or when converted to percentage values, reasoning that the reference is represented by 100%, the entrapped artemisone Pheroid vesicle was 357% more bioavailable. When calculating the absolute bioavailability (F) it was reasoned that an intravenous injection had a bioavailability of 100% since it was directly introduced into the blood, that scenario is represented by F = 1.00. The absolute bioavailability of the reference was calculated as F = 0.10. Artemisone entrapped in Pheroid vesicles performed much

better, the absolute bioavailability was calculated as F = 0.48. The difference translates to an astounding 4.80 factorial increase in the absolute bioavailability of artemisone.

5.7.2 Evaluation of intravenous formulations

An overlay of the results obtained with the intravenous artemisone reference and Pheroid formulations are presented in the form of an overlay of the mean concentration versus time graphs in figure 5.14.

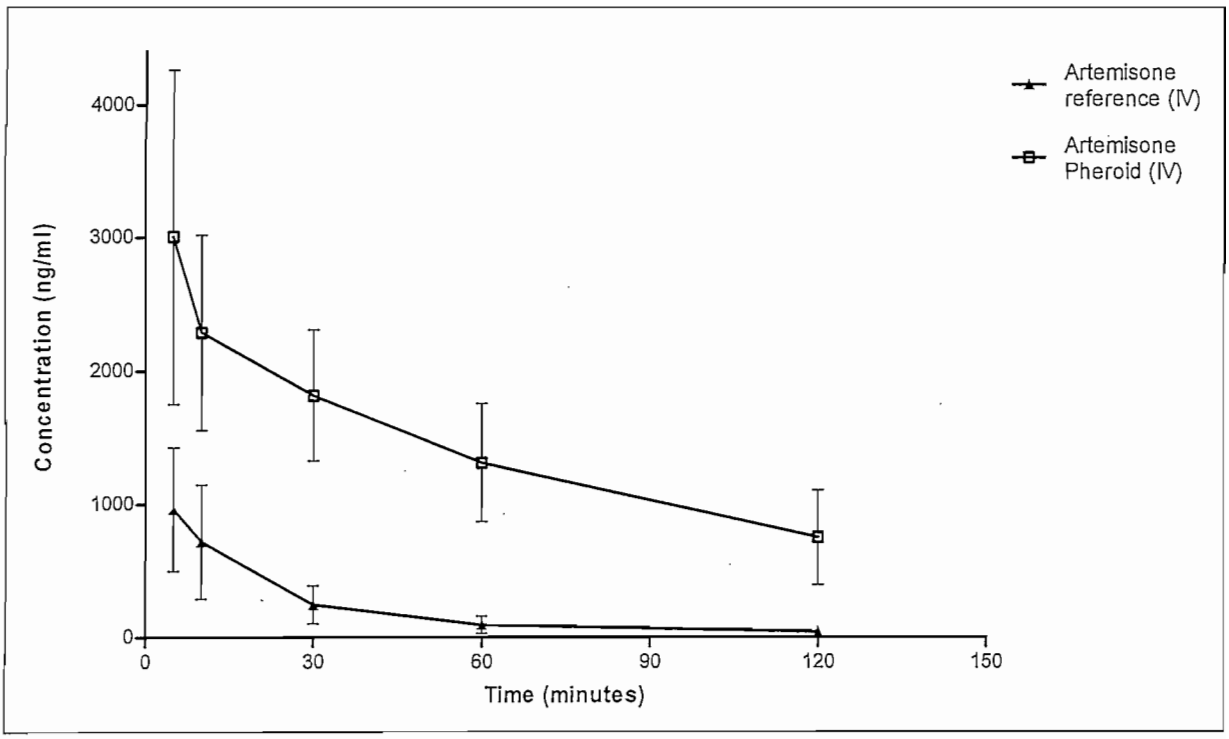

Figure 5:14: An overlay of the mean concentration vs. time graphs of the intravenous artemisone reference and Pheroid vesicle data

The incorporation of artemisone in a Pheroid vesicle formulation for intravenous administration produced outstanding results. The differences in all the following values were scrutinized and subjected to the Mann-Whitney non-parametric test. The C_{max} achieved by incorporating artemisone in a Pheroid vesicle formulation was significantly greater than that achieved by the reference. The reference produced a C_{max} of 962.5 ng/ml while the Pheroid vesicles produced a C_{max} of 3006.0 ng/ml ($p < 0.0001$). The increase in artemisone's C_{max} was calculated at approximately 210% with the aid of the Pheroid delivery system. The incorporation of artemisone in Pheroid vesicles also had a major impact on the $T_{1/2}$ of the

drug, the reference had a $T_{1/2}$ of approximately 30 minutes ($T_{1/2}$ = 0.49 hours) while the Pheroid vesicles extended the $T_{1/2}$ to more than 70 minutes ($T_{1/2}$ = 1.18 hours) ($p < 0.005$). The difference amounts to an increase of more than double the value achieved by the reference formulation. There was also a very significant difference when comparing the calculated AUC values. The reference group had an arithmetic mean $AUC_{0\text{-last}}$ value of 464.8 ng.h/ml while the Pheroid formulation had a value of 3009.0 ng.h/ml ($p < 0.0001$). The calculated $AUC_{0\text{-Inf}}$ values also reflected the same significant difference that was observed in the mean $AUC_{0\text{-last}}$ value, the reference was represented by an arithmetic mean $AUC_{0\text{-Inf}}$ value of 496.0 ng.h/ml while the Pheroid formulation had a value of 4431.0 ng.h/ml ($p < 0.0001$).

Table 5.8: **Pharmacokinetic summary. Artemisone reference administration = 50.0 mg/kg (p.o.) and artemisone Pheroid vesicle administration = 50.0 mg/kg (p.o.)**

Subject	T_{max} (h)		C_{max} (ng/ml)		$T_{1/2}$ (h)		$AUC_{0\text{-}last}$ (ng.h/ml)		$AUC_{0\text{-}inf}$ (ng.h/ml)	
	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid
1	0.083	0.5	1300	1400	0.206	0.974	317.9	2035	337.2	2735
2	0.166	1.0	829	1780	0.297	-	459.5	2789	511.8	-
3	0.166	0.5	1010	1520	0.400	0.306	1090.3	1586	1133	1611
4	0.166	0.5	961	2150	0.851	0.503	748.9	2277	1360	2471
5	0.083	0.5	473	2020	0.327	0.646	207.4	2783	243.8	3190
6	0.166	0.5	607	1250	0.264	2.690	384.1	2053	412.2	5356
7	0.166	0.5	1240	1570	0.470	1.426	969.0	2296	1300	3842
8	0.083	0.5	616	1180	0.191	1.206	195.8	1880	236.3	2793
9	0.083	0.5	649	1270	0.369	1.049	364.5	1994	433.2	2752
10	0.083	0.5	410	1360	0.199	-	119.9	2492	148.2	-
Arithmetic Mean	0.1245	0.55	809.5	1550	0.358	1.100	485.7	2219	604.6	3094
Geometric Mean	0.1174	0.5359	756.1	1520	0.322	0.910	388.7	2188	467.5	2934
Lower 95% CI (Arithmetic)	0.09321	0.4369	587.5	1312	0.2171	0.4794	245.2	1941	279.2	2167
Upper 95% CI (Arithmetic)	0.1558	0.6631	1031	1788	0.4978	1.721	726.3	2496	929.9	4021
SEM	0.01383	0.0500	98.12	105.4	0.06203	0.2624	106.3	122.7	143.8	392.1
%CV	35.14	28.75	38.33	21.49	54.88	67.48	69.23	17.49	75.22	35.85
Mann-Whitney non-parametric test										
	$p < 0.0001$		$p = 0.0001$		$p = 0.0021$		$p < 0.0001$		$p < 0.0001$	

Table 5.9: **Pharmacokinetic summary. Artemisone reference administration = 5.0 mg/kg (IV) and artemisone Pheroid vesicle administration = 5.0 mg/kg (IV)**

Subject	Tmax (h)		Cmax (ng/ml)		$T_{1/2}$ (h)		$AUC_{0\text{-last}}$ (ng.h/ml)		$AUC_{0\text{-inf}}$ (ng.h/ml)	
	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid
1	N/A	N/A	1810	2180	0.397	1.237	877.9	2296.9	906.5	3458.9
2	N/A	N/A	1400	2030	0.253	1.017	655.9	1946.3	696.1	2555.0
3	N/A	N/A	1440	2130	0.400	2.112	771.4	2798.4	806.0	6118.8
4	N/A	N/A	1030	2280	0.396	1.175	434.2	2597.7	455.4	3823.2
5	N/A	N/A	1090	2840	0.381	1.213	525.1	2885.5	546.1	4167.0
6	N/A	N/A	563	2120	0.291	0.772	218.3	2524.3	244.3	3025.6
7	N/A	N/A	651	5050	0.446	1.239	360.4	4646.6	380.8	6755.6
8	N/A	N/A	589	3080	0.617	0.439	287.3	2421.1	319.8	2498.4
9	N/A	N/A	439	2850	0.689	1.213	232.8	3046.8	268.2	4683.4
10	N/A	N/A	613	5500	0.989	1.329	285.0	4927.8	336.7	7228.9
Arithmetic Mean	N/A	N/A	962.5	3006	0.4860	1.175	464.8	3009	496.0	4431
Geometric Mean	N/A	N/A	866.3	2818	0.4488	1.099	415.1	2885	449.9	4143
Lower 95% CI (Arithmetic)	N/A	N/A	612.6	2176	0.3353	0.8216	290.2	2336	323.1	3143
Upper 95% CI (Arithmetic)	N/A	N/A	1225	3648	0.6007	1.470	593.9	3564	626.5	5462
SEM	N/A	N/A	147.2	396.8	0.06987	0.1350	74.17	312.9	74.07	545.3
%CV	N/A	N/A	48.37	41.74	45.46	36.35	50.46	32.88	47.23	38.91
Mann-Whitney non-parametric test										
	N/A		$p < 0.0001$		$p = 0.0002$		$p < 0.0001$		$p < 0.0001$	

5.8 METHODOLOGY: ARTEMISIDE

A sensitive and selective LC/MS/MS method was developed to analyze the artemiside concentration in plasma samples collected from C57 BL6 mice.

5.8.1 Mass spectrometry

The detection of artemiside and artemisinin, internal standard (ISTD), was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode, MRM). The settings on the apparatus are summarized in tables 5.10 and 5.11. The Q1 (MS) spectrum of artemiside is presented in figure 5.14 and the Q3 (MS/MS) spectrum is presented in figure 5.15. The Q1 (MS) spectrum of the ISTD is presented in figure 5.16 and the Q3 (MS/MS) spectrum is presented in figure 5.17.

Table 5.10: ESI settings

Curtain gas	20
Collision gas	5
Ionspray voltage (V)	5500
Source temperature (°C)	500
Gas 1 (psi)	50
Gas 2 (psi)	60

Table 5.11: MS/MS settings

	Artemiside	**ISTD**
Q1 mass $[M+H]^+$	370.2	283.2
Q3 mass	163.2	151.1
Dwell time (ms)	150	150
Declustering potential (V)	26	26
Entrance potential (V)	5	9.5
Collision energy (V)	25	21
Collision cell exit potential (V)	4	4
Scan type	MRM	MRM
Polarity	positive	positive
Pause time (ms)	5	5

