

Pheroid™-based anti-malarial formulations – HPLC method development and stability studies

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ABBREVIATIONS

% RSD	% Relative Standard Deviation
DDT	Dichloro-diphenyl-trichloroethane
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
ICH	International Conference of Harmonisation
IPT	Intermittent preventive treatment
ITN	Insecticide-treated nets
PEG	Polyethylene glycol
RH	Relative Humidity
SAMF	South African Medicines Formulary
SD	Standard Deviation
STD	Standard
USP	United States Pharmacopoeial Convention
V _d	Volume of Distribution
WHO	World Health Organization

ABSTRACT

Malaria remains one of the world's most common infectious diseases with 300 to 500 million cases every year. The current treatment of malaria is restricted due to ever increasing drug resistance, cost of treatment and the availability of treatment. When looking at solutions for these problems, new drug delivery systems should be taken into consideration. The Pheroid™ drug delivery system which incorporates Pheroid™ technology can be placed in this category and the stability of anti-malarial drugs in combination with the Pheroid™ drug delivery system was investigated.

The main objectives of this study were the development of HPLC methods for the analysis of the anti-malarials in the Pheroid™ drug delivery system, the validation of the HPLC methods developed and the determination of drug stability of the formulated products during accelerated stability conditions.

Mefloquine HCl and chloroquine phosphate were each separately formulated in both the Pheroid™ and pro-Pheroid systems. HPLC analysis was done after each month according to the different assay methods.

Accelerated stability studies, conducted on the four products formulated, indicate that only chloroquine phosphate in Pheroid™ complies with specifications. This product may be further developed for possible commercialisation. It was found that chloroquine phosphate cannot be formulated with pro-Pheroid, because of solubility issues.

Results for mefloquine HCl in microsponges were inconclusive. Assay values for months 1 and 3 were low, whilst values for month 2 were much higher. Despite the fact that mefloquine HCl in pro-Pheroid did not comply with assay specifications, the results obtained were consistent.

With the HPLC assay methods used, peak interference from the Pheroid™ system was significant. It would be advisable to further develop the methods to eliminate this problem. Questionable results may then be re-examined to distinguish between poor product performance and analytical issues.

ABSTRAK

Malaria bly nog steeds een van die wêreld se mees algemene infektiewe siektes met 300 tot 500 miljoen gevalle jaarliks. Die huidige malaria behandeling word beperk deur toenemende geneesmiddelweerstandigheid, die koste van behandeling en die beskikbaarheid van behandeling. Wanneer daar na oplossings gekyk word vir hierdie probleme moet nuwe geneesmiddelafleweringsisteme ook in ag geneem word. Die Pheroid™ geneesmiddelafleweringsstelsel wat Pheroid™ tegnologie inkorporeer kan in hierdie kategorie geplaas word en die stabiliteit van die anti-malaria middels in die Pheroid™ stelsel was ondersoek.

Die hoofdoelwitte van hierdie studie was om HPLC metodes te ontwikkel vir die analise van anti-malaria middels in die Pheroid™ geneesmiddelafleweringsstelsel, die validasie van hierdie HPLC metodes en die bepaling van geneesmiddelstabiliteit van die geformuleerde produkte gedurende versnelde stabiliteitskondisies.

Mefloquine HCl en chloroquine fosfaat was elk apart geformuleer in beide die Pheroid™ en pro-Pheroid stelsel. Na elke maand was HPLC analises uitgevoer volgens die verskillende metodes.

Versnelde stabiliteitstudies, uitgevoer op die vier geformuleerde produkte, dui daarop dat slegs die chloroquine fosfaat in Pheroid™ aan die vereistes vir gehaltebepaling voldoen het. Hierdie produk kan verder ontwikkel word vir moontlike kommersialisering. Daar was bevind dat chloroquine fosfaat nie in die pro-Pheroid geformuleer kan word nie as gevolg van oplosbaarheidsprobleme.

Die resultate vir die mefloquine HCl in mikrosponsies was nie beslissend nie. Gehaltewaardes vir maande 1 en 3 was laag, terwyl die waardes vir maand 2 baie hoër was. Ten spyte van die feit dat mefloquine HCl in die pro-Pheroid nie voldoen aan die spesifikasies nie, het die resultate konstant gebly.

Pieksteuring van die Pheroid™ stelsel met die HPLC metodes wat gebruik is, was betekenisvol. Dit sal raadsaam wees om die metodes verder te ontwikkel om die probleem te elimineer. Twyfelagtige resultate mag dan herevalueer word om te onderskei tussen swak produkvertoning en analitiese probleme.