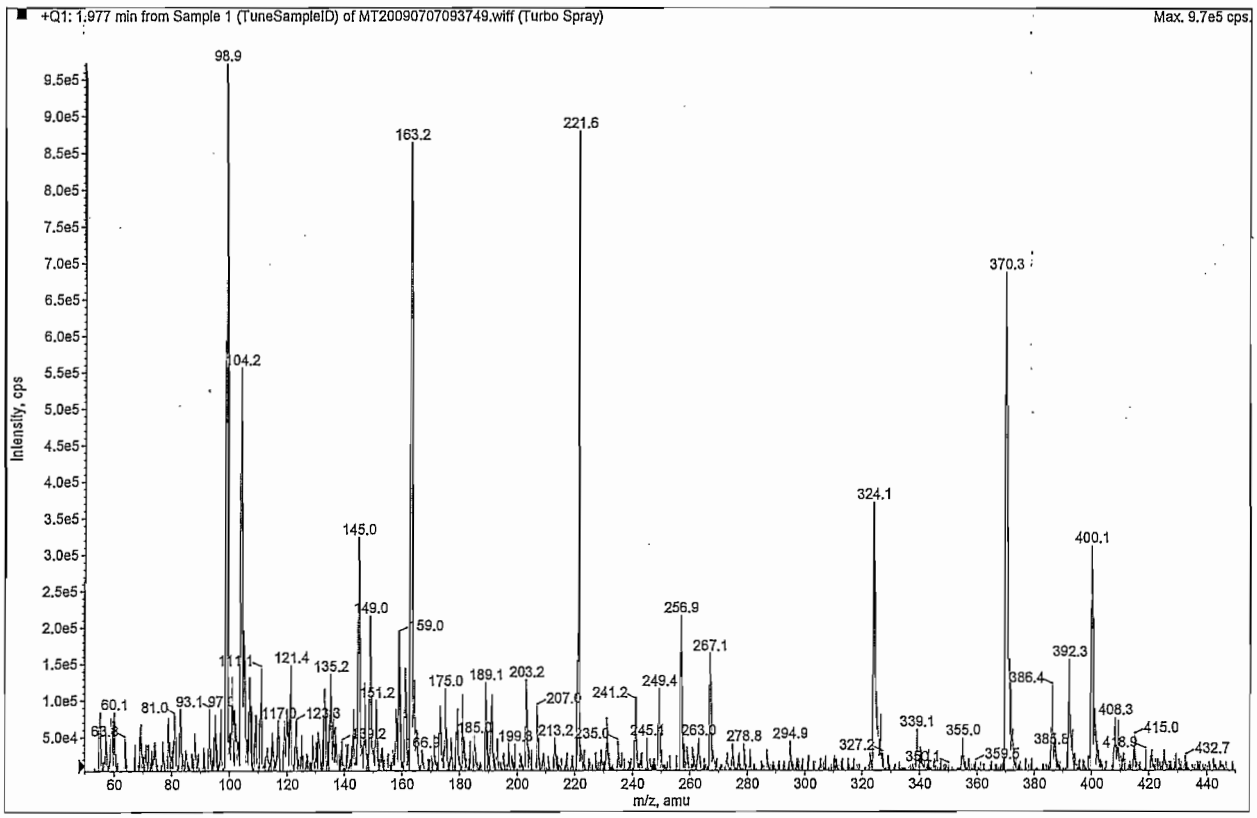

Figure 5.14: MS spectrum of artemiside

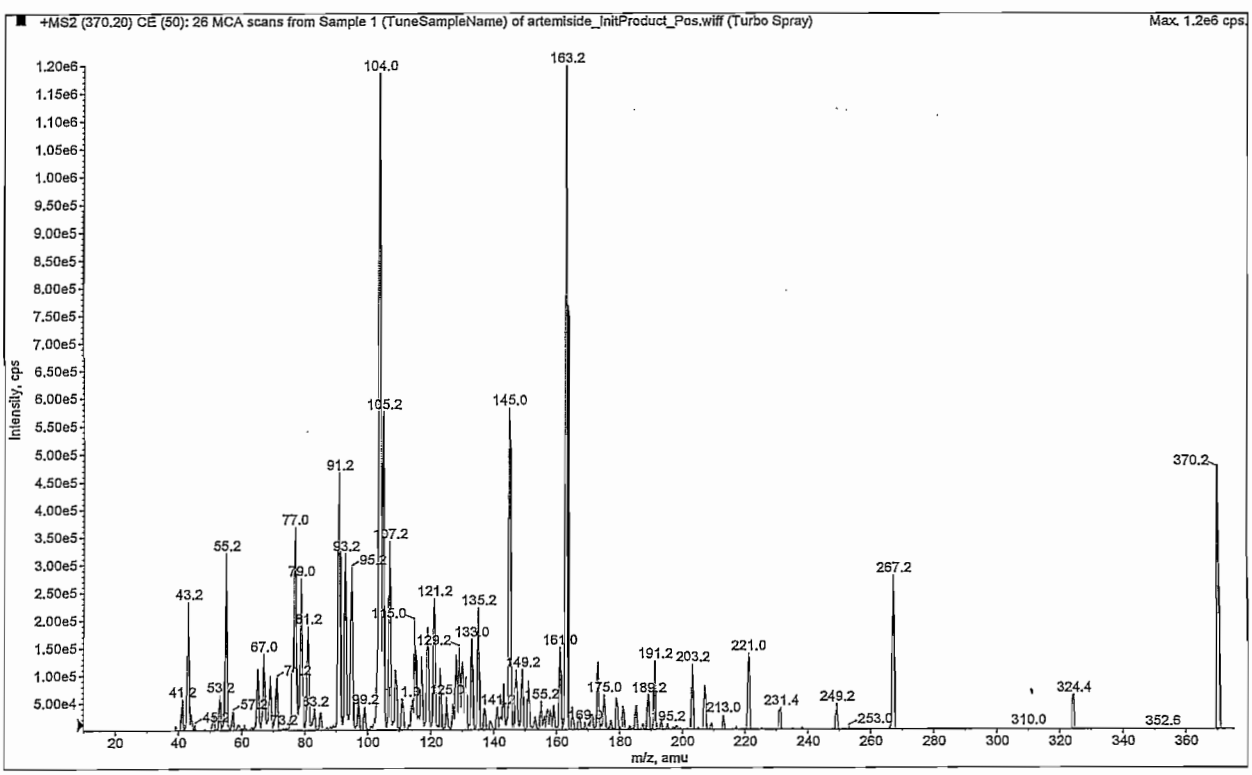

Figure 5.15: MS/MS spectrum of artemiside

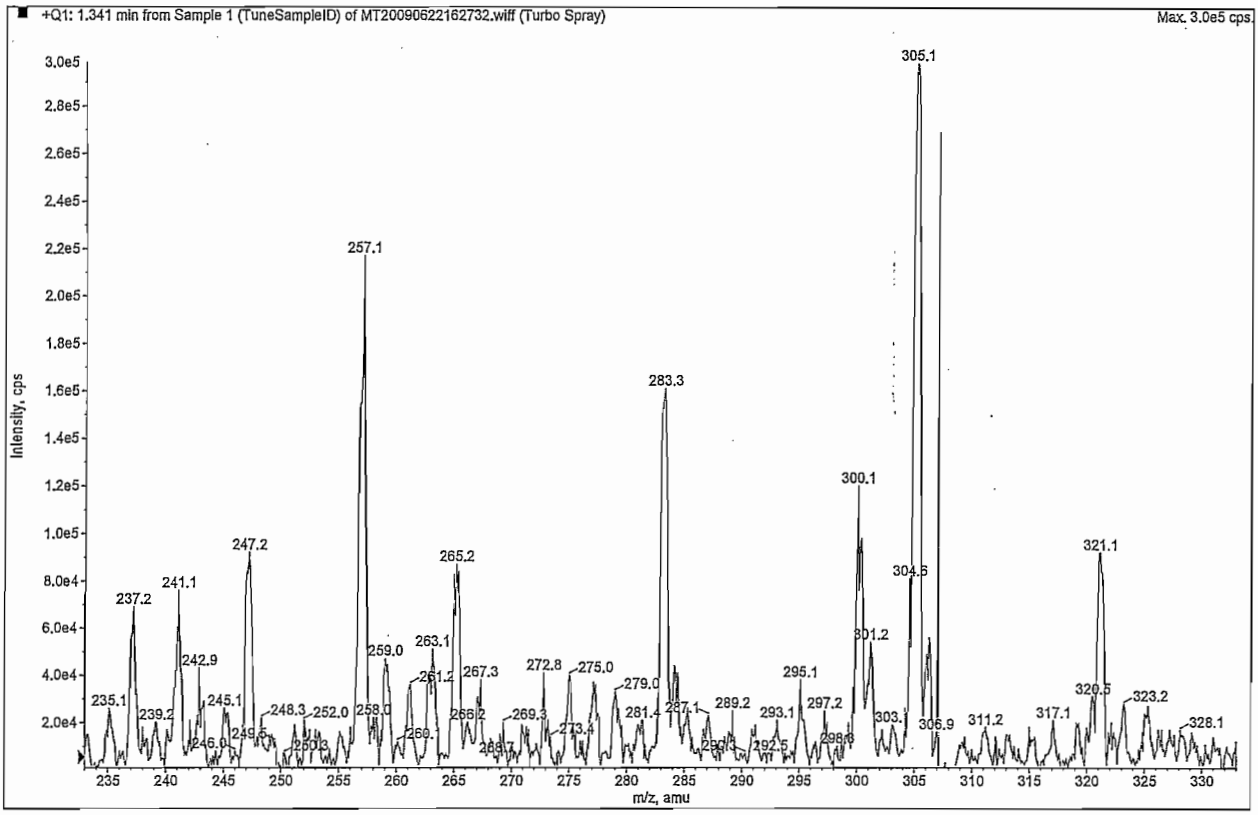

Figure 5.16: MS spectrum of the ISTD

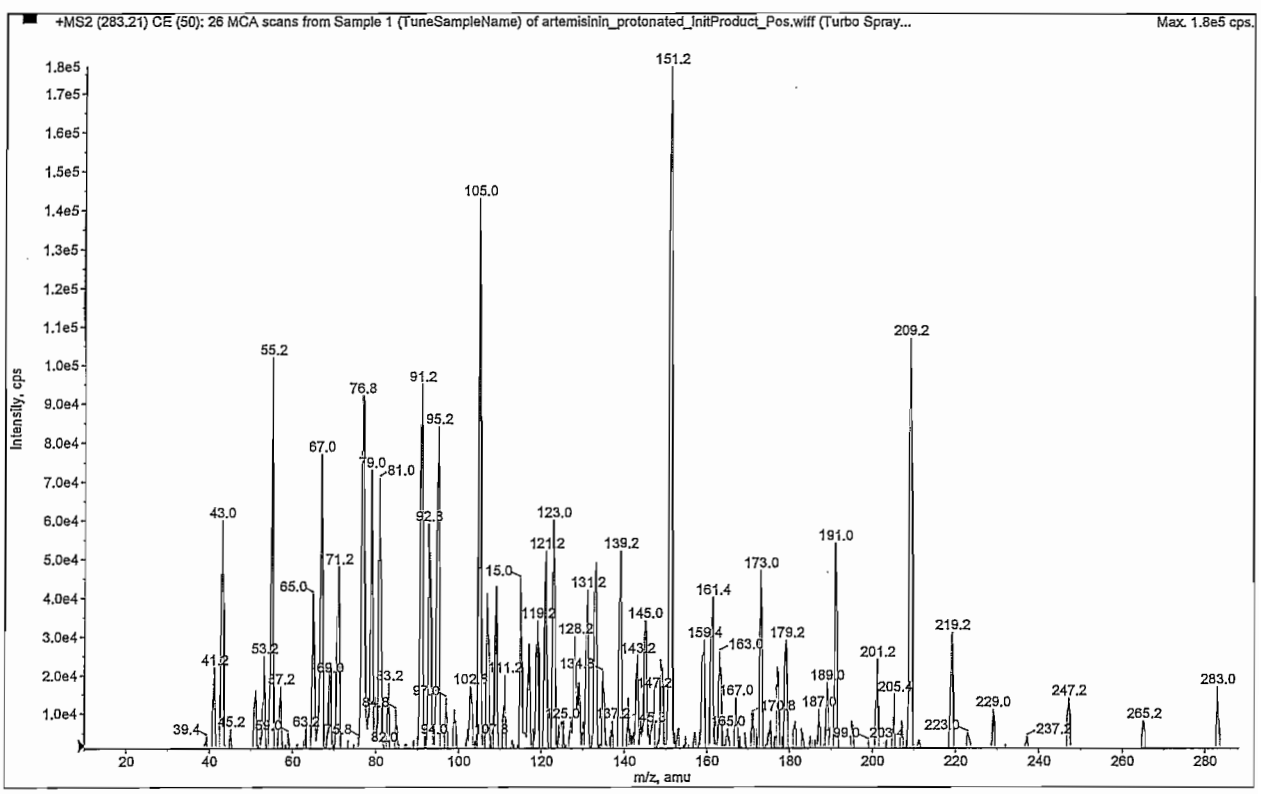

Figure 5.17: MS/MS spectrum of the ISTD

5.8.2 Chromatography

Chromatography was performed on a Phenomenex Gemini-NX (5µ, C18, 110A, 50x2 mm) analytical column using an Agilent 1200 series HPLC. The mobile phase consisted of methanol and ammonium acetate (10 mM with 0.1% acetic acid) (75:25) and was delivered at 0.5 ml/min for 3 minutes. The column was kept in a column compartment at 35 °C. An autosampler injected 5 µl onto 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 cooled to 5 °C while awaiting injection. Representative chromatograms are presented in figures 5.17 (STD 5), 5.18 (Mouse 2, reference, oral, 120 minutes) and 5.19 (blank).

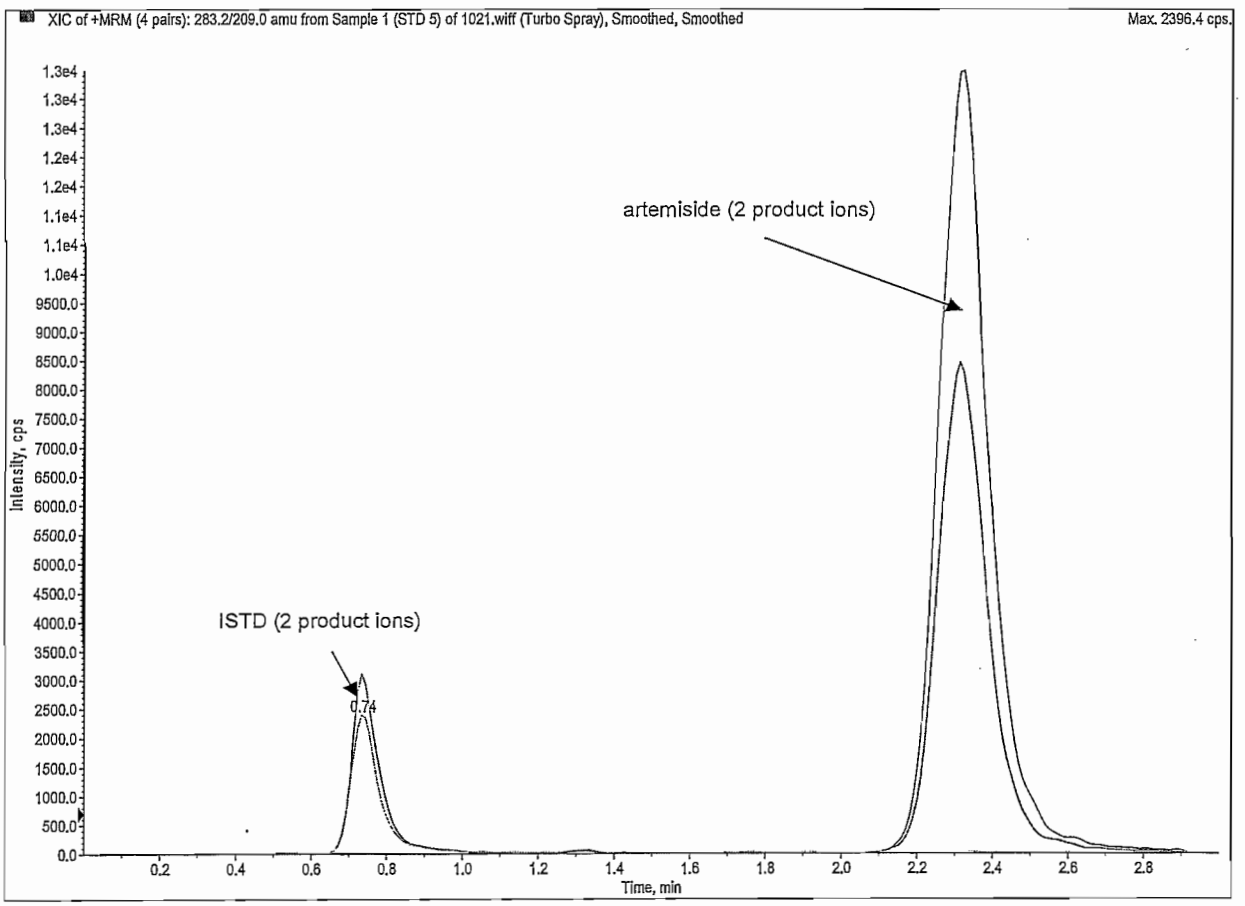

Figure 5.18: Representative chromatogram of STD 5 (500 ng/ml)

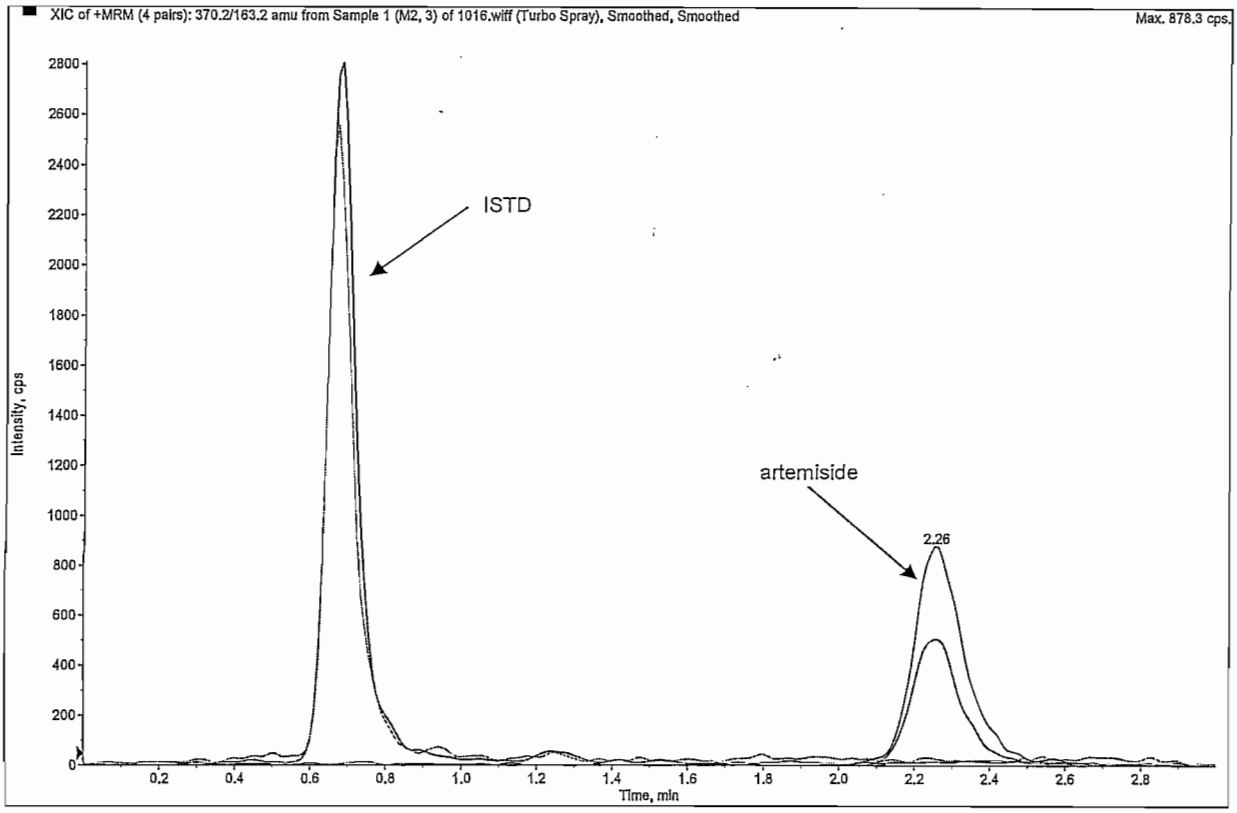

Figure 5.19: Representative chromatogram of Mouse 2 (reference, oral) 120 minute sample

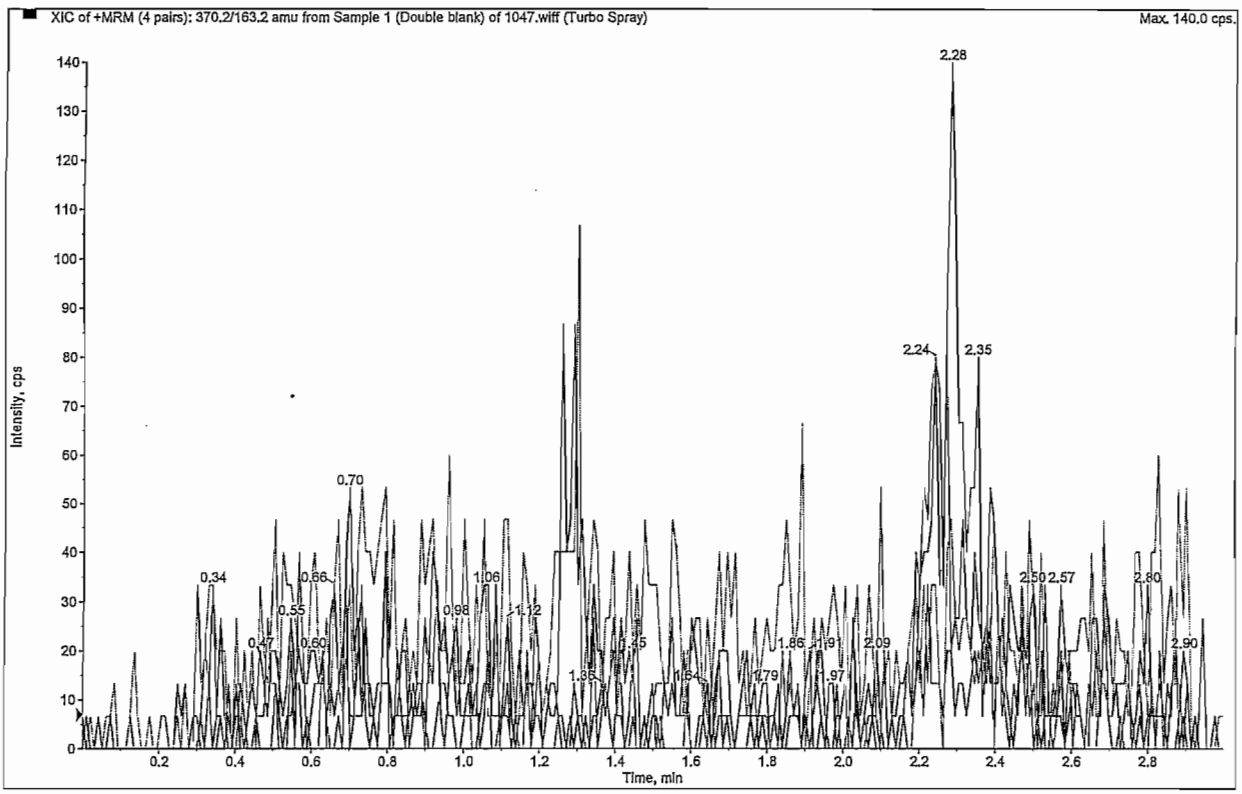

Figure 5.20: Representative chromatogram of a blank sample

5.8.3 Liquid-liquid extraction procedure

The extraction procedure was performed on ice using polypropylene test tubes. The plasma samples were thawed on ice and briefly vortexed. Twenty five microlitres of a universal Britton Robinson buffer (pH 9) was aliquotted into clean eppendorf tubes and 15 µl of the plasma sample was added. The ISTD was spiked at an appropriate concentration into the universal buffer and 25 µl was subsequently added to the extraction tubes. 1-Chlorobutane (350 µl) was added to each tube to function as an organic solvent, the samples were vortexed for 1.5 minutes and centrifuged for 5 minutes at high speed. The organic phase (300 µl) was transferred to clean polypropylene tubes and 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 (50:50, v/v) was added to the dry samples. The samples were vortexed for 30 seconds and then transferred to 96 well polypropylene plates. Five microlitres of each sample was then injected onto the HPLC column.

5.8.4 Calibration standards preparation

A stock solution of artemiside was prepared in ethanol at a concentration of 1 mg/ml. Blank mouse plasma (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 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). The calibration standards were briefly vortexed, aliquotted into labelled polypropylene tubes and stored at approximately -20 °C.

5.9 RESULTS: ARTEMISIDE

5.9.1 Calibration curve

The calibration standards were analyzed with the study samples. A representative calibration curve is presented in figure 5.21. The accuracy and precision statistics of this calibration curve are presented in table 5.12.

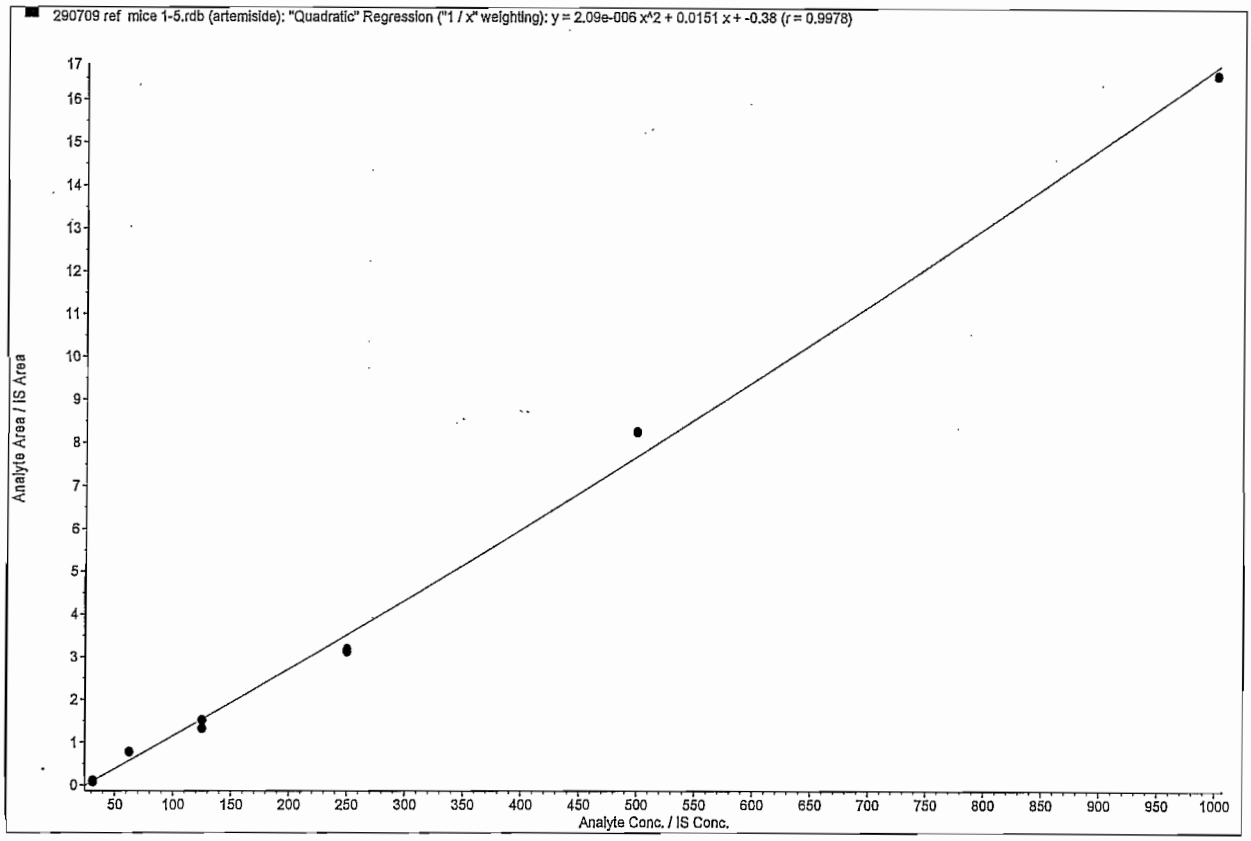

Figure 5.21: Representative calibration curve of artemiside

Table 5.12: Accuracy data of a representative calibration curve

Expected concentration (ng/ml)	STD	Mean (ng/ml)	% CV	% Accuracy
31.3	STD 9 (N=2)	31.0	4.5	99.0
62.5	STD 8 (N=1)	75.5	N/A	120.9
125	STD 7 (N=2)	117	7.4	93.8
250	STD 6 (N=2)	227	1.3	90.9
500	STD 5 (N=2)	533	0.1	106.7
1000	STD 4 (N=2)	990	0.0	99.0

5.9.2 Artemiside reference (p.o.)

The samples were analyzed in two batches, the results are presented in table 5.13 and graphically depicted in figure 5.22 in the form of a mean drug concentration versus time graph (N = 10).