AIM AND OBJECTIVES

Malaria remains one of the world's most common infectious diseases with 300 to 500 million cases every year. *P. falciparum* is the most common form found in Southern Africa including South Africa and also the most difficult to treat since it has developed resistance to nearly all anti-malarials currently in use. To combat drug resistance the WHO suggests the use of combination therapy although mismatched pharmacokinetics can also play a role in the development of resistance. A strategy to prevent drug resistance that has received much attention recently, is the combination of anti-malarial drugs such as mefloquine, sulfadoxine-pyrimethamine or amodiaquine with an artemisinin derivative. Developing new drugs and new therapeutic options are also being looked at. A possible new therapeutic option is the use of a drug delivery system that can bypass the method of current drug resistance.

The Pheroid™ drug delivery system is a novel, patented, colloidal delivery system, using Pheroid™ technology that consists mainly of modified essential fatty acids. It increases the amount of drug at the target site; it increases bioavailability and drug absorption and most important, in the case of malaria, it decreases drug resistance. It shows great potential for use with current anti-malarials. The anti-malarials used in this study were chloroquine phosphate and mefloquine HCl.

The aim of this study was to determine whether chloroquine phosphate and mefloquine HCl were stable in the Pheroid™ drug delivery system. The main objectives of this study were:

- Development of HPLC methods for the analysis of the anti-malarials in the Pheroid™ drug delivery system.
- Validation of the HPLC methods developed.
- Determination of drug stability of the formulated products during accelerated stability conditions.
- To establish the effectiveness of the preservatives chosen.

CHAPTER 1

Malaria

1.1 Introduction

Malaria is a disease that is caused by the *Plasmodium* parasite of which there are four species that effect humans, namely: *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae* (Anon, 2007a). These four species differ in geographical distribution, microscopic appearance, clinical features (periodicity of infection, potential for severe disease and ability to cause relapses) and potential for development of resistance to anti-malarials (Bloland, 2001). Resistance to anti-malarials has been reported for *P. falciparum*, *P. vivax* and, recently, *P. malariae* (World Health Organization, 2006).

The distribution of the malaria species are as follows (Anon, 2007a):

- *P. falciparum*: Africa (85 to 90% of cases), South East Asia, South America;
- *P. vivax*: South Asia, Central America, Central and East Africa;
- *P. ovale*: South and West Africa, West Pacific;
- *P. malariae*: infrequent all over.

P. falciparum is the most common form found in Southern Africa including South Africa. Mixed infections are possible although *P. falciparum* often overrides other species which is why the other species are often overlooked, remain untreated and can flare up once *P. falciparum* is removed. Malaria is transmitted by certain *Anopheles* mosquitoes (Anon, 2007a).

1.2 Life cycle of the malaria parasite

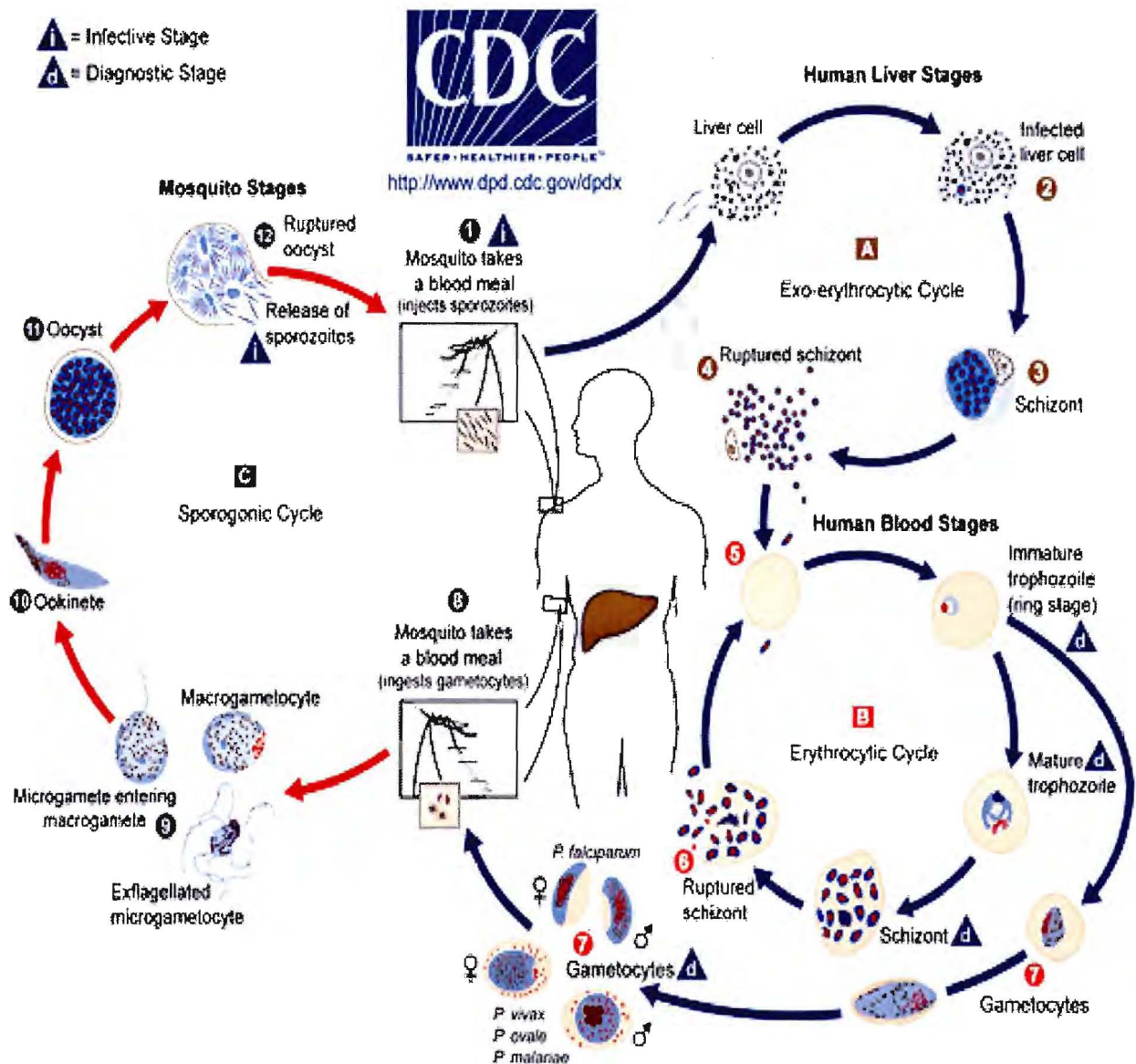


Figure 1.1: The life cycle of the malaria parasite (Anon, 2008a).

The malaria parasite life cycle involves two hosts (Figure 1.1). During a blood meal, a malaria infected female *Anopheles* mosquito inoculates sporozoites into the human host¹. Sporozoites infect liver cells² and mature into schizonts³, which rupture and release merozoites⁴. (Of note, in *P. Vivax* and *P. Ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony^A), the parasites undergo asexual multiplication in the erythrocytes

(erythrocytes schizogony⁸). Merozoites infect red blood cells⁵. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites⁶. Some parasites differentiate into sexual erythrocytic stages (gametocytes)⁷. Blood stage parasites are responsible for the clinical manifestations of the disease.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal³. The parasites' multiplication in the mosquito is known as the sporogonic cycle⁴. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes⁹. The zygotes in turn become motile and elongated (ookinetes)¹⁰ which invade the midgut wall of the mosquito where they develop into oocysts¹¹. The oocysts grow, rupture, and release sporozoites¹², which make their way to the mosquito's salivary glands. Inoculation of the sporozoites¹ into a new human host perpetuates the malaria life cycle (Anon, 2008a).

1.3 Malaria risk areas

Malaria occurs in 90 countries at present with 1.62 billion people living in areas where malaria is now increasing after having been reduced previously (Figure 1.2). 400 million people live with endemic malaria unchanged by control (Anon, 2007a).

Every year there are about 300 to 500 million cases of malaria making it one of the most common infectious diseases world wide (Bloland, 2001) with about 100 million deaths, of which 90 % is in Africa, south of the Sahara (Anon, 2007a). Young children, pregnant women and non-immune visitors to malaria-endemic areas are at great risk of contracting this severe or fatal illness (Bloland, 2001).

In South Africa the most affected areas are KwaZulu-Natal, Limpopo, Mpumalanga and the Northern Province (Anon, 2007b). According to an article on Health 24, health Minister Dr Manto Tshabalala-Msimang said deaths in South Africa have skyrocketed from 14 in 1992 to 423 in 2005 (Anon, 2007b). The number of reported cases of malaria have also increased from 2 872 in 1992 to 61 934 in 2005. Between June 2005 and March 2006 10 000 cases of malaria were reported in South Africa, this number dropped by 65 % during the corresponding period between 2006 and 2007 when only 3000 cases were reported.