Table 5.13: Plasma concentrations (ng/ml) of the artemiside reference group

Time (minutes)	0	10	30	60	120	180
Mouse 1	0	122	38.0	138	46.6	64.4
Mouse 2	0	53.7	59.6	60.2	BLQ	BLQ
Mouse 3	0	47.6	45.5	41.1	31.6	40.0
Mouse 4	0	83.4	42.8	31.8	49.8	56.3
Mouse 5	0	39.8	91.2	35.4	131	46.7
Mouse 6	0	87.6	77.4	50.7	34.1	28.3
Mouse 7	0	166	183	115	80.2	31.6
Mouse 8	0	159	81.2	BLQ	BLQ	BLQ
Mouse 9	0	145	95.5	98.1	43.7	41.7
Mouse 10	0	129	BLQ	BLQ	BLQ	54.7
Mean	0	103	79.4	71.3	59.6	45.5
SD	N/A	47.2	44.3	40.3	35.3	12.5

BLQ = Below the limit of quantitation.

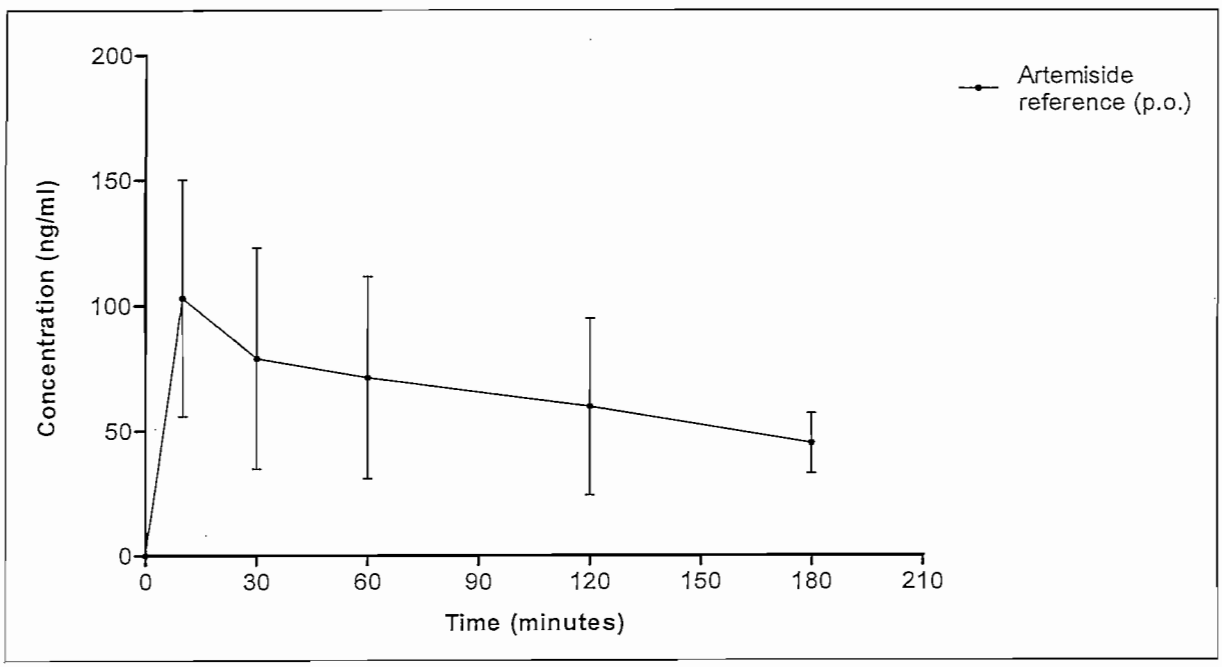

Figure 5.22: Mean concentration vs. time graph of the artemiside reference group

The first samples were collected just before administration, all of those tested negative for artemiside. Ten minutes after administration the mean plasma concentration of artemiside was 103.0 ng/ml. At 30 minutes the recorded plasma concentration was 79.4 ng/ml. There was a gradual decrease in plasma concentration beyond 30 minutes, at 60 minutes the concentration was 71.3 ng/ml and 59.6 ng/ml at 120 minutes post administration. The last samples were collected at 180 minutes post administration, they represented a mean drug concentration of 45.5 ng/ml.

5.9.3 Artemiside in Pheroid vesicles (p.o.)

The samples were analyzed in two batches, the results are presented table 5.14 and graphically depicted in figure 5.23 in the form of a mean drug concentration versus time graph (N = 9).

Table 5.14: Plasma concentrations (ng/ml) of artemiside in Pheroid vesicles

Time (minutes)	0	10	30	60	120	180
Mouse 1	0	50.6	63.1	41.5	38.7	40.2
Mouse 2	0	174	69.9	35.9	35.9	34.6
Mouse 3	0	66.1	108	60.4	34.2	32.5
Mouse 4	0	61.0	169	149	53.1	61.4
Mouse 5	0	40.5	87.3	35.2	37.8	34.4
Mouse 6	*	*	*	*	*	*
Mouse 7	0	101	135	54.2	101	36.6
Mouse 8	0	93.3	107	120	48.4	BLQ
Mouse 9	0	115	132	89.3	41.6	46.5
Mouse 10	0	93.4	164	251	118	34.8
Mean	0	88.3	115.0	92.9	56.5	40.1
SD	N/A	40.6	38.1	71.3	30.9	9.7

BLQ = Below the limit of quantitation

* dose error

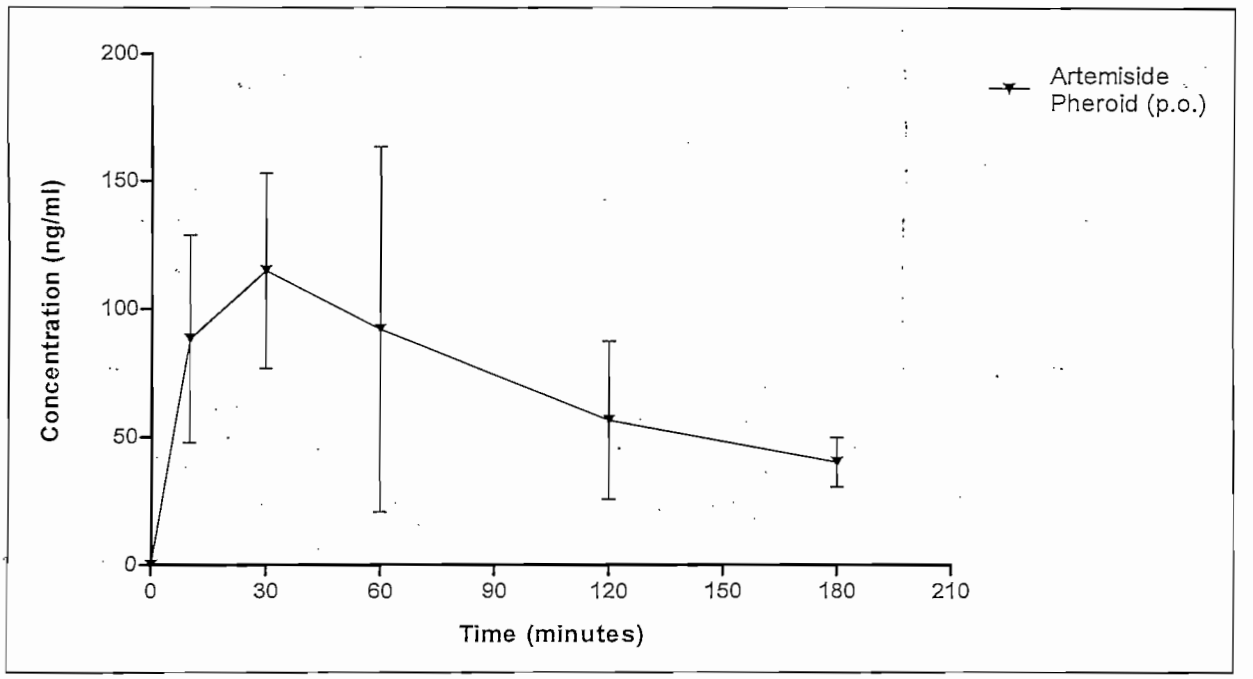

Figure 5.23: Mean concentration vs. time graph of artemiside in Pheroid vesicles

The oral artemiside in Pheroid vesicles did not perform much better than the reference formulation. The first samples were collected just before administering the vesicles, all those samples tested negative for artemiside. Ten minutes after administration the mean recorded plasma concentration was 88.3 ng/ml while the reference gave a concentration of 103.0 ng/ml at the same time point. At 30 minutes the recorded plasma concentration was 115.0 ng/ml for the Pheroid formulation in contrast to the reference formulations' 79.4 ng/ml. A gradual decrease was observed after 30 minutes, a concentration of 92.9 ng/ml was achieved at 60 minutes and 56.5 ng/ml at 120 minutes post administration. At 180 minutes it decreased further to 40.1 ng/ml.

5.9.4 Artemiside reference formulation (IV)

When the intravenous reference formulation was prepared it was observed that the drug had precipitated to some extent and it was decided to first evaluate the feasibility of the experiment in 5 test animals. The samples were collected and analyzed and it was obvious that artemiside had precipitated to such an extent that the administered dose fell short of the intended dose of 5.0 mg/kg. The insufficient dosing could be attributed to the complexity of the procedure. It should be kept in mind that a very thin needle was required to intravenously administer the drug formulation to the mice. The combination of the thin needle together with

the size of the precipitated drug particles caused non-uniform dosing. After evaluating the results it was decided not to continue with the experiment in the 5 remaining test animals. The results are presented in table 5.15 and graphically depicted in figure 5.24 in the form of a mean drug concentration versus time graph (N = 5).

Table 5.15: Plasma concentrations (ng/ml) of the artemiside reference group (IV)

Time (minutes)	0	10	30	60	120	180
Mouse 1	0	49.8	23.6	16.0	BLQ	BLQ
Mouse 2	0	42.6	24.5	BLQ	BLQ	BLQ
Mouse 3	0	22.3	BLQ	17.9	BLQ	BLQ
Mouse 4	0	28.5	BLQ	BLQ	BLQ	BLQ
Mouse 5	0	83.6	44.6	BLQ	BLQ	BLQ
Mean	0	45.0	31.0	17.0	N/A	N/A
SD	N/A	24.0	12.0	1.0	N/A	N/A

BLQ = Below the limit of quantitation

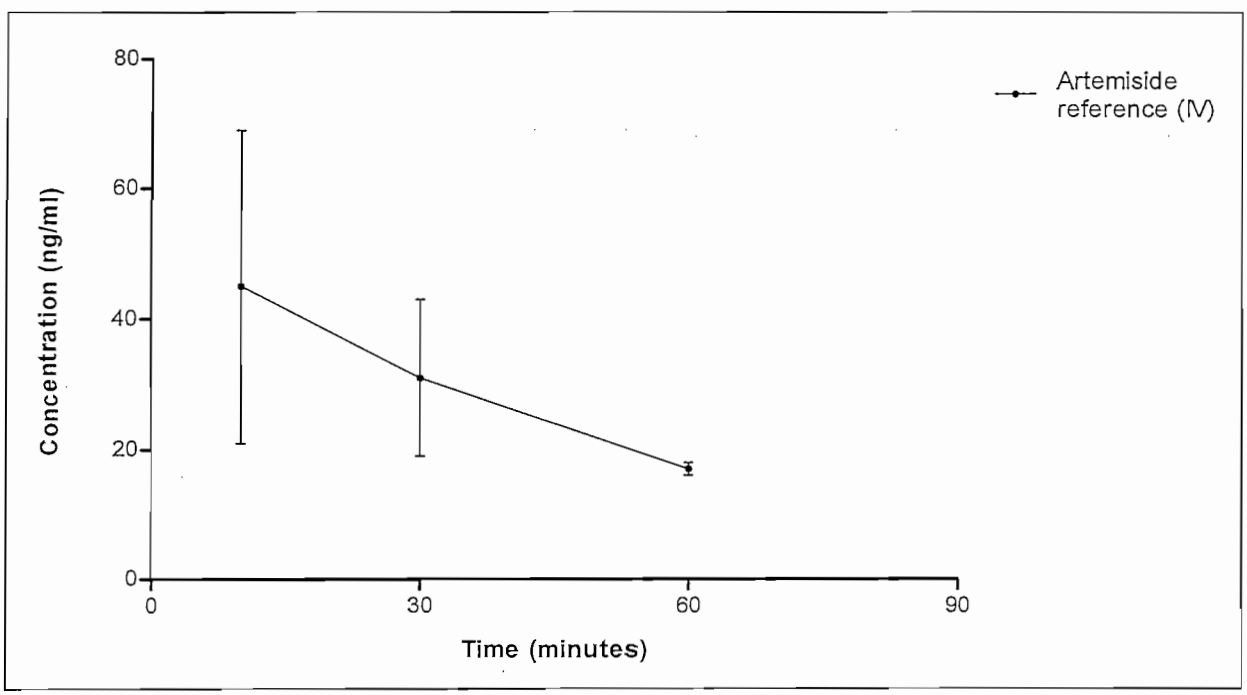

Figure 5.24: Mean concentration vs. time graph of artemiside in the reference formulation (IV)

The intravenous artemiside reference formulation was injected at a dose of 5.0 mg/kg. The first samples were collected just before administration, all of those tested negative for artemiside. Due to the poor solubility characteristics of artemiside in the reference formulation and the subsequent non-uniform dosing the aquired results were far from ideal. The 10 minute samples contained a mean artemiside concentration of 45.0 ng/ml while the 30 minute samples contained a mean concentration of 31.0 ng/ml. A further decrease was observed at 60 minutes with a concentration of 17.0 ng/ml. Beyond 60 minutes the artemiside plasma levels were below the limit of quantitation and could not be accurately detected.

5.9.5 Artemiside in Pheroid vesicles (IV)

The intravenous reference formulation produced questionable results, it was suspected that the plasma levels could be increased by improving the solubility of the drug with the addition of a more favourable vehicle. Artemiside is a very hydrophobic compound by nature and it was observed that both the reference and intravenous formulations formed a flakey precipitate upon the addition of water to the DMSO-drug solutions. In order to address the solubility issues, artemiside was encapsulated in a sterilized Pheroid vesicle formulation and also administered intravenously. The samples were analyzed in two batches, the results are presented in table 5.16 and graphically depicted in figure 5.25 (N = 10).

Table 5.16: Plasma concentrations (ng/ml) of artemiside in Pheroid vesicles (IV)

Time (minutes)	0	10	30	60	120	180
Mouse 1	0	2740	738	208	72.7	44.7
Mouse 2	0	3440	557	171	60.5	46.2
Mouse 3	0	1730	268	199	106	65.9
Mouse 4	0	2300	469	245	119	84.5
Mouse 5	0	2510	472	193	67.4	63.3
Mouse 6	0	2370	685	350	84.7	54.4
Mouse 7	0	2880	663	257	112	55.8
Mouse 8	0	3530	738	312	122	112
Mouse 9	0	1740	656	253	96.4	81.9
Mouse 10	0	2090	551	201	115	84.6
Mean	0	2533	580	239	95.6	69.3
SD	N/A	626	148	56.7	22.8	21.2

BLQ = Below the limit of quantitation

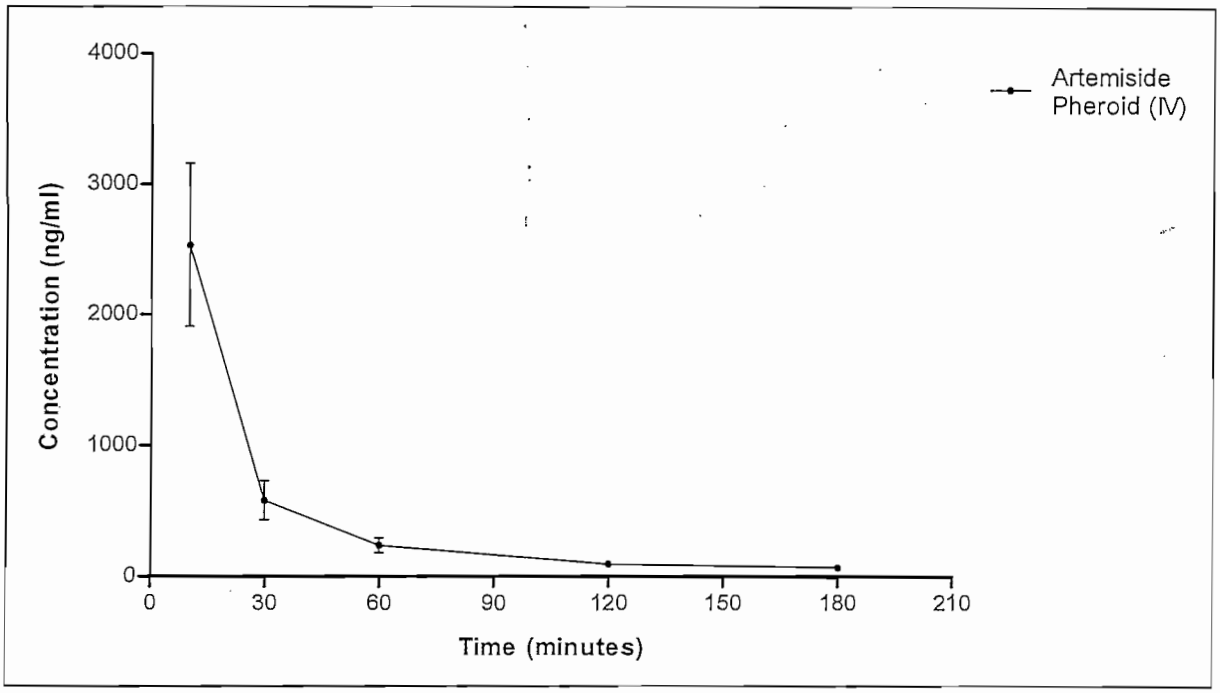

Figure 5.25: Mean concentration vs. time graph of artemiside in Pheroid vesicles (IV)

The intravenous artemiside Pheroid vesicle formulation was injected at a dose of 5.0 mg/kg. The first samples were collected just before administration, all of those tested negative for artemiside. The 10 minute samples contained a mean artemiside concentration of 2533.0 ng/ml while the 30 minute samples contained a mean concentration of 580.0 ng/ml. A further decrease was observed at 60 minutes with a concentration of 239.0 ng/ml. Beyond 60 minutes a more gradual decrease was observed, at 120 minutes the concentration was 95.6 ng/ml and 69.3 ng/ml at 180 minutes.

5.9.6 Metabolic conversion of artemiside to artemisone

After careful evaluation of the molecular structure of artemiside it was suspected that a slight possibility exist that artemiside could, to some extent, be metabolically converted to artemisone. The same samples collected from the group which received the intravenously administered artemiside in a sterilized Pheroid vesicle formulation was also analyzed for its artemisone content (N = 10). The results were very surprising, it was obvious that a fraction of the administered artemiside was indeed converted to artemisone. The recorded plasma concentrations of artemisone are presented in table 5.17 and graphically depicted in figure 5.26.

Table 5.17: Plasma concentrations (ng/ml) of artemisone after the intravenous administration of artemiside in Pheroid vesicles

Time (minutes)	0	10	30	60	120	180
Mouse 1	0	16.3	61.2	21.8	BLQ	BLQ
Mouse 2	0	BLQ	82.9	46.5	BLQ	BLQ
Mouse 3	0	17.0	60.6	46.8	17.0	BLQ
Mouse 4	0	25.0	75.6	77.3	18.9	BLQ
Mouse 5	0	17.7	59.2	39.3	BLQ	BLQ
Mouse 6	0	BLQ	45.9	41.8	BLQ	BLQ
Mouse 7	0	BLQ	28.2	30.2	BLQ	BLQ
Mouse 8	0	BLQ	19.1	23.2	BLQ	BLQ
Mouse 9	0	BLQ	26.7	22.1	BLQ	BLQ
Mouse 10	0	BLQ	31.0	20.5	BLQ	BLQ
Mean	0	19.0	49.0	37.0	18.0	-
SD	N/A	4.0	22.0	18.0	1.3	-

BLQ = Below the limit of quantitation

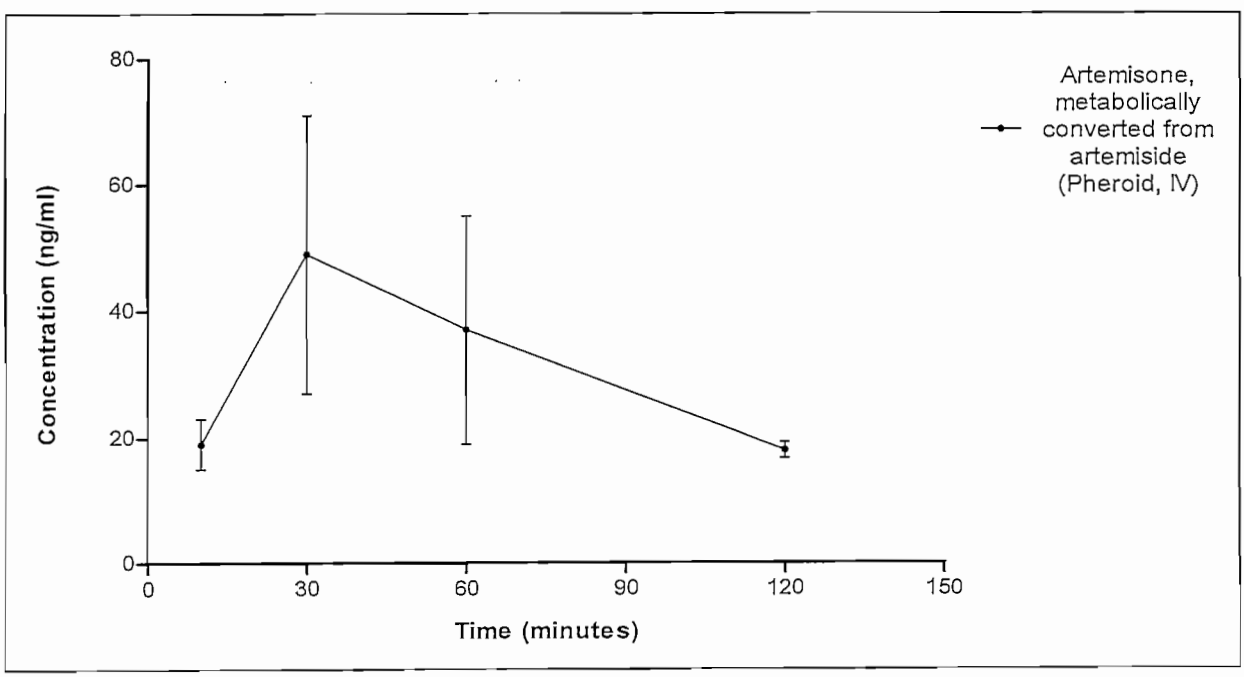

Figure 5.26: Mean concentration vs. time graph of artemisone after administration of artemiside in Pheroid vesicles (IV)

The intravenous artemiside Pheroid vesicle formulation was injected at a dose of 5.0 mg/kg. The first samples were collected just before administration, all of those tested negative for artemiside and artemisone. The 10 minute samples contained a mean artemiside concentration of 2533.0 ng/ml while the mean artemisone concentration was recorded as 19.0 ng/ml. The 30 minute samples contained a mean artemiside concentration of 580.0 ng/ml while the artemisone concentration was 49.0 ng/ml. It became very apparent at that stage that a decrease in the artemiside concentration was followed by a subsequent increase in the plasma levels of artemisone. This points to an inversely proportional relationship between the drug concentrations which suggest that artemisone was formed during the metabolic degradation of artemiside. A further decrease in the artemiside levels was observed at 60 minutes with a concentration of 239.0 ng/ml. Artemisone concentrations also decreased, the mean plasma concentration was 37.0 ng/ml at 60 minutes. Artemiside levels gradually declined beyond 60 minutes, at 120 minutes the concentration was 95.6 ng/ml and 69.3 ng/ml at 180 minutes. Artemisone levels were 18.0 ng/ml at 120 minutes and at 180 minutes the levels were below the limit of quantitation.

5.10 STATISTICAL EVALUATION: ARTEMISIDE

Noncompartmental analysis was also used to calculate the *PK* parameters for artemiside in C57 BL 6 mice (WinNonlin version 5.2, Pharsight Corporation, California, USA). Linear interpolation was used to determine the area under the concentration time curve. $AUC_{0\text{-last}}$ is defined as the AUC computed from time zero to the time of the last Y-value above the lower limit of quantitation of the assay. All values below this limit were treated as "missing". $AUC_{0\text{-inf}}$ 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 (λz). This constant was also used to determine the observed elimination half-life ($T_{1/2}$) of the compound. The difference seen between the $AUC_{0\text{-last}}$ and $AUC_{0\text{-inf}}$ values resulted due to incomplete sampling periods, especially in the oral administration groups (i.e. the concentration not returning to zero during the sampling period). The summary statistics and Mann-Whitney non-parametric test were performed using Prism version 4 (GraphPad Software Inc., California, USA).

5.10.1 Evaluation of oral formulations

The *PK* parameters of the oral reference and the oral Pheroid vesicle groups were calculated, the results are presented in the form of an overlay of the mean concentration

versus time graphs in figure 5.27. A summary of the pharmacokinetic data are presented in tables 5.18 and 5.19.

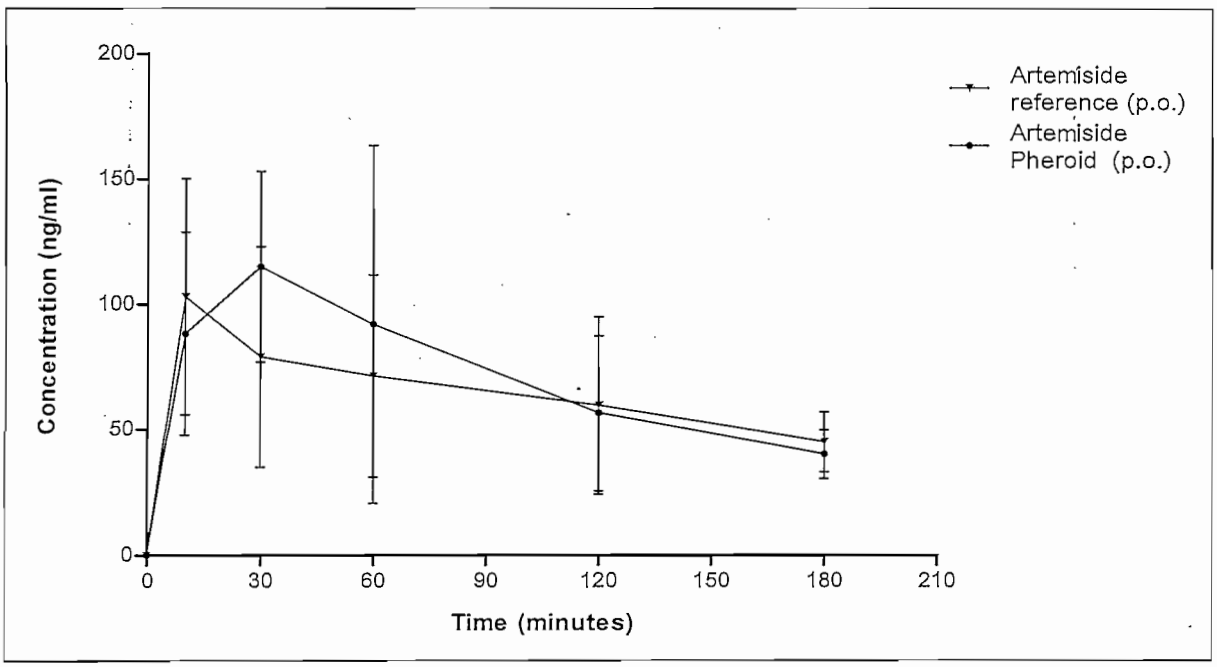

Figure 5.27: Mean concentration vs. time graph overlay of the artemiside reference group and the Pheroid group

The incorporation of artemiside in a Pheroid vesicle formulation did not produce the expected results. The differences in all the following values were scrutinized and subjected to the Mann-Whitney non-parametric test. The T_{max} of artemiside, when incorporated in Pheroid vesicles, was not dramatically increased as was the case with artemisone. When evaluating and comparing the T_{max} values of the two data sets, the reference rendered a T_{max} of approximately 30 minutes (0.55 hours) while the Pheroid vesicles rendered a T_{max} of approximately 35 minutes (0.57 hours) ($p > 0.05$). The C_{max} was also not greatly increased by the Pheroid vesicle formulation, the reference had a C_{max} of 116.4 ng/ml while the Pheroid vesicles had a C_{max} of 137.7 ng/ml ($p > 0.05$). The difference amounts to an increase in artemiside's C_{max} by approximately 18% with the aid of the Pheroid delivery system.

The data generated from this study did not lend itself to the accurate determination of either the elimination half-life ($T_{1/2}$) or the area under the curve from zero to infinity, $AUC_{0\text{-}inf}$. The reason for this could be in the explanation of how the elimination rate constant (λz) was calculated. A least squares regression was calculated using the last few points of the concentration curve, assuming that the elimination phase had been reached in the mouse.

The slope of that regression then represents λz. Due to the fact that so few subjects were sampled, and due to some of the detected concentrations which did not return to zero, the estimation for certain subjects (especially numbers 2 and 4 of the reference group) were exaggerated. The elimination rate constant for certain subjects could not be calculated due to that occurrence. For example, the concentrations detected in the last three samples collected from subject 2 (Pheroid vesicle group) were 36.0, 36.0 and 34.0 ng/ml respectively. That would not translate into a useful $T_{1/2}$ or $AUC_{0\text{-}Inf}$, and subsequently result in grossly inflated estimations. The calculated values for these pharmacokinetic parameters are still included in this chapter mainly as a point of interest but should be seen in the context described above. Extended sampling, increased assay sensitivity and increased subject numbers may be the route of choice to follow in future studies to correct these irregularities. Nevertheless, the incorporation of artemiside in Pheroid vesicles did not have a major impact on the calculated $T_{1/2}$ of the drug, the reference had a calculated $T_{1/2}$ of approximately 450 minutes ($T_{1/2}$ = 7.52 hours) while the Pheroid vesicles had a $T_{1/2}$ of approximately 400 minutes ($T_{1/2}$ = 6.49 hours) ($p > 0.05$). The $AUC_{0\text{-}Inf}$ for the reference was calculated as 749.80 ng.h/ml and 554.20 ng.h/ml for the Pheroid vesicle formulation ($p > 0.05$).

The relative bioavailability was also determined by using the arithmetic mean of the calculated area under the curve ($AUC_{0\text{-}last}$) values of both the oral reference and the oral Pheroid vesicle data ($p > 0.05$). The relative bioavailability was calculated with the aid of *Eq. 5.*

The relative bioavailability of artemiside entrapped in Pheroid vesicles was RA = 1.21 in comparison to the reference which was represented by RA = 1.00. These calculated values imply that artemiside entrapped in Pheroid vesicles was not significantly more bioavailable than that in the reference formulation.

The absolute bioavailability of artemiside was not determined. Due to the poor solubility of artemiside in the reference formulation the data obtained from the intravenously administered reference was not accurate nor sufficient to accurately calculate the absolute bioavailability.

5.10.2 Evaluation of intravenous formulations

The *PK* parameters of the intravenous reference and intravenous Pheroid vesicle groups were calculated, the corresponding graphs are presented in figures 5.28 and 5.29. For clarity purposes the graphs are presented separately to compensate for the huge differences in the

plasma concentrations. A summary of the pharmacokinetic data is presented in tables 5.20 and 5.21.

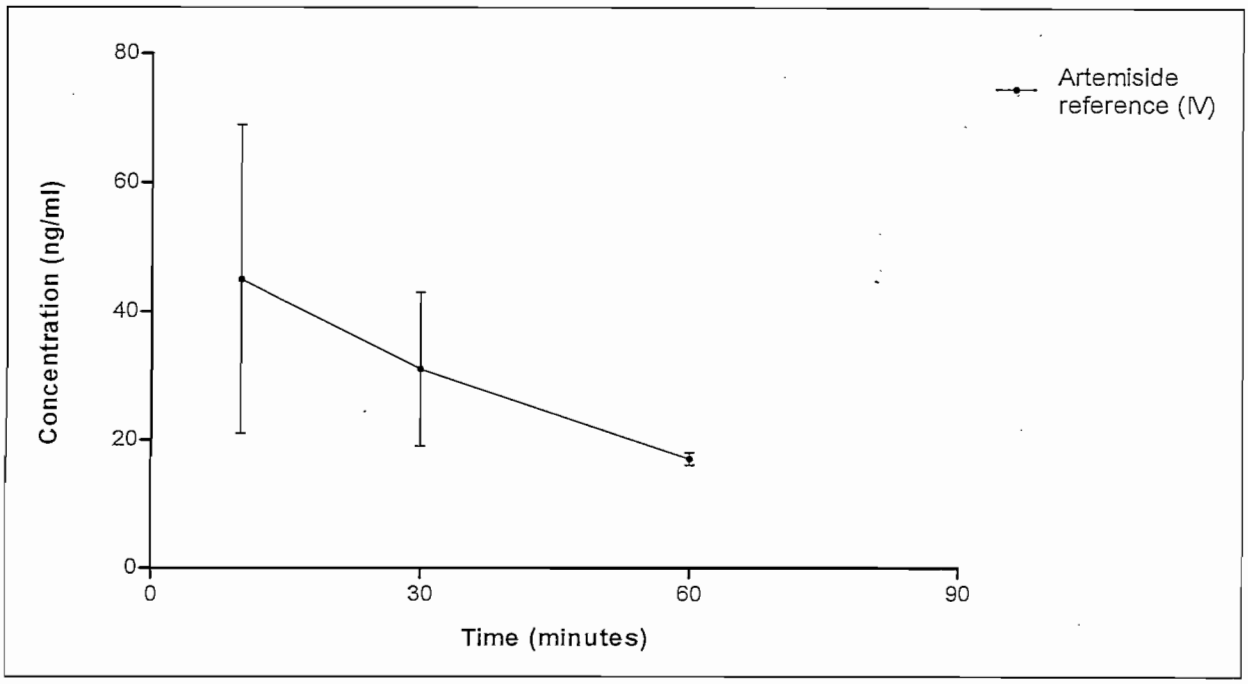

Figure 5.28: Mean concentration vs. time graph of artemiside in the reference formulation (IV)

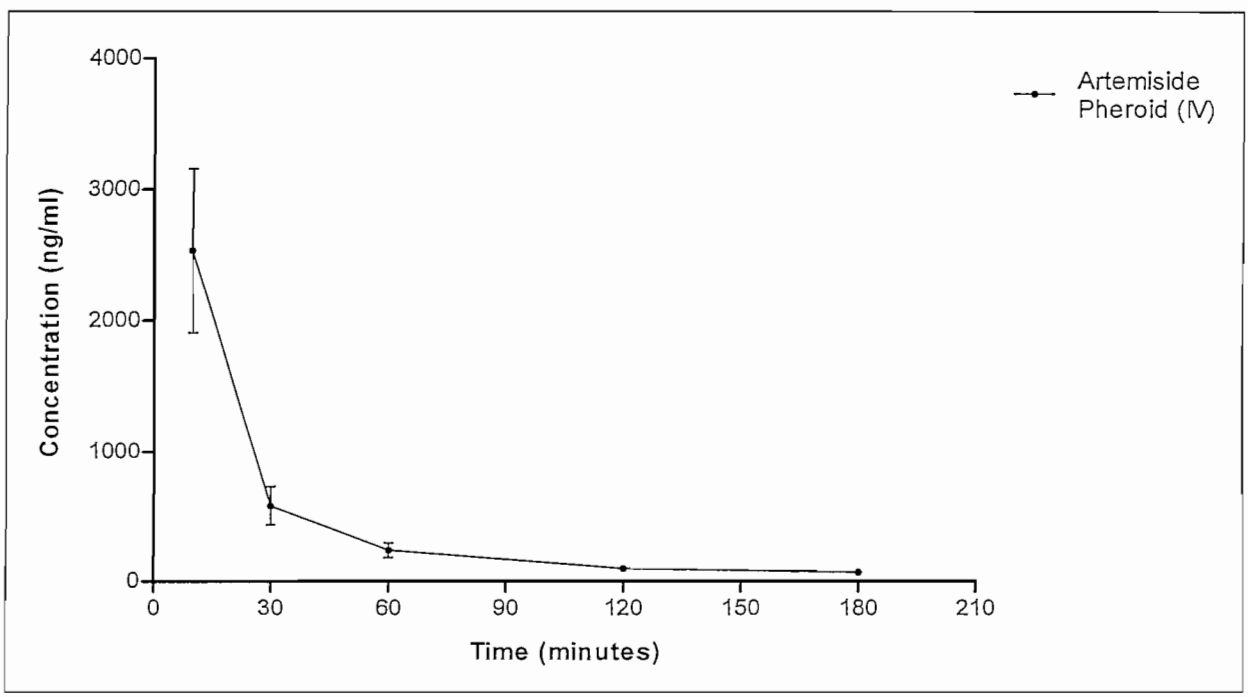

Figure 5.29: Mean concentration vs. time graph of artemiside in Pheroid vesicles (IV)

It should be kept in mind that the reference formulation formed a flakey suspension which led to non-uniform dosing of the test animals, it is also important to note that the reference formulation was only administered to 5 test animals (N = 5). These shortcomings rendered the derived data virtually unusable. The data was only included to illustrate the difference in the obtained plasma levels due to the improvement of artemisides' solubility when added to the Pheroid formulation. The incorporation of artemiside in a sterilized Pheroid vesicle formulation for intravenous administration produced outstanding results. The C_{max} achieved by incorporating artemiside in a Pheroid vesicle formulation was significantly higher than that achieved by the reference. The reference produced a C_{max} of 45.0 ng/ml while the Pheroid vesicles produced a C_{max} of 2533.0 ng/ml. The $T_{1/2}$ of the reference formulation was not calculated since the recovered data was very limited and questionable due to solubility issues and a limited amount of samples. The Pheroid vesicle formulation had a $T_{1/2}$ of approximately 60 minutes ($T_{1/2}$ = 0.98 hours). There was also a very significant difference when comparing the calculated AUC values. The difference between the arithmetic mean $AUC_{0\text{-last}}$ reference and Pheroid values were scrutinized and subjected to the Mann-Whitney non-parametric test. The reference group had an arithmetic mean $AUC_{0\text{-last}}$ value of 25.7 ng.h/ml while the Pheroid formulation had a value of 1350.0 ng.h/ml ($p < 0.0001$). The $AUC_{0\text{-inf}}$ values were not determined.

The evaluation of the metabolic conversion of artemiside to artemisone was rather interesting. Only a limited amount of data was collected since it was considered to be a "proof of concept" investigation only. Artemiside was intravenously administered at a dose of 5.0 mg/kg in a sterilized Pheroid vesicle formulation. Metabolic conversion of artemiside rendered a small amount of artemisone, the arithmetic mean $AUC_{0\text{-last}}$ was calculated as 51.0 ng.h/ml. It was interesting to note that the amount of artemisone encountered in the plasma after Pheroid administration was more than double the amount of artemiside recorded in the plasma after administration with the reference formulation.