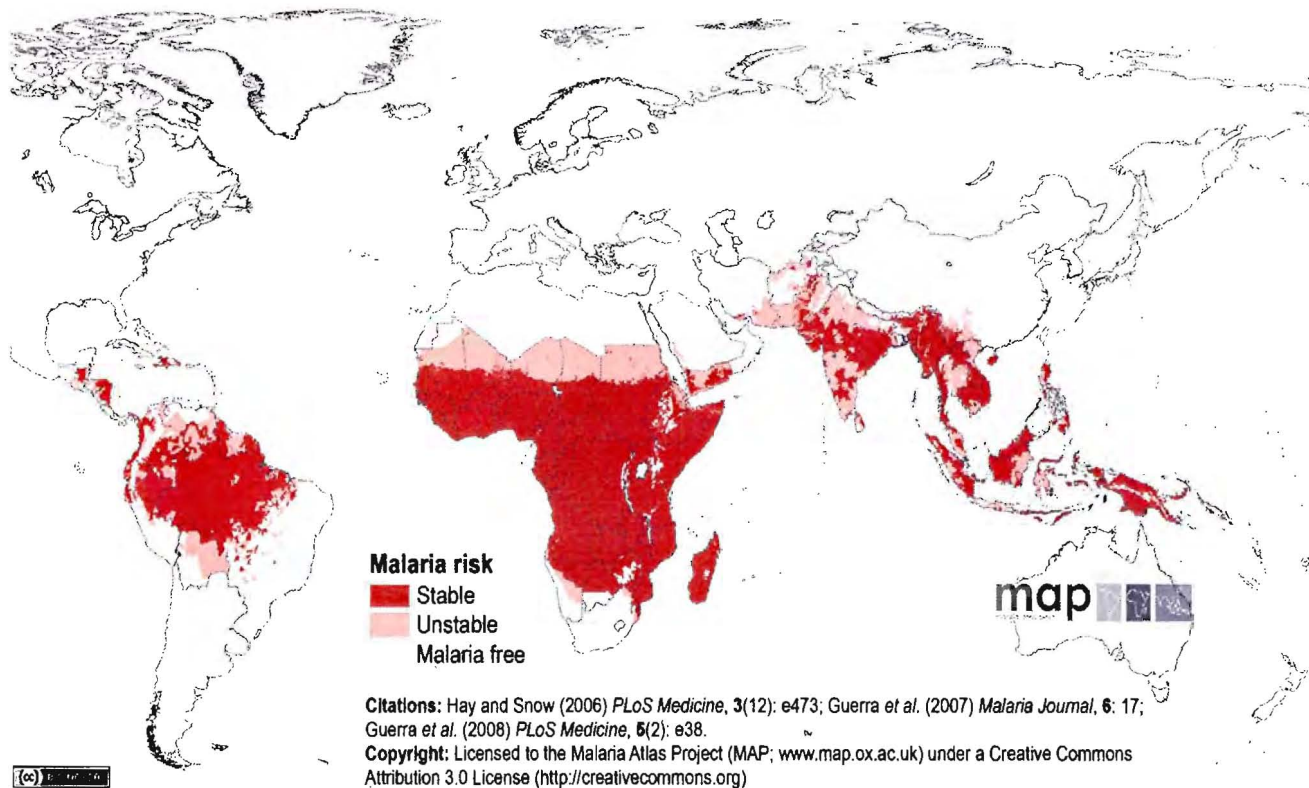


Figure 1.2: Map indicating malaria risk areas (Anon, 2008b).

The number of deaths has also been reduced by 73 % (Anon, 2008c). The South African health department has attributed this sharp decline to several interventions including:

- An increase in the indoor residual spraying of dichloro-diphenyl-trichloroethane (DDT) with the overall coverage of more than 80 % and with the spraying being completed before the peak in malaria transmission;
- The use of artemisinin – based combination therapy;
- Advocacy with mass community mobilisation and training of health care workers in the malaria affected areas (Anon, 2008c).

The Cape Times quoted Abdoulie Jack from the WHO office in Harare saying that malaria transmission from November 2007 to May 2008 was expected to be above normal in most parts of southern Africa because of the heavy rains and the likelihood of flooding in certain areas from December and thus precautionary measures were advised (Anon, 2008d).

1.4 Malaria prophylaxis

Prevention of mosquito bites is the mainstay of malaria prophylaxis. Non-drug measures should always be strictly applied (even when chemoprophylactic agents are used). These non-drug measures include:

- Visiting endemic areas during the dry season;
- Wearing light-coloured clothes, long sleeves and long trousers if outside between dusk and dawn;
- Applying insect repellent to clothing and exposed skin;
- Using mosquito nets, screens and coils/pads;
- Impregnating nets and clothing with pyrethroid insecticide (Gibbon, 2005).