Table 5.18: **Pharmacokinetic summary. Artemiside reference administration = 50.0 mg/kg (p.o.) and artemiside Pheroid vesicle administration = 50.0 mg/kg (p.o.)**

Subject	T_{max} (h)		C_{max} (ng/ml)		$T_{1/2}$ (h)		$AUC_{0\text{-last}}$ (ng.h/ml)		$AUC_{0\text{-inf}}$ (ng.h/ml)	
	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid	Reference	Pheroid
1	1.00	0.50	138.00	63.10	1.82	4.72	228.57	128.76	397.57	402.58
2	1.00	0.17	60.20	174.00	-	37.59	53.21	152.63	-	2028.81
3	0.17	0.50	47.60	108.00	7.84	1.49	113.21	157.10	565.42	226.80
4	0.17	0.50	83.40	169.00	31.17	1.46	140.41	280.94	2672.10	410.49
5	2.00	0.50	131.00	87.30	-	2.49	228.70	127.75	-	251.30
6	0.17	-	87.60	-	1.68	-	140.30	-	208.74	-
7	0.50	0.50	183.00	135.00	1.05	1.92	299.70	241.23	347.57	342.46
8	0.17	1.00	159.00	120.00	-	-	53.15	181.93	-	-
9	0.17	0.50	145.00	132.00	1.56	1.57	214.01	215.36	307.65	320.98
10	0.17	1.00	129.00	251.00	-	0.70	270.90	415.06	-	450.29
Arithmetic Mean	0.552	0.5744	116.40	137.7	7.52	6.493	174.2	211.2	749.8	554.2
Geometric Mean	0.3454	0.5174	107.50	128.3	3.271	2.59	149.9	196.4	487.4	418.8
Arithmetic/Geometric	1.60	1.11	1.08	1.07	2.30	2.51	1.16	1.08	1.54	1.32
Lower 95% CI (Arithmetic)	0.1724	0.3485	78.30	93.99	0.8341	0.9439	95.66	145.4	190.5	237
Upper 95% CI (Arithmetic)	0.692	0.7681	147.60	175	12.82	7.109	234.9	265.2	1247	740
SEM	0.1937	0.08811	14.03	18.44	4.842	4.463	27.47	30.8	387.4	212.4
%CV	110.96	46.02	38.12	40.16	157.72	194.41	49.86	43.76	126.56	108.41
Mann-Whitney non-parametric test										
	0.9203	nsd	0.3644	nsd	0.8797	nsd	0.3812	nsd	0.6445	nsd

* nsd = no significant difference, sd = significant difference

Table 5.19: Artemiside $AUC_{0\text{-last}}$ (5.0 mg/kg, reference and Pheroid, IV) and artemisone $AUC_{0\text{-last}}$ after metabolic conversion from artemiside

Subject	Artemiside reference, IV, $AUC_{0\text{-last}}$ (ng.h/ml)	Artemiside Pheroid, IV, $AUC_{0\text{-last}}$ (ng.h/ml)	Artemiside Pheroid, IV, $T_{1/2}$ (h)	Artemisone (converted from artemiside) $AUC_{0\text{-last}}$ (ng.h/ml)
1	29.67	1422	0.9016	38.26
2	17.79	1532	0.7266	90.02
3	20.30	948	1.2226	76.34
4	-	1266	1.3023	109.38
5	34.13	1233	0.8713	42.13
6	N/A	1407	0.6644	46.00
7	N/A	1517	0.9077	28.70
8	N/A	1833	0.9365	20.13
9	N/A	1151	0.678	26.94
10	N/A	1196	1.602	32.34
Arithmetic Mean	25.74	1350	0.981	51.02
S.D	7.71	246.92	0.3036	30.214
%CV	30.27	18.28	30.94	59.22
Mann-Whitney non-parametric test				
	$p < 0.0001$		N/A	N/A

5.11 GENERAL DISCUSSION OF PHARMACOKINETIC RESULTS

The acquired pharmacokinetic results of artemisone were very promising indeed. The graphs obtained from the reference and the Pheroid formulation data were pharmacokinetically sound in terms of the basic configuration with well defined data points during both the absorption and elimination phases of the drug. The C_{max} and T_{max} were also well defined in these graphs. The calculated C_{max} and T_{max} values correlate very well with the apparent corresponding points on the concentration versus time curve of both the reference and Pheroid formulations.

The reference formulation produced significantly lower C_{max} and T_{max} values than the Pheroid formulation, this difference may likely be explained by less favourable absorption characteristics of the reference formulation. The reference formulation appeared to form a micro-suspension after the addition of water to the DMSO-drug solution. It was also

previously reported that artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration (Wong and Yuen, 2001). The probability exist that the Pheroid system was able to improve the solubility of artemisone and subsequently enhance the usual erratic absorption characteristics associated with this drug class by encapsulating the drug in a lipophilic layer. This was achieved by first dissolving artemisone in the oil-phase of the Pheroid system, which basically consist only of essential fatty acids, and then adding the appropriate amount of NW to promote the formation of a lipophilic shielding layer on the surface of the drug particles (Grobler, 2004).

Various studies concerning the interaction of lipophilic compounds with intestinal fatty acid-binding protein (I-FABP) suggest that the binding of these lipophilic entities (Pheroid vesicles) to I-FABP may lead to an increase in the cytosolic solubility of that entities (Velkov *et al.,* 2005). Thus increased cytosolic solubility may also subsequently facilitate the transport of the artemisone entrapped in Pheroid vesicles from the intestinal lumen across the enterocytes to sites of drug distribution. This occurrence in conjunction with the improved solubility of artemisone in the Pheroid vesicles may explain the observed increase in the C_{max} and T_{max} values. The dramatic increase in C_{max} and T_{max} values also explain why the $T_{1/2}$ had more than doubled and the bioavailability increased with the use of Pheroid technology. This did not hold true for the reference formulation which was not able to completely dissolve the drug nor was it able to provide the same, well defined, lipophilic surface characteristics as the Pheroid system to promote an interaction with I-FABP.

The acquired *PK* results of artemiside were less promising. Artemiside appeared to be more hidrophobic than artemisone. The reference formulation also formed a precipitate with the addition of water to the DMSO-drug solution but the precipitate had a more flakey appearance than the artemisone reference. The graph obtained from the water-based reference formulation was difficult to interpret, due to poor solubility characteristics of the drug and due to insufficient sampling intervals. The poor solubility predisponed to erratic absorption which in turn led to irregularities in the concentration versus time curve which had relatively large SEM values and were indicative of erratic absorption. The graph did not contain a representative drug concentration value during the absorption phase. This can be attributed to the fact that the first samples were collected at 10 minutes instead of 5 minutes. Since no previous *PK* studies had been conducted on this drug in a mouse model which could be used as a reference, the sample intervals were chosen based on the relatively short $T_{1/2}$ history of the drug class. Another consideration was the small blood volume of the test animals. Only 5 samples could be collected from each animal before the reduction in blood volume would start to have a concentrating effect of the drug in the plasma due to the

subsequent decrease in the total blood volume of the test animals. The sample intervals were chosen to ensure the best possible utilization of the limited amount of samples. In retrospect the first samples should rather have been collected at 2 - 5 minutes post administration than at 10 minutes in order to correct this situation. Despite this shortcoming the T_{max} and C_{max} values on the reference formulation curve were still in relative accordance with the calculated values.

The calculated $T_{1/2}$ values of both the Pheroid and the reference formulations presented irregularities. Mice numbers 3 and 4 of the reference group and 1 and 2 of the Pheroid group presented with inconsistent drug plasma profiles which led to misleadingly high mean $T_{1/2}$ values (table 5.20) of the drug in both formulations. The reason for these unusually high $T_{1/2}$ values were explained in the paragraph concerning the statistical analysis. Due to the limited number of test animals in the study it was thought to be relevant to still include these values in the study to emphasize the reality of the erratic absorption characteristics of the artemisinin drug class (Wong & Yuen, 2001). By omitting the apparent outliers the $T_{1/2}$ of artemiside in the reference formulation would most probably be more in the vicinity of 90 minutes (1.50 hours) rather than the calculated 450 minutes and that of the Pheroid formulation would be 96 minutes (1.60 hours) instead of the calculated 390 minutes.

The drug concentration versus time curve of the artemiside Pheroid vesicle formulation was more favourable in terms of the basic configuration with definite data points during both the absorption and elimination phases of the drug. The C_{max} and T_{max} were both well defined in the corresponding graph. The calculated C_{max} and T_{max} values correlated well with the corresponding points on the concentration versus time curve of the Pheroid formulation. Artemiside was encapsulated by first dissolving the drug in the oil-phase of the Pheroid system, which basically consist only of essential fatty acids, and then adding the appropriate amount of NW to promote the formation of a lipophilic shielding layer on the surface of the drug particles (Grobler, 2004). Although a small improvement was observed in the results of this formulation in terms of the C_{max} and T_{max} over that of the reference there was not a very apparent increase in the bioavailability of artemiside when administered in conjunction with Pheroid vesicles. Artemiside entrapped in Pheroid vesicles, however, took longer to reach its C_{max} in comparison to the reference formulation which made it possible to record definite data points during the absorption phase of the curve. This may probably be explained by the usually rapid conversion of most artemisinin derivatives to DHA or other metabolites (Haynes *et al.,* 2006). In the case of artemiside it was proven that the drug was metabolically converted to artemisone and possibly also to a few other unidentified metabolites. Figure 5.30 graphically depicts the conversion of artemiside to artemisone.

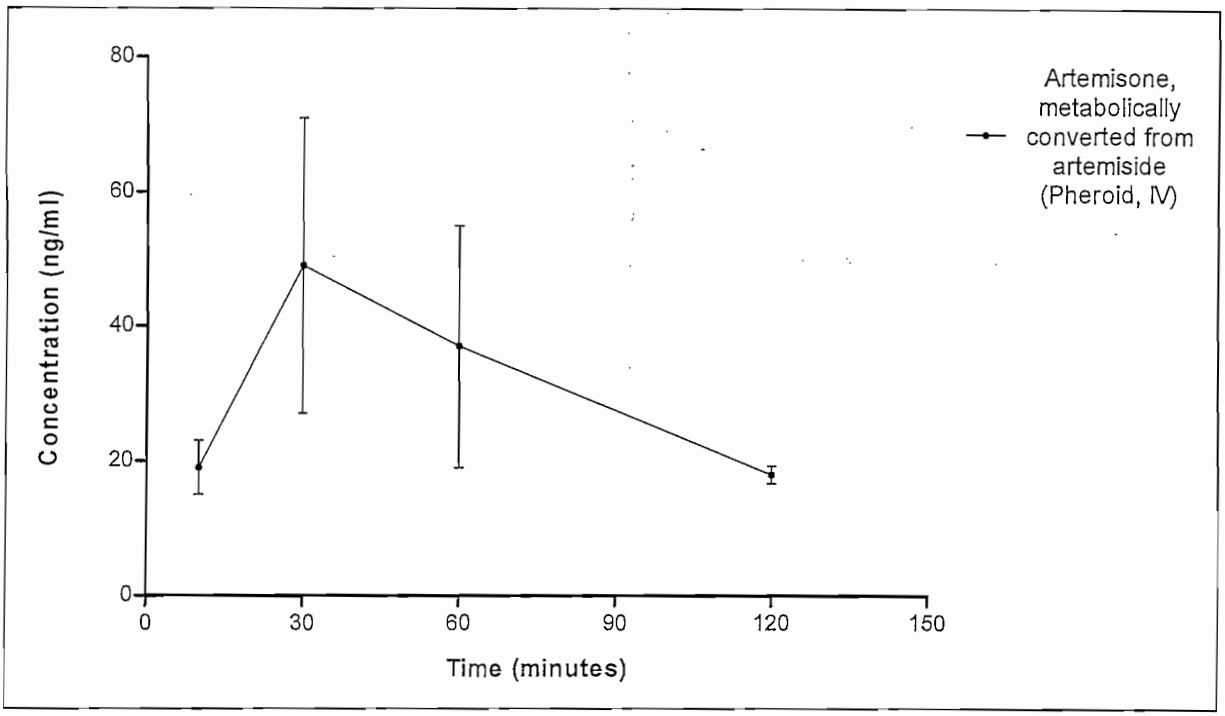

Figure 5.30: Mean concentration vs. time graph of artemisone after administration of artemiside in Pheroid vesicles (IV)

It is very likely that the lipophilic shielding layer of the Pheroid system was able to partially protect the drug against this rapid metabolic conversion. This in turn would explain the increase in the T_{max} and C_{max} of artemiside (figure 5.27). Note that the first samples collected at 10 minutes represented the T_{max} for the reference formulation while the same samples of the Pheroid formulation represented a point during the absorption phase, thus suggesting a slight delay in absorption.

When comparing the bioavailability results of artemisone to that of artemiside the differences become very apparent and a few questions arise. Both compounds were orally administered at a dose of 50.0 mg/kg and yet the artemisone Pheroid formulation rendered a peak plasma concentration of approximately 1500.0 ng/ml in contrast to the 137.0 ng/ml of the artemiside Pheroid formulation. The most probable explanation for this occurrence may lie in differences in the metabolic conversion and degradation of these drugs. Most artemisinin class drugs are very quickly converted to DHA or other metabolites after oral administration while artemisone, in contrast to other artemisinins, are not converted to DHA. Artemisone is metabolized to the products M1 - M5 as described in chapter 2 (section 2.7). The conversion of artemiside, to artemisone, seem to occur at a more rapid pace in comparison to the metabolic conversion rate of artemisone itself. These differences in metabolic products and

the varied rapidness of the conversion rates may shed some light on the differences encountered in the *PK* results of the two compounds. The sensitive and selective LC/MS/MS method was developed to analyze either the artemisone or artemiside concentration of the plasma samples and not that of the metabolic products. Since the first plasma samples were collected at 10 minutes post administration the possibility exist that a large fraction of the artemiside was already converted to either artemisone or other products in the acidic milieu of the stomach before or during the absorption phase of the drug. A large fraction of this conversion had most likely occured before the first samples were collected which would explain the low artemiside plasma levels. Had the initial samples of the oral reference and Pheroid groups been collected at 5 minutes post administration and had the LC/MS/MS been set up to rather analyze both the artemisone and the artemiside content of the plasma samples the results might have indicated a proportional increase in the plasma concentration of artemisone in relation to a proportional decrease in the plasma levels of artemiside. In the case of artemisone, which is converted to products M1 - M5, the possibility exist that the drug was absorbed unchanged and that the metabolic transformation to products M1 – M5 was less rapid than in the case of artemiside. The possibility also exist that this conversion may only occur during the elimination phase of the drug which would explain its much higher peak plasma concentrations in relation to that of artemiside.

5.12 CONCLUSION

Artemisone has proven its antimalarial activity in both the *in vivo* and *in vitro* activity studies. The *PK* analysis of artemisone was instrumental in explaining its remarkable *in vivo* efficacy. The data also delivered compelling evidence in favour of the ability of the Pheroid delivery system to enhance the bioavailability of artemisone. All the experiments indicated a very dramatic improvement in the T_{max}, C_{max} and $T_{1/2}$ of the drug when administered in a Pheroid vesicle formulation. It effectively translates to a scenario where the drug concentration could be significantly decreased and still achieve therapeutic drug plasma concentrations. The combination of artemisone and Pheroid technology may perhaps prove to be an essential component in antimalarial combination therapy regimens in the very near future.

The Pheroid delivery system did not produce such spectacular results with artemiside as it did with artemisone. Only a marginal increase was observed in the T_{max} and C_{max} values with no added benefit to the $T_{1/2}$ of the drug. The sharp contrast between the artemisone and artemiside *PK* results may be attributed to varying solubility characteristics and perhaps the different metabolic pathways and metabolic products of the drugs.

SUMMARY AND FUTURE PROSPECTS

The aim of this study was to investigate the potential application of Pheroid technology, a novel drug delivery system, in combination with novel artemisinin derivatives against *Plasmodium* infections. Artemisone and artemiside were evaluated in a reference formulation and also in Pheroid vesicles. *In vitro* and *in vivo* antimalarial efficacy studies were conducted. Pharmacokinetic parameters for artemisone and artemiside were also determined, both in the presence and absence of Pheroid technology.

In general, it was previously reported that artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration (Wong & Yuen, 2001). The poor solubility and erratic absorption of these compounds usually translate to low bioavailability. Enzymatic degradation and physical barriers are also amongst the challenges which must be overcome to ensure effective delivery of these novel derivatives. Artemisinin-based monotherapy and combination therapies are essential for the management and treatment of uncomplicated as well as severe malaria. Any possible improvement in the delivery of these derivatives 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 Pheroid delivery system may play a key role in ensuring effective delivery and enhanced bioavailability of these novel antimalarial compounds. Pheroid technology is a patented colloidal type drug delivery system. It primarily consists of plant and fundamental fatty acids which have the ability to capture, transport and deliver pharmacologically active compounds and other valuable molecules (Grobler, 2004). The Pheroid delivery system is superior to most other delivery systems and is able to improve the delivery of dynamic complexes, reduce the time to onset of action, decrease the minimal effective drug concentration and enhance therapeutic efficacy. It is also able to indirectly decrease the cytotoxicity of therapeutic compounds and to infiltrate virtually all known barriers in the body. The system is further capable of targeting specific treatment areas, to transport genetic material to the cellular nucleus and to decrease drug resistance (Grobler, 2004).

Chapter 4 describes the *in vitro* antimalarial efficacy of these compounds when evaluated in cultures containing *Plasmodium falciparum* (strain RSA 11). Very promising results were obtained. Artesunate was also included in the study based on the fact that it is currently the most widely used artemisinin derivative against acute and severe malaria. It served as a

"commercial reference" against which the efficacy of artemisone and artemiside was measured. Artemiside produced very promising results and had proven to be a very potent antimalarial compound. Artemisone also produced very promising results although it was not as potent as artemiside. Artesunate was not as potent as artemisone or artemiside and the IC_{50} of artesunate was much higher than that of the two other compounds. Artemiside had IC_{50} values of 0.54 ± 0.03 nM (reference) and 0.10 ± 0.05 nM (Pheroid) ($p = 0.009$) while artemisone had values of 0.94 ± 0.04 nM (reference) and 0.21 ± 0.04 nM (Pheroid) ($p < 0.0001$). Artesunate had IC_{50} values of 29.65 ± 0.05 nM (reference) and 10.20 ± 0.04 nM (Pheroid) ($p < 0.0001$). The incorporation of the compounds in Pheroid vesicles were obviously even more effective in reducing the parasitaemia and presented with much lower IC_{50} values than that achieved by the reference formulations. Solubility of the drugs did not present a problem at this stage since only low concentrations were needed to conduct the studies. The acquired results prompted further investigation and in this regard artemisone and artemiside were then further evaluated in terms of their *in vivo* antimalarial efficacy and pharmacokinetic profiles.

An *in vivo* efficacy study was conducted in a mouse model with artemisone and artemiside. C57 BL6 mice were infected with *Plasmodium berghei* and the Peter's 4-day suppressive test was used as a basis for the evaluation. The compounds were formulated in DMSO/water (1:9, v/v) and also in Pheroid vesicles. The compounds were tested at a low and a high concentration in both vehicles. The vehicles were also administered on their own to serve as drug-free references. Artemisone dissolved freely in analytical grade DMSO but the addition of water produced a micro-suspension. The suspension precipitated after a while but was easily resuspended, this was indicative to artemisones' relatively hidrophobic nature. The formulation of artemisone in the Pheroid system did not present any problems. The compound was first dissolved in the Pheroid oil-phase which was then followed by the addition of the desired amount of NW, there was no sign of precipitated drug particles. Artemiside did not dissolve freely, the DMSO-drug mixture had to be vortexed for 1 minute, at the maximum setting, to achieve a clear solution. The addition of water produced a suspension which precipitated more rapidly than in the case of artemisone. It was also more difficult to resuspend the formed precipitate. The incorporation of artemiside in Pheroid vesicles did not present any difficulties. The same procedure was followed as for artemisone and there was no sign of precipitated drug particles. The formulations were administered at a volume of 200 µl via an oral gavage tube. Blood samples were collected on microscope slides at pre-determined time points. The slides were stained with a Giemsa solution and microscopically evaluated to determine the percentage parasitaemia. Artemisone performed very well, the high dose formulations A (reference) and B (Pheroid) (20.0 mg/kg) kept the

parasitaemia below 6% until 16 days post infection. Recrudescence occurred beyond 16 days with both formulations. When comparing the effect of the different vehicles (reference versus Pheroid vesicles), there was no significant difference in terms of parasite reduction or in the achieved treatment outcomes. The low dose formulations C (reference) and D (Pheroid) (2.5 mg/kg) kept the parasitaemia below 9% until 12 days post infection. Recrudescence occurred beyond 12 days with both formulations. When comparing the effect of the different vehicles (reference versus Pheroid vesicles), there was no significant difference in terms of parasite reduction or in the achieved treatment outcomes. The drug-free reference formulations E and F did not have any noticeable effect on the parasite burden and a dramatic increase in parasitaemia was observed beyond 4 days post infection. Artemiside did not perform according to expectations based on the *in vitro* antimalarial efficacy results. The high dose formulations A (reference) and B (Pheroid) (10 mg/kg) kept the parasitaemia below 2% until 8 days post infection. Recrudescence occurred beyond 8 days with both formulations. When comparing the effect of the different vehicles (reference versus Pheroid vesicles), there was no significant difference in terms of parasite reduction or in the achieved treatment outcomes. The low dose formulations C (reference) and D (Pheroid) (2.5 mg/kg) kept the parasitaemia below 3% until 8 days post infection. Recrudescence occurred beyond 8 days with both formulations. When comparing the effect of the different vehicles (reference versus Pheroid vesicles), there was no significant difference in terms of parasite reduction or in the achieved treatment outcomes. The drug-free reference formulations did not have any noticeable effect on the parasite burden and a dramatic increase in the parasitaemia was observed beyond 5 days post infection which was in accordance with the results of the artemisone study.

In general it can be concluded that the *in vitro* results suggested that artemiside was slightly more potent than artemisone and much more potent than artesunate. Incorporation of the compounds in Pheroid vesicles proved to be more effective in reducing the parasitaemia in all instances. The *in vivo* results were rather surprising and in contrast to the *in vitro* results which indicated that artemiside was more potent than artemisone. The *in vivo* activity study had shown that artemisone was able to increase the time to recrudescence by a large margin in comparison to artemiside. It was previously reported that artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration (Wong & Yuen, 2001). The most probable explanation for artemisides' poor performance during the *in vivo* activity study may be attributed to its low bioavailability which was also demonstrated during its pharmacokinetic evaluation. Recrudescence is a very common occurrence when implementing artemisinin-based monotherapy and is the main reason for the implementation of combination therapy regimens. In situations where

artemisinin-based monotherapy is considered to be the last resort it is recommended that treatment should be given for 7 consecutive days (WHO, 2006a). The 4-day treatment regimen of the Peter's 4-day suppressive test falls short of the, WHO recommended, 7-day treatment regimen for artemisinin-based monotherapy and may explain the occurrence of recrudescence during both drug treatments. The effect of auto-induction with the repeated administration of artemisinins and the subsequent increase in artemisinin clearance may also shed some light on the observed recrudescence (Ashton *et al.*, 1997).

Chapter 5 described the pharmacokinetic analysis obtained in a C57 BL6 mouse model. Two formulations were prepared with each compound, a reference formulation with DMSO/water (1:9 v/v) and also a Pheroid vesicle formulation. The same procedures were followed to prepare the drug formulations as described for the *in vivo* efficacy study. Both artemisone and artemiside formed a micro-suspension in the reference formulations. It was more difficult to resuspend the precipitated artemiside than it was to resuspend the artemisone. The compounds were both administered at a dose of 50.0 mg/kg via an oral gavage tube at a volume of 200 µl. Blood samples were colleted by means of tail-bleeding at pre-determined time points. The blood samples were centrifuged and the plasma extracted. Sensitive and selective LC/MS/MS methods were developed to analyze the drug concentrations in the plasma samples. The absorption of artemisone was dramatically enhanced by the Pheroid delivery system. The T_{max} was extended from 0.12 hours in the reference formulation to 0.55 hours in the Pheroid vesicle formulation ($p < 0.0001$) and the C_{max} was increased from 809.5 ng/ml to 1550.0 ng/ml respectively ($p = 0.0001$). The $T_{1/2}$ was increased from 0.36 hours in the reference formulation to 0.55 hours in the Pheroid formulation ($p = 0.0076$). The relative bioavailability of the reference formulation was RA = 1.0 in contrast to that of the Pheroid formulation with RA = 4.57 ($p < 0.0001$). The absolute bioavailability of the reference formulation was calculated as F = 0.10 and that of the Pheroid formulation was F = 0.48 ($p < 0.0001$). The absorption of artemiside was not dramatically enhanced by the Pheroid delivery system. The T_{max} was marginally extended from 0.55 hours in the reference formulation to 0.57 hours in the Pheroid vesicle formulation ($p > 0.05$) and the C_{max} was increased from 116.4 ng/ml to 137.7 ng/ml respectively ($p > 0.05$). The $T_{1/2}$ could not be quantified accurately. The relative bioavailability of the Pheroid formulation was calculated as RA = 1.21 in comparison to the reference formulation which was represented by an RA = 1.00 ($p > 0.05$). These calculated values imply that artemiside was not significantly more bioavailable in the Pheroid formulation than in the reference formulation. Due to the poor solubility of artemiside in the reference formulation the data obtained from the intravenously administered reference formulation was not accurate nor sufficient to calculate the absolute bioavailability. It was also discovered that a fraction of the administered artemiside was

metabolically converted to artemisone, the extent of this conversion is, however, still unknown.

In general it can be concluded that the *PK* data delivered compelling evidence in favour of the ability of the Pheroid delivery system to enhance the bioavailability of artemisone. All the experiments indicated a very dramatic improvement in the T_{max}, C_{max} and $T_{1/2}$ of the drug when administered in a Pheroid vesicle formulation, this effectively translates to a scenario where the drug concentration could be significantly decreased to still achieve therapeutic drug concentrations. The combination of artemisone and Pheroid technology may perhaps prove to be an essential component in antimalarial combination therapy regimens in the very near future. The Pheroid delivery system did not produce such spectacular results with artemiside as it did with artemisone. Only a marginal increase was observed in the T_{max} and C_{max} values with no added benefit to the $T_{1/2}$ of the drug. The sharp contrast between the artemisone and artemiside *PK* results may be attributed to varying solubility characteristics and also to different metabolic pathways and metabolic products of the drugs.

Recommendations to consider for future studies on artemisone, artemiside and Pheroid technology may include the following:

- Further studies should be conducted to establish the exact mechanism of action of the Pheroid delivery system. Such studies should be conducted on a cellular and molecular level.
- Complete toxicity studies should be conducted on all Pheroid formulations.
- The stability profile of different artemisinin derivatives, entrapped in Pheroid formulations, should be investigated.
- The characteristics of artemiside should be investigated more closely. Chemical manipulation will most likely resolve the solubility issues.
- The metabolic degradation and subsequent metabolic products of artemiside should be investigated. The conversion of artemiside to artemisone may prove to be of great importance as artemiside may act as a pro-drug for artemisone.
- Artemiside is a very potent antimalarial compound and may very well be an excellent candidate for the treatment of severe malaria if the solubility issues are resolved.

- Possible artemisone-based combinations should be investigated. Piperaquine is a very promising candidate and should be a perfect partner drug for artemisone.
- Artemisone should be subjected to more extensive phase I trials in humans to establish its safety profile and other alternative partner drugs.

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ANNEXURE 1

Response: ethics application

NORTH-WEST UNIVERSITY
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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 satisfacory answers to all the questions posed by the Ethics panel and has therefore found the ethical aspects to be in order.

PROF. J. DU PLESSIS
DIRECTOR

Artemisone study - Calculation for infection with *P. berghei* (*in vivo* antimalarial activity evaluation)

Dilution ratio: 10 µl primary stock + 990 µl PBS (1/100); take 500 µl and add 500 µl PBS (1/2) = 1/200

Parasitaemia:	159 pRBC from 634 RBC total		
	= 25.08%		
Cell count:	808 cells/5 fields		
	= 4040 cells/25 fields	x1e4	→ /ml
	= 4.04e7 RBC/ml	x200	→ diln ratio
	= 8.08e9 RBC/ml	x0.2508	→ pst
	= 2.026e9 pRBC/ml		
	= 2.026e6 pRBC/µl		
Injection:	= 2e6 pRBC/mouse		
	= 1µl/mouse	x 30	→ total #mice
	= 30µl from primary stock		

At 200 µl/mouse, need min. 6ml secondary stock; use 9 ml (enough for 45 mice)

Secondary stock: 8.955 ml PBS + 45 µl primary stock. Use 200 µl secondary stock/mouse i.p.

Artemiside study - Calculation for infection with *P. berghei* (*in vivo* antimalarial activity evaluation)

Dilution ratio: 10 µl primary stock + 990 µl PBS (1/100)

Parasitaemia:	198 pRBC from 608 RBC total		
	= 32.57%		
Cell count:	357 cells/5 fields	x5	→ full count
	= 1785 cells/25 fields	x1e4	→ /ml
	= 1.785e7 RBC/ml	x100	→ diln ratio
	= 1.785e9 RBC/ml	x0.3257	→ pst
	= 5.814e8 pRBC/ml		
	= 5.814e5 pRBC/µl		
Injection:	= 2e6 pRBC/mouse		
	= 3.44 µl/mouse	x 30	→ total #mice
	= 103.2 µl from primary stock		

At 200 µl/mouse, need min. 6 ml secondary stock; use 9 ml (enough for 45 mice)

Secondary stock: 8.845 ml PBS + 155 µl primary stock. Use 200 µl secondary stock/mouse i.p.

ANNEXURE 2

Proposed article for publication in the

Journal of drug delivery science and technology

(In preparation to be submitted)

Pharmacokinetic analysis of novel artemisinin derivatives with Pheroid™ technology for the treatment of malaria.

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Abstract

Artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration. The poor solubility and erratic absorption usually translate to low bioavailability. Artemisinin-based monotherapy and combination therapies are essential for the management and treatment of uncomplicated as well as cerebral malaria. Artemisone and artemiside are novel artemisinin derivatives with reported antimalarial activity. Pheroid™ technology is a patented drug delivery system which has the ability to entrap, transport and deliver pharmacologically active compounds. Pharmacokinetic models were constructed for artemisone and artemiside in Pheroid™ vesicle formulations.

The compounds were administered at a dose 50.0 mg/kg bodyweight to C57 BL6 mice via an oral gavage tube and blood samples were collected by means of tail-bleeding. Drug concentrations in the samples were determined with a LC/MS/MS method. The relative bioavailability of artemisone was RA = 1.0 (reference) and RA = 4.57 (Pheroid™) ($p < 0.0001$). The absolute bioavailability was calculated as F = 0.10 (reference) and F = 0.48 (Pheroid™) ($p < 0.0001$). The bioavailability of artemiside was not dramatically enhanced by the Pheroid™ delivery system. It was also established that artemiside was metabolically converted to artemisone.

1. Introduction

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 which 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 [1].

Drug-resistant malaria is a huge threat and desperate measures should be taken to ensure the preservation of effectiveness of current antimalarial drugs. Drug-resistant malaria materializes with evolutionary, single or multiple, point-mutations in the *Plasmodium* genome rendering parasites that are drug insensitive [2]. The emergence and spread of this phenomenon has greatly affected the control and treatment of malaria in endemic countries, specifically concerning *P. falciparum* infections [3]. Combination drug therapy is currently the mainstay approach in preventing the development of further resistance to current antimalarials. Artemisinin-based combination therapy is the treatment of choice and it is of great importance that the efficacy of those therapeutic regimens is maintained. There is presently no other effective alternatives to surmount the ever increasing problem of drug resistance, it is thus essential to focus all efforts on the research and development of novel antimalarial compounds [4] [5].

Artemisinins are natural products and was first developed in China in the 1960's. Artemether, an artemisinin derivative, is known to be as effective as quinine in the treatment of severe *P. falciparum* malaria. Other artemesinin derivatives include artesunate, artemotil and dihydro-artemisinin. One key advantage of these agents is the fact that they are active against all of the red blood cell stages of *P. falciparum* [6]. At this stage there is limited resistance to these agents. Due to the short elimination half-life of artemisinin-based drugs it is recommended that they are used in combination with other drugs such as mefloquine, lumefantrine or amodiaquine as first-line therapies for the treatment of uncomplicated malaria [2]. New artemisinin derivatives such as artemisone and artemiside are reported to be much more potent than the existing derivatives and it would be of great value to optimize the delivery of these compounds by using novel drug delivery systems [7].

Alternative drug delivery options such as the Pheroid™ delivery system may play a key role in ensuring effective delivery and enhanced bioavailability of these novel antimalarial compounds. Pheroid™ technology is a patented, novel, colloidal type drug delivery system.

It primarily consists of plant and fundamental fatty acids which have the ability to capture, transport and deliver pharmacologically active compounds and other valuable molecules. The Pheroid™ delivery system is superior to most other delivery systems and is able to improve the delivery of dynamic complexes, reduce the time to onset of action, decrease the minimal effective drug concentration and enhance therapeutic efficacy. It is also able to indirectly decrease the cytotoxicity of therapeutic compounds and to infiltrate virtually all known barriers in the body. The system is further capable of targeting specific treatment areas, to transport genetic material to the cellular nucleus and to decrease drug resistance [8]. The aim of this study was to evaluate the oral absorption profile of artemisone and artemiside in Pheroid™-based formulations.

2. Materials and Methods

2.1 Materials

Artemisone and artemiside were kindly donated by The Department of Chemistry, Open Laboratory of Chemical Biology, Institute of Molecular Technology for Drug Discovery and Synthesis, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, P.R. China. Vitamin F ethyl ester was obtained from Kurt Richter Pharma (Germany) and Cremaphor® EL was obtained from BASF (Germany). *D*-α-tocopherol, polyethylene glycol (PEG) and butylated hydroxyanisole (BHA) were purchased from Chempure (South Africa). Analytical grade artemisinin and dimethyl sulfoxide (DMSO) were obtained from Merck (South-Africa). All other reagents used were also of analytical grade.

2.2 Drug administration

Artemisone and artemiside were evaluated for their pharmacokinetic properties in a mouse model. The drugs were tested in both a reference formulation and a Pheroid™ vesicle formulation. The animals utilized were male C57 BL6 mice, weighing approximately 25 g each. The study and all procedures were approved by the Ethics Committee of the University of Cape Town, approval number 009/034. The drugs were administered via the oral and intravenous routes. Test animals were randomly allocated to each group. The exact procedure is described below:

Artemisone

Reference group (p.o.), (N = 10): Artemisone was administered orally at a dose of 50.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 5, 10, 30, 60 and 120 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at –20 °C.

Pheroid™ group (p.o.), (N = 10): Artemisone in Pheroid™ vesicles was also administered orally at 50.0 mg/kg. The same protocol was used as described for the oral reference group.

Reference group (IV), (N = 10): Artemisone was administered intravenously at a dose of 5.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 5, 10, 30, 60 and 120 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at –20 °C.

Pheroid™ group (IV), (N = 10): Artemisone in Pheroid™ vesicles was administered intravenously at a dose of 5.0 mg/kg. The same protocol was used as described for the IV reference group.

Artemiside

Reference group (p.o.), (N = 10): Artemiside was administered orally at a dose of 50.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 10, 30, 60, 120 and 180 minutes after drug administration. The samples were centrifuged and 15 µl plasma was recovered and stored at –20 °C.

Pheroid™ group (p.o.), (N = 10): Artemiside in Pheroid™ vesicles was administered orally at 50.0 mg/kg. The same protocol was used as described for the oral reference group.

Reference group (IV), (N = 5): Artemiside was administered intravenously at a dose of 5.0 mg/kg in DMSO / water (1:9, v/v). The total volume per administration was 200 µl. Blood samples (50 µl) were collected via tail-bleeding at 10, 30, 60, 120 and 180 minutes after drug administration. The samples were centrifuged and 15 µl plasma was collected from each sample and stored at –20 °C.

Pheroid™ group (IV), (N = 10): Artemiside in Pheroid™ vesicles was administered intravenously at a dose of 5.0 mg/kg. The same protocol was used as described for the IV reference group. The metabolic conversion of artemiside to artemisone was also investigated in this group.

2.3 Reference formulations

An intravenous dose of 5.0 mg/kg bodyweight of either artemisone or artemiside was administered to serve as reference standards for calculation of the absolute bioavailability of the compounds. Oral reference formulations were also prepared at a dose of 50.0 mg/kg bodyweight for each compound to compare against the Spheroid™ vesicle (entrapped drug) formulations. The reference formulations were prepared immediately before injection/administration by dissolving the appropriate amount of each drug in analytical grade DMSO. Purified water was then added until the desired volume was reached. The final DMSO to water ratio was 1:9 v/v. Both of the reference formulations formed a micro-suspension after the addition of water to the DMSO-drug solution. This hydrophobic interaction is not uncommon and it was previously reported that most artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration [9].

2.4 Pheroid™ formulations

Pheroid™ vesicles were prepared by melting vitamin F ethyl ester (66.2g), Cremaphor® EL (27.6 g) and *D*-α-tocoferol (1.0 g). PEG was then added (5.0 g) together with BHA (0.2 g). This mixture constituted the oil-phase of the Pheroid™ vesicle formulations. Nitrous oxide water was also prepared by saturating purified water with N_2O under high pressure. The required amount of drug was added to 1.0 ml of the prepared oil-phase (at room temperature). This mixture was then agitated for approximately 1 - 2 minutes until all the drug particles had dissolved. The prepared nitrous oxide water was then added the oil-phase to achieve a total volume of 10.0 ml (oil : water, 1:9 v/v). The mixture was homogenised with a Heidolph Diax 600 homogeniser (Labotec, South Africa) at 8000 rpm for 1 minute. The homogenous mixtures were then transferred to amber glass bottles.

2.5 Measurement of drug content

2.5.1 Instrumentation

A sensitive and selective LC/MS/MS method was developed to determine the drug concentrations in the collected plasma samples. An Agilent 1200 series HPLC system and an Applied Biosystems API 3200 triple quadrupole mass spectrometer from Applied Biosystems were used.

2.5.2 Calibration standards

Stock solutions of artemisone and artemiside were prepared in ethanol at a concentration of 1 mg/ml. Blank mouse plasma (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 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). Artemisinin was added to act as an internal standard (ISTD). The calibration standards were briefly vortexed, aliquotted into labelled polypropylene tubes and stored at -20 °C.

2.5.3 Mass spectrometry

The detection of artemisone, artemiside and artemisinin (internal standard, ISTD) was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode, MRM). The settings on the apparatus are summarized in tables I and II.

Table I: ESI settings

Curtain gas	20
Collision gas	5
Ionspray voltage (V)	5000
Source temperature (°C)	500
Gas 1 (psi)	50
Gas 2 (psi)	60

Table II: MS/MS settings

Setting	Artemisone	ISTD	Artemiside
Q1 mass $[M+H]^+$	402.2	283.2	370.2
Q3 mass	163.2	151.1	163.2
Dwell time (ms)	150	150	150
Declustering potential (V)	46	26	26
Entrance potential (V)	2	9.5	5
Collision energy (V)	25	21	25
Collision cell exit potential (V)	4	4	4
Scan type	MRM	MRM	MRM
Polarity	positive	positive	positive
Pause time (ms)	5	5	5

2.5.4 Chromatography

Chromatography was performed on a Phenomenex Gemini-NX (5 µ, C18, 110A, 50 x 2 mm) analytical column using an Agilent 1200 series HPLC. For artemisone the mobile phase consisted of methanol and ammonium acetate (10 mM with 0.1% acetic acid) (60:40) and was delivered at 0.5 ml/min for 3 minutes. The organic phase was increased after 3 minutes to 95% for another 2 minutes to clean the column and was then brought back to 60% organic phase for 3 minutes to equilibrate the column. For artemiside the mobile phase consisted of methanol and ammonium acetate (10 mM with 0.1% acetic acid) (75:25) and was delivered at 0.5 ml/min for 3 minutes. The column was kept in a column compartment at 35 °C. An autosampler injected 10 µl (artemisone) and 5 µl (artemiside) 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 cooled to 5 °C whilst awaiting injection.

2.5.5 Liquid-liquid extraction

The extraction procedure was performed on ice using polypropylene test tubes. The plasma samples were thawed on ice and briefly vortexed. Twenty five microlitres of a universal Britton Robinson buffer (pH 9) was aliquotted into clean polypropylene tubes and 15 µl of the plasma sample was added. The ISTD was spiked at an appropriate concentration into

the universal buffer and 25 µl was subsequently added to the extraction tubes. 1-Chlorobutane (350 µl) was also added to each tube to function as an organic solvent. The samples were vortexed for 1.5 minutes and centrifuged for 5 minutes at high speed. The organic phase (300 µl) was transferred to clean polypropylene tubes and evaporated under vacuum in a rotor evaporation system at 30 °C for 45 minutes. Mobile phase (50 µl) was added to the dry samples. The samples were then vortexed for 30 seconds and transferred to 96 well polypropylene plates. Five or ten microlitres of each sample was then injected into the HPLC column and analyzed.

2.6 Pharmacokinetic parameters and statistical evaluation

The recovered experimental data was evaluated in terms of the drug plasma concentration versus time. The following pharmacokinetic (*PK*) parameters were calculated:

- the peak drug plasma concentration (C_{max}) in ng/ml;
- time to peak plasma concentration (T_{max}), only for the oral dose experiments;
- apparent elimination half-life ($T_{1/2}$);
- area under the plasma concentration-time curve between time zero and the time of last sample collection ($AUC_{0\text{-}last}$) in ng.h/ml;
- area under the plasma concentration-time curve from time zero to infinity ($AUC_{0\text{-}inf}$) in ng.h/ml and
- the absolute and relative bioavailability was then subsequently calculated.

Noncompartmental analysis was used to calculate the *PK* parameters for artemisone and artemiside (WinNonlin version 5.2, Pharsight Corporation, California, USA). Linear interpolation was used to determine the area under the concentration time curve. $AUC_{0\text{-}last}$ is defined as the AUC computed from time zero to the time of the last Y-value above the lower limit of quantitation of the assay. All values below this limit were treated as "missing". $AUC_{0\text{-}Inf}$ was calculated by extrapolating the concentration time curve from time zero to infinity, using the last three concentration time points to estimate the elimination rate constant (λz). This constant was also used to determine the observed elimination half-life ($T_{1/2}$) of the compounds. The summary statistics and Mann-Whitney non-parametric test were performed using Prism version 4 (GraphPad Software Inc., California, USA).

3. Results

3.1.1 Intravenous artemisone formulations

The results obtained with the intravenous artemisone reference formulation and Pheroid™ vesicle formulation are presented in the form of an overlay of the mean concentration versus time graphs in figure 1. The pharmacokinetic (*PK*) parameters are presented in Table III.

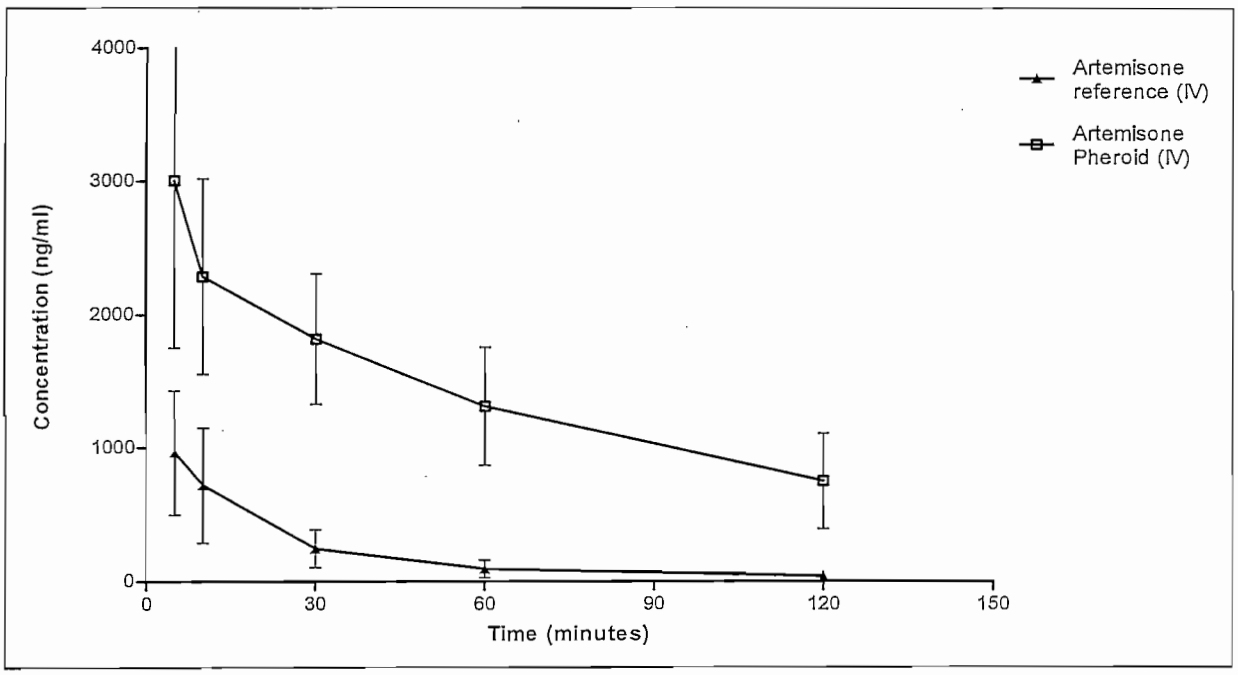

Figure 1: Mean concentration vs. time graph of the intravenous artemisone reference formulation and Pheroid™ vesicle formulation

The incorporation of artemisone in a Pheroid™ vesicle formulation for intravenous administration produced very promising results. The C_{max} achieved by incorporating artemisone in a Pheroid™ vesicle formulation was significantly greater than that achieved by the reference formulation. The reference formulation had a C_{max} of 962.5 ng/ml while the Pheroid™ vesicles produced a C_{max} of 3006.0 ng/ml ($p < 0.0001$). The difference translates to an increase in artemisone's C_{max} by approximately 210% with the aid of the Pheroid™ delivery system. The incorporation of artemisone in Pheroid™ vesicles also had a major impact on the $T_{1/2}$ of the drug, the reference had a $T_{1/2}$ of approximately 30 minutes ($T_{1/2}$ = 0.49 hours) while the Pheroid™ vesicles extended the $T_{1/2}$ to more than 70 minutes ($T_{1/2}$ = 1.18 hours) ($p < 0.005$). The difference amounts to an increase of more than twice the value achieved by the reference formulation. There was also a very significant difference

when comparing the calculated AUC values. The reference group presented with an arithmetic mean $AUC_{0\text{-last}}$ value of 464.8 ng.h/ml while the Pheroid™ formulation presented with a value of 3009.0 ng.h/ml ($p < 0.0001$). The calculated $AUC_{0\text{-inf}}$ values also reflected the same significant difference that was observed in the mean $AUC_{0\text{-last}}$ values, the reference was represented by an arithmetic mean $AUC_{0\text{-inf}}$ value of 496.0 ng.h/ml while the Pheroid™ formulation presented with a value of 4431.0 ng.h/ml ($p < 0.0001$).

Table III: Summary of the pharmacokinetic parameters

Formulation: 50.0 mg/kg p.o. and 5.0 mg/kg IV	Mean T_{max} (h)	Mean C_{max} (ng/ml)	Mean $T_{1/2}$ (h)	Mean $AUC_{0\text{-last}}$ (ng.h/ml)	Mean $AUC_{0\text{-inf}}$ (ng.h/ml)
Artemisone (reference, p.o.)	0.12 ± 0.01	809.50 ± 98.12	0.36 ± 0.06	485.7 ± 106.30	604.6 ± 143.80
Artemisone (Pheroid™, p.o.)	0.55 ± 0.05	1550.00 ± 105.40	1.10 ± 0.26	2219.00 ± 122.70	3094.00 ± 392.10
Artemisone (reference, IV)	N/A	962.50 ± 147.20	0.49 ± 0.07	646.80 ± 74.17	496.00 ± 74.70
Artemisone (Pheroid™, IV)	N/A	3006.00 ± 396.80	1.18 ± 0.13	3009.00 ± 312.90	4431.00 ± 545.30
Artemiside (reference, p.o.)	*0.55 ± 0.19*	*116.40 ± 14.03*	*7.52 ± 4.84*	*174.20 ± 27.47*	*749.80 ± 387.40*
Artemiside (Pheroid™, p.o.)	*0.57 ± 0.09*	*137.70 ± 18.44*	*6.50 ± 4.46*	*211.20 ± 30.80*	*554.20 ± 212.4*
Artemiside (reference, IV)	*N/A*	*N/D*	*N/D*	*25.74 ± 7.71*	*N/D*
Artemiside (Pheroid™, IV)	*N/A*	*N/D*	*0.98 ± 0.30*	*1350.00 ± 246.92*	*N/D*
Artemisone converted from Artemiside	*N/A*	*N/D*	*N/D*	*51.02 ± 30.21*	*N/D*

* N/A - Not applicable
* N/D - Not determined

3.1.2 Oral artemisone formulations

The *PK* parameters of the oral reference and the oral Pheroid™ vesicle group were calculated and are given in table III. The results are graphically presented in the form of an overlay of the mean concentration versus time graphs in figure 2.

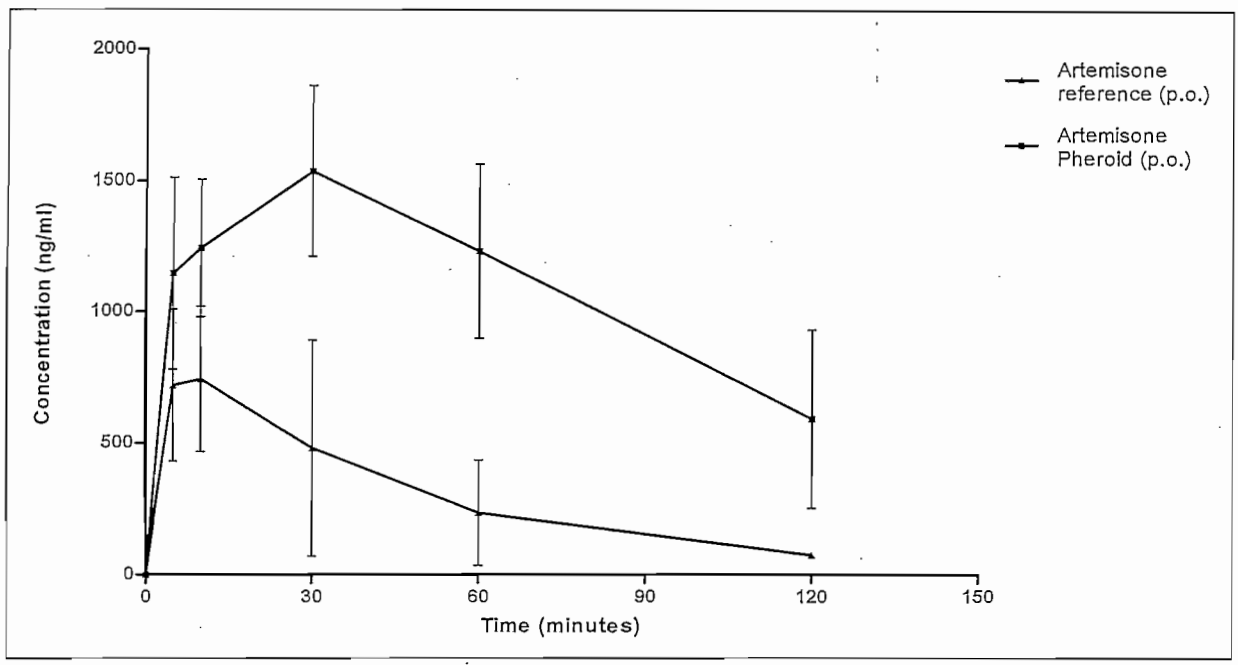

Figure 2: Mean concentration vs. time graph of the oral artemisone reference and Pheroid™ vesicle formulations

The incorporation of artemisone in a Pheroid™ vesicle formulation produced very promising results. The T_{max} of artemisone, when incorporated in Pheroid™ vesicles, was increased dramatically. When comparing the T_{max} of the two data sets, the reference formulation rendered a T_{max} of just 7 minutes (0.12 hours) while a time delay was observed with the Pheroid™ vesicle formulation for which the T_{max} was approximately 30 minutes (0.55 hours) ($p < 0.0001$). The T_{max} of artemisone, when incorporated in the Pheroid™ vesicle formulation, was delayed by a time-factor of 4 in comparison to that of the reference formulation. The C_{max} was greatly increased by the Pheroid™ vesicle formulation, the reference formulation produced a C_{max} of 809.5 ng/ml while the Pheroid™ vesicle formulation gave a C_{max} of 1550.0 ng/ml ($p < 0.005$). That amounts to an increase in artemisone's C_{max} by approximately 90% with the aid of the Pheroid™ delivery system. The incorporation of artemisone in a Pheroid™ vesicle formulation also had a major impact on the $T_{1/2}$ of the drug. The reference formulation had a $T_{1/2}$ of approximately 20 minutes

($T_{1/2}$ = 0.36 hours) while the Pheroid™ vesicle formulation extended the $T_{1/2}$ to more than 60 minutes ($T_{1/2}$ = 1.10 hours) ($p < 0.005$). This difference amounts to an increase of more than 3-times that of the reference formulation.

The bioavailability of artemisone was also determined. The data obtained from the intravenously administered artemisone reference formulation was required to determine the absolute bioavailability. This data is presented in table III and are graphically depicted in figure 1. The relative bioavailability was determined by using the calculated arithmetic mean of the area under the curve ($AUC_{0\text{-last}}$) values of both the oral reference formulation and the oral Pheroid™ vesicle formulation data. There was a significant difference between the two values (reference, 458.7 ng.h/ml and Pheroid™, 2219.0 ng.h/ml) ($p < 0.0001$). The $AUC_{0\text{-Inf}}$ for the reference formulation was calculated as 604.6 ng.h/ml and 3094.0 ng.h/ml for the Pheroid™ vesicle formulation ($p < 0.0001$). Equations *1* and *2* were used to calculate the relative and the absolute bioavailability:

$$\text{Relative availability (RA)} = \frac{[AUC]_A}{[AUC]_B} \qquad \textit{Eq. 1}$$

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

$$\text{Absolute availability (F)} = \frac{[AUC]_{po} * dose_{IV}}{[AUC]_{IV} * dose_{po}} \qquad \textit{Eq. 2}$$

where $[AUC]_{po}$ is represented by the arithmetic mean of the $AUC_{0\text{-last}}$ values of either the oral reference formulation or the oral Pheroid™ vesicle formulation. The arithmetic mean of the $AUC_{0\text{-last}}$ values of the intravenous reference is represented by $[AUC]_{IV}$. The dose, IV and p.o., are respectively represented by the intravenous reference dose of 5.0 mg/kg and the oral dose of 50.0 mg/kg.

The relative bioavailability of the Pheroid™ vesicle formulation was RA = 4.57 in comparison to the reference formulation which was represented by RA = 1.00. These calculated values imply that the Pheroid™ vesicle formulation was 4.57 times more bioavailable than the reference formulation, or when converted to percentage values, reasoning that the reference

is represented by 100%, the Pheroid vesicle formulation was 357% more bioavailable. When calculating the absolute bioavailability (F) it was reasoned that an intravenous injection had a bioavailability of 100% since it was directly introduced into the blood, that scenario is represented by F = 1.00. The absolute bioavailability of the reference formulation was calculated as F = 0.10. The Pheroid™ vesicle formulation gave an absolute bioavailability of F = 0.48. The difference translates to a 4.80 factorial increase in the absolute bioavailability of artemisone.

3.1.3 Intravenous artemiside formulations

The *PK* parameters of the intravenous reference formulation and intravenous Pheroid™ vesicle formulation are given in Table III. The corresponding graphs are presented in figures 3 and 4. For clarity purposes the graphs are presented separately to compensate for the huge differences in the plasma concentrations.

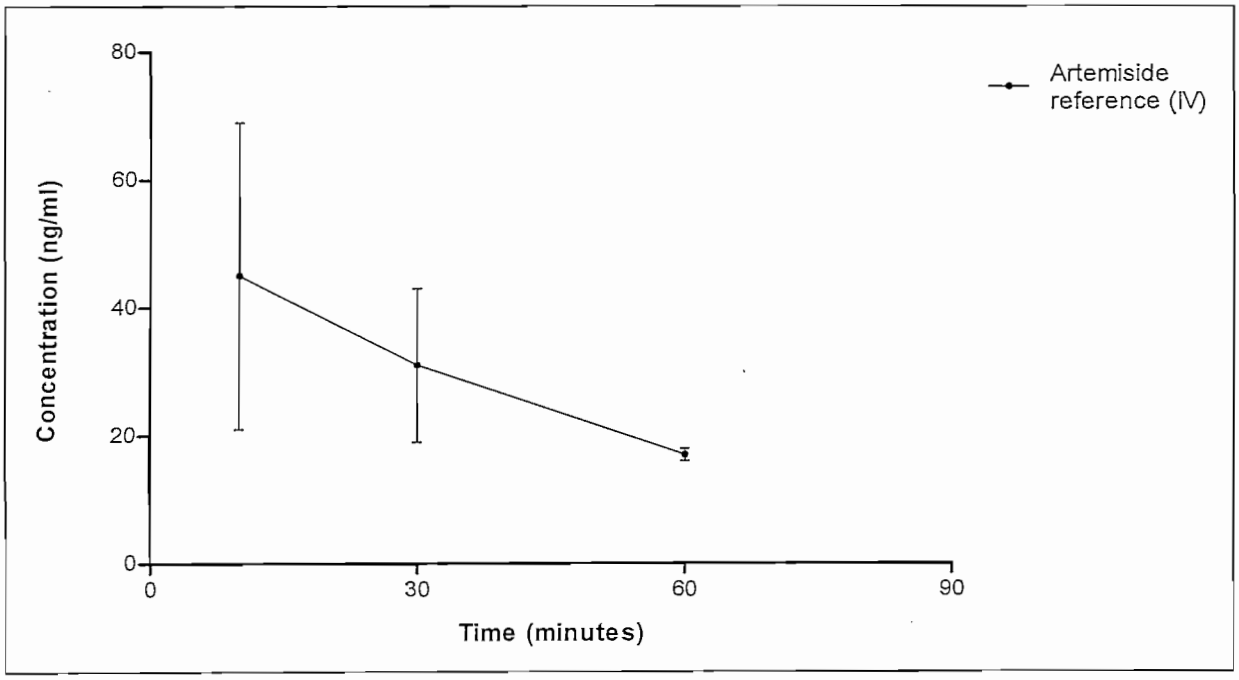

Figure 3: Mean concentration vs. time graph of artemiside in the reference formulation (IV)

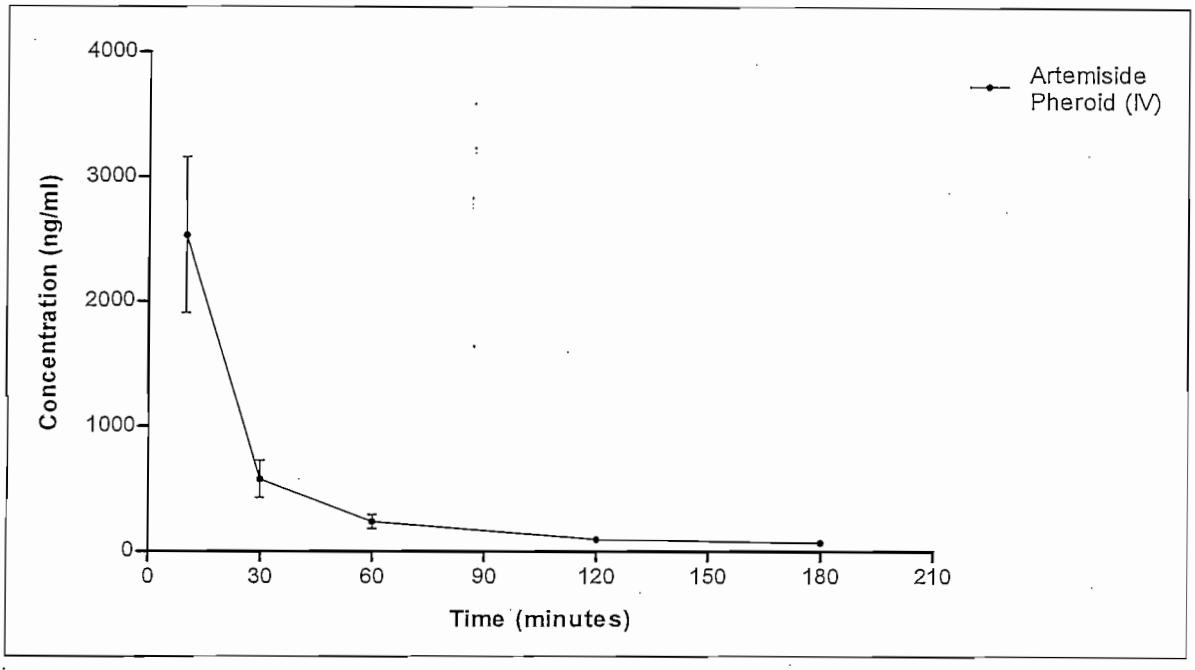

Figure 4: Mean concentration vs. time graph of artemiside in a Pheroid™ vesicle formulation (IV)

It should be kept in mind that the reference formulation formed a flakey suspension which led to non-uniform dosing of the test animals. It is also important to note that the reference formulation was only administered to 5 test animals (N = 5). These shortcomings rendered the derived data virtually unusable. The data is only included to illustrate the difference in the obtained plasma levels due to the improvement of artemisides' solubility when added to the Pheroid™ vesicle formulation. The incorporation of artemiside in a sterilized Pheroid™ vesicle formulation for intravenous administration rendered promising results. The C_{max} obtained by incorporating artemiside in a Pheroid™ vesicle formulation was significantly greater than that obtained with the reference formulation. The reference formulation had a C_{max} of 45.0 ng/ml while the Pheroid™ vesicle formulation had a C_{max} of 2533.0 ng/ml. The $T_{1/2}$ of the reference formulation was not calculated since the recovered data was very limited and questionable due to solubility issues and a limited amount of samples. The Pheroid™ vesicle formulation produced a $T_{1/2}$ of approximately 60 minutes ($T_{1/2}$ = 0.98 hours). Statistically there was also a very significant difference when comparing the calculated AUC values. The reference formulation had an arithmetic mean $AUC_{0\text{-last}}$ value of 25.7 ng.h/ml while the Pheroid™ vesicle formulation had a value of 1350.0 ng.h/ml ($p<0.0001$). The $AUC_{0\text{-inf}}$ values were not determined.

The evaluation of the metabolic conversion of artemiside to artemisone was rather interesting (figure 5). Artemiside was intravenously administered at a dose of 5.0 mg/kg in a Pheroid™ vesicle formulation. Metabolic conversion of artemiside rendered a small amount of artemisone, the arithmetic mean $AUC_{0\text{-last}}$ was calculated as 51.0 ng.h/ml. It was interesting to note that the amount of artemisone encountered in the plasma after Pheroid™ administration was more than double the amount of artemiside recorded in the plasma after administration with the reference formulation.

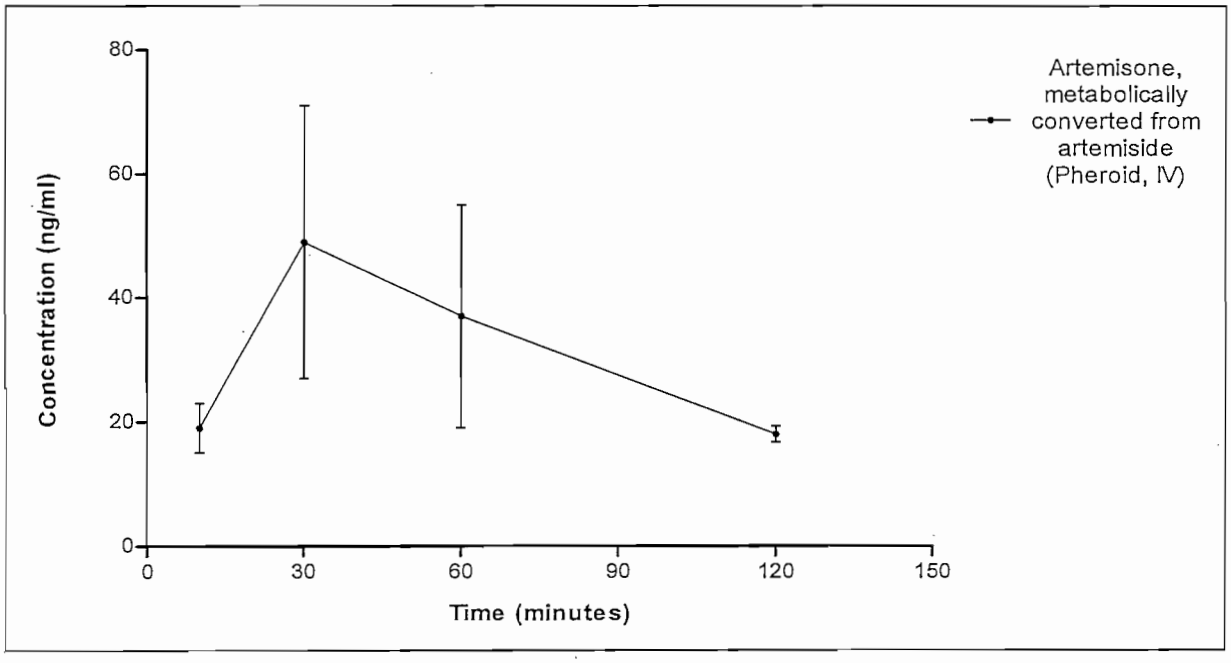

Figure 5: Mean concentration vs. time graph of artemisone after administration of artemiside in a Pheroid™ vesicle formulation (IV)

3.1.4 Oral artemiside formulations

The *PK* parameters of the oral reference formulation and the oral Pheroid™ vesicle formulation are presented in table III. The mean concentration versus time graphs are presented in figure 6.

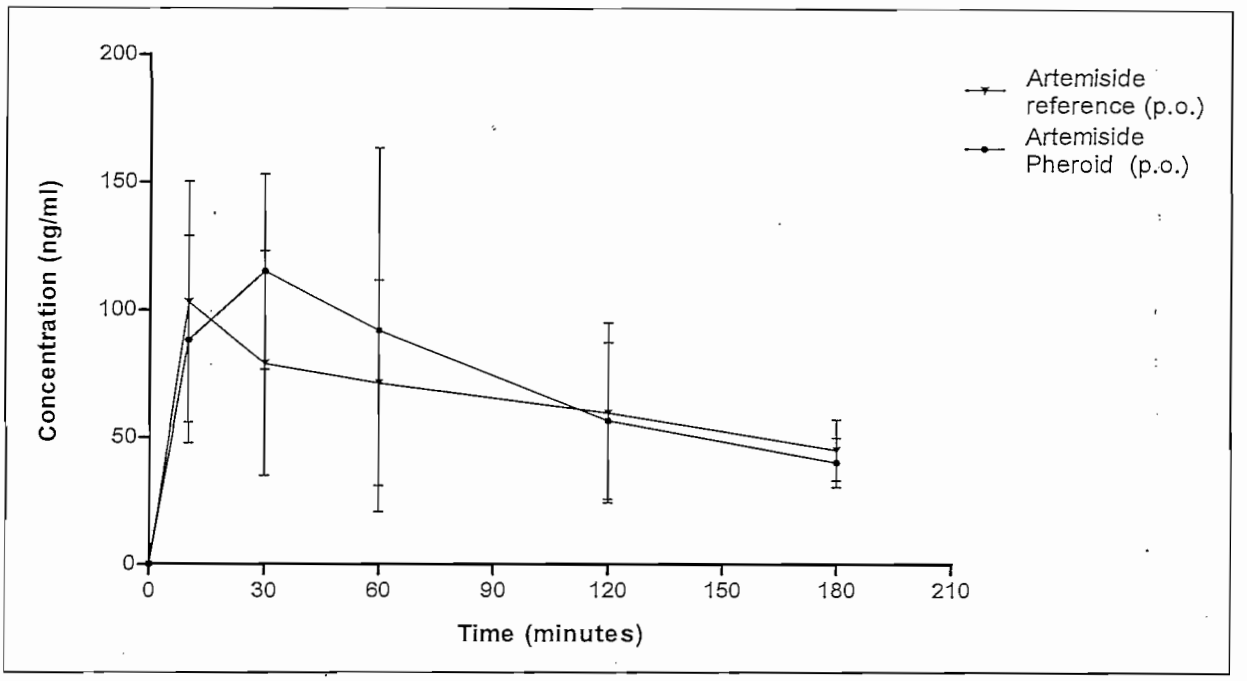

Figure 6: Mean concentration vs. time graph overlay of the oral artemiside reference formulation and Pheroid™ vesicle formulation

The incorporation of artemiside in a Pheroid™ vesicle formulation produced less promising results. The T_{max} of artemiside, when incorporated in a Pheroid™ vesicle formulation, was not extended as was the case with artemisone. When comparing the T_{max} values of the two data sets, the reference formulation had a T_{max} of approximately 30 minutes (0.55 hours) while the Pheroid™ vesicle formulation delayed the T_{max} to approximately 35 minutes (0.57 hours) ($p > 0.05$). The C_{max} was also not greatly increased by the Pheroid™ vesicle formulation, the reference formulation had a C_{max} of 116.4 ng/ml while the Pheroid™ vesicle formulation had a C_{max} of 137.7 ng/ml ($p > 0.05$). The difference amounts to an increase in artemiside's C_{max} by approximately 18% with the aid of the Pheroid™ delivery system. The data generated from this study did not lend itself to the accurate determination of either the $T_{1/2}$ or the $AUC_{o\text{-}inf}$. The reason for this would be in the explanation of how the elimination rate constant (λz) was calculated. A least squares regression was calculated using the last few points of the concentration curve, assuming that the elimination phase had been reached in the mouse. The slope of that regression then represents λz. Due to the fact that so few subjects were sampled, and due to some of the detected concentrations which did not return to zero, the estimation for certain subjects were exaggerated. The elimination rate constant for certain subjects could not be calculated due to that occurrence. For example, the concentrations detected in the last three samples collected from subject 2 (Pheroid™ vesicle formulation) were 36.0, 36.0 and 34.0 ng/ml respectively. The values would not

translate to the calculation of a useful $T_{1/2}$ or $AUC_{0\text{-}inf}$ value and subsequently result in grossly inflated estimations. Nevertheless, the incorporation of artemiside in Pheroid™ vesicles did not have a major impact on the calculated $T_{1/2}$ of the drug, the reference formulation had a calculated $T_{1/2}$ of approximately 450 minutes ($T_{1/2}$ = 7.52 hours) while the Pheroid™ vesicle formulation had a $T_{1/2}$ of approximately 400 minutes ($T_{1/2}$ = 6.49 hours) ($p > 0.05$). The $AUC_{0\text{-}inf}$ for the reference formulation was calculated as 749.80 ng.h/ml and 554.20 ng.h/ml for the Pheroid™ vesicle formulation ($p>0.05$).

The relative bioavailability was also determined by using the arithmetic mean of the calculated area under the curve ($AUC_{0\text{-}last}$) values of both the oral reference formulation and the oral Pheroid vesicle formulation data ($p > 0.05$).

The relative bioavailability of the Pheroid™ vesicle formulation was RA = 1.21 in comparison to the reference formulation which was represented by RA = 1.00. These calculated values imply that the Pheroid™ vesicle formulation was not significantly more bioavailable compared to the reference formulation.

Due to the poor solubility of artemiside in the reference formulation the data obtained from the intravenously administered reference formulation was neither accurate nor sufficient to accurately calculate the absolute bioavailability.

4. Discussion

The acquired *PK* results of artemisone were very promising. The concentration versus time graphs obtained from the reference formulation and the Pheroid™ vesicle formulation data were pharmacokinetically sound in terms of the basic configuration with well defined data points during both the absorption and elimination phases of the drug. The C_{max} and T_{max} were also well defined in these graphs. The calculated C_{max} and T_{max} values correlate very well with the apparent corresponding points on the concentration versus time curve of both the reference and Pheroid™ formulations.

The reference formulation produced significantly lower C_{max} and T_{max} values than the Pheroid™ formulation, the difference may likely be explained by less favourable absorption characteristics of the reference formulation. The reference formulation appeared to form a micro-suspension after the addition of water to the DMSO-drug solution. It was also previously reported that most artemisinins are known for their low aqueous solubility and resultant poor and erratic absorption upon oral administration [9]. The probability exist that

the Pheroid™ system was able to improve the solubility of artemisone and subsequently enhance the usual erratic absorption characteristics associated with this drug class by encapsulating the drug in a lipophilic layer. This was achieved by first dissolving artemisone in the oil-phase of the Pheroid™ system and then adding the appropriate amount of nitrous oxide water to promote the formation of a lipophilic shielding layer on the surface of the drug particles [10].

Various studies concerning the interaction of lipophilic compounds with intestinal fatty acid-binding protein (I-FABP) suggest that the binding of these lipophilic entities (Pheroid™ vesicles) to I-FABP may lead to an increase in the cytosolic solubility of those entities [10]. Thus increased cytosolic solubility may also subsequently facilitate the transport of the Pheroid™ vesicles from the intestinal lumen across the enterocytes to sites of drug distribution. This occurrence in conjunction with the improved solubility of artemisone in the Pheroid™ vesicles may explain the observed increase in the C_{max} and T_{max} values. The dramatic increase in C_{max} and T_{max} values also explain why the $T_{1/2}$ had more than doubled and the bioavailability increased with the use of Pheroid™ technology. This did not hold true for the reference formulation which was not able to completely dissolve the drug nor was it able to provide the same, well defined, lipophilic surface characteristics as the Pheroid™ system to promote an interaction with I-FABP.

The acquired *PK* results of artemiside were less promising. Artemiside appeared to be more hydrophobic than artemisone, the reference formulation also formed a precipitate with the addition of water to the DMSO-drug solution but the precipitate had a more flakey appearance than the artemisone reference. The graph obtained from the (water-based) reference formulation was difficult to interpret due to poor solubility characteristics of the drug. The poor solubility predisposed to erratic absorption which in turn led to irregularities in the concentration versus time curve which had relatively large SEM-values and were indicative of erratic absorption. The graph did not contain a representative drug concentration value during the absorption phase. This can be attributed to the fact that the first samples were collected at 10 minutes instead of 5 minutes. Since no previous *PK* studies had been conducted on this drug in a mouse model which could be used as a reference, the sample intervals were chosen based on the relatively short $T_{1/2}$ history of the drug class. Another consideration was the small blood volume of the test animals. Only 5 samples could be collected from each animal before the reduction in blood volume would start to have a concentrating effect of the drug in the plasma due to the subsequent decrease in the blood volume as a result the sample collection. The sample intervals were chosen to ensure the best possible utilization of the limited amount of samples. For future

studies the first samples should rather be collected at 2 - 5 minutes post administration than at 10 minutes. However, the T_{max} and C_{max} values on the reference formulation curve were still in relative accordance with the calculated values.

The drug concentration versus time curve of the artemiside Pheroid™ vesicle formulation was more favourable in terms of the basic configuration with definite data points during both the absorption and elimination phases of the drug. The C_{max} and T_{max} were both well defined in the corresponding graph. The calculated C_{max} and T_{max} values correlated well with the corresponding points on the concentration versus time curve of the Pheroid™ formulation. Artemiside was encapsulated by first dissolving the drug in the oil-phase of the Pheroid™ system and then adding the appropriate amount of nitrous oxide water to promote the formation of a lipophilic shielding layer on the surface of the drug particles [10]. Although a small improvement was observed in the results of this formulation in terms of the C_{max} and T_{max} over that of the reference there was not a very apparent increase in the bioavailability of artemiside when administered in conjunction with Pheroid™ technology. The Pheroid™ vesicle formulation, however, took a bit longer to reach its C_{max} in comparison to the reference formulation which made it possible to record definite data points during the absorption phase of the curve. This may probably be explained by the usually rapid conversion of most artemisinin derivatives to DHA or other metabolites [7]. In the case of artemiside it was proven that the drug was metabolically converted to artemisone and possibly also to a few other unidentified metabolites (figure 5).

It is very likely that the lipophilic shielding layer of the Pheroid™ system was also able to partially protect the drug against this rapid metabolic conversion. This in turn would explain the increase in the T_{max} and C_{max} of artemiside, the first samples were collected at 10 minutes and represented the T_{max} for the reference formulation while the same samples of the Pheroid™ vesicle formulation represented a point during the absorption phase, thus suggesting a slight delay in absorption.

When comparing the bioavailability results of artemisone to that of artemiside the differences become very apparent. Both compounds were orally administered at a dose of 50.0 mg/kg and yet the artemisone Pheroid™ vesicle formulation rendered a peak plasma concentration of approximately 1500.0 ng/ml in contrast to the 137.0 ng/ml of the artemiside Pheroid™ vesicle formulation. The most probable explanation for this occurrence may lie in differences in the metabolic conversion and degradation of these drugs. Most artemisinin class drugs are very quickly converted to DHA or other metabolites after oral administration while artemisone, in contrast to other artemisinins, are not converted to DHA. Artemisone is

metabolized to the products M1 - M5 [7]. The conversion of artemiside, to artemisone, seems to occur at a more rapid pace in comparison to the metabolic conversion rate of artemisone itself. These differences in metabolic products and the varied rapidness of the conversion rates may shed some light on the differences encountered in the *PK* results of the two compounds. The sensitive and selective LC/MS/MS method was developed to determine either the artemisone or artemiside concentration of the plasma samples and not that of the metabolic products. Since the first plasma samples were collected at 10 minutes post administration the possibility exist that a large fraction of the artemiside was already converted to either artemisone or other products in the acidic milieu of the stomach before or during the absorption phase of the drug. A large fraction of this conversion had most likely occurred before the first samples were collected which would explain the low artemiside plasma levels. Had the initial samples of the oral reference and Pheroid™ formulations been collected at 5 minutes post administration and had the LC/MS/MS been set up to rather determine both the artemisone and the artemiside content of the plasma samples the results might have indicated a proportional increase in the plasma concentration of artemisone in relation to a proportional decrease in the plasma levels of artemiside. In the case of artemisone, which is converted to products M1 - M5, the possibility exist that the drug was absorbed unchanged and that the metabolic transformation to products M1 – M5 was less rapid than in the case of artemiside. The possibility also exist that this conversion only occurred during the elimination phase of the drug which would explain its much higher peak plasma concentrations in relation to that of artemiside. However, with these arguments in mind it is clear that further studies are required to fully elucidate the obtained results.

5. Conclusion

Artemisone was proven to be relatively bioavailable. The *PK* analysis of artemisone was instrumental in explaining its previously reported, remarkable, *in vivo* efficacy [7]. The data also delivered compelling evidence in favour of the ability of the Pheroid™ delivery system to further enhance the bioavailability of artemisone. All the experiments indicated a very dramatic improvement in the C_{max} and $T_{1/2}$ of the drug and a time delay in T_{max} when administered in a Pheroid™ vesicle formulation. This effectively translates to a scenario where the drug concentration could be significantly decreased and still achieve therapeutic drug plasma concentrations. The combination of artemisone and Pheroid™ technology may perhaps prove to be an essential component in antimalarial combination therapy regimens in the very near future.

The Pheroid™ delivery system did not produce results to the same extent with artemiside as it did with artemisone. Only a marginal increase was observed in the T_{max} and C_{max} values with no added benefit to the $T_{1/2}$ of the drug. The sharp contrast between the artemisone and artemiside *PK* results may be attributed to varying solubility characteristics and the different metabolic pathways and metabolic products of the drugs.

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