A person at an increased risk of severe malaria should be discouraged from entering a malaria area. High-risk persons are:

- Pregnant women;
- Children under 5 years old;
- The elderly;
- Immunocompromised patients (Gibbon, 2005).

No anti-malarial agent is 100 % effective so travellers should be warned to seek medical attention immediately should flu-like symptoms develop (Gibbon, 2005).

Tables 1.1 and 1.2 shows the chemoprophylactic agents and the recommended dosages respectively.

TABLE 1.1: CHEMOPROPHYLACTIC AGENTS AVAILABLE IN SOUTH AFRICA

Drug	Drug of choice	Pharmacokinetics	Adverse effects	Special Prescriber's Points
Mefloquine	Short-term travellers who are at risk of contracting <i>P. falciparum</i> , pregnant women in their second & third trimesters. Recommended for children above 5 kg (Gibbon, 2005)..	Protein binding: 98 % Half-life elimination: 13 – 33 days. Metabolism: Hepatic Eliminated: Mainly in bile and faeces. Excretion: Urine (5 % unchanged) (Gibbon, 2005).	Dizziness, vertigo, gastro-intestinal disturbances, neuro-psychiatric disturbances, chest pain, oedema and sinus bradycardia (Gibbon, 2005).	Should be avoided in travellers requiring fine motor co-ordination. Tablets should be taken after a meal with plenty of water (Gibbon, 2005).
Doxycycline	Short-term travellers who are at risk of contracting <i>P. falciparum</i> . It is the preferred short-termed prophylaxis for travellers to multidrug-resistant areas. Not	Absorption: Oral, almost complete. Distribution: Widely into tissues and fluids, crosses placenta and enters breast milk. Protein binding: 90 % Metabolism: Not hepatic; inactivated in gastro-intestinal	Haematological and hypersensitivity reactions may occur. Gastro-intestinal effects may occur. May cause teeth discolouration in children (Gibbon, 2005).	To reduce the risk of oesophageal irritation and ulceration, doses should be taken with adequate amounts of water, in an upright position and well before retiring at night (Gibbon, 2005).

	recommended for children under 12 years of age (Gibbon, 2005).	tract by chelate formation. Half-life elimination: 12 – 15 hours (usually increases to 22 – 24 hours with multiple doses). Time to peak, serum: 1.5 – 4 hours. Excretion: Faeces (30 %) urine (23 %) (Porter, 2006)..		
Atovaquone-proguanil	Only registered in South Africa as prophylaxis for adults (Gibbon, 2005).	Atovaquone: Absorption: Significantly increased with a high fat meal. Distribution: 3.5 L/kg Protein binding: > 99 % Metabolism: Undergoes enterohepatic recirculation. Bioavailability: Tablet: 23 %; Suspension: 47 %. Half-life elimination: <u>adults:</u> 2-3 days <u>children:</u> 1-2 days. Excretion: Faeces (94 % as unchanged drug) (Porter, 2006).	Gastric intolerance, mouth ulcers, stomatitis. Skin rash, hair loss, anaemia and neutropenia, hyponatraemia, elevated liver enzymes and amylase, headache, insomnia, fever and angio-edema have been reported (Gibbon, 2005).	If vomiting occurs within one hour of dosing repeat the dose. The daily dose should be taken with food/milk, and at the same time each day (Gibbon, 2005).

		<u>Proguanil:</u> Protein binding: 75 % Half-life elimination: 12 – 15 hours. Metabolism: Hepatic (only partially). Excretion: Urine (less than 40 % unchanged) (Gibbon, 2005).		
Chloroquine	As a prophylactic agent the use is limited because of high levels of <i>P. falciparum</i> resistance (Gibbon, 2005).	<u>Hydroxychloroquine:</u> Absorption: Complete. Metabolism: Hepatic. Half-life elimination: 32 – 50 days. Excretion: Urine (as metabolites and unchanged drug); may be enhanced by urinary acidification (Porter, 2006).	Gastrointestinal effects, skin rash, pruritus, headaches, vertigo and blurred vision may occur with higher doses. Irreversible ocular damage, notable retinal damage has been reported with long-term prophylactic use, particularly in patients with underlying retinal disease (Gibbon, 2005).	Gastrointestinal effects are minimised when taken with food. In G6PD deficiency monitor for haemolysis. Six-monthly opthalmological examinations are recommended in all patients on long-term prophylaxis or therapy (Gibbon, 2005).

TABLE 1.2: DOSAGES FOR THE CHEMOPROPHYLACTICS OF MALARIA FOR ADULTS AND PAEDIATRICS (Gibbon, 2005)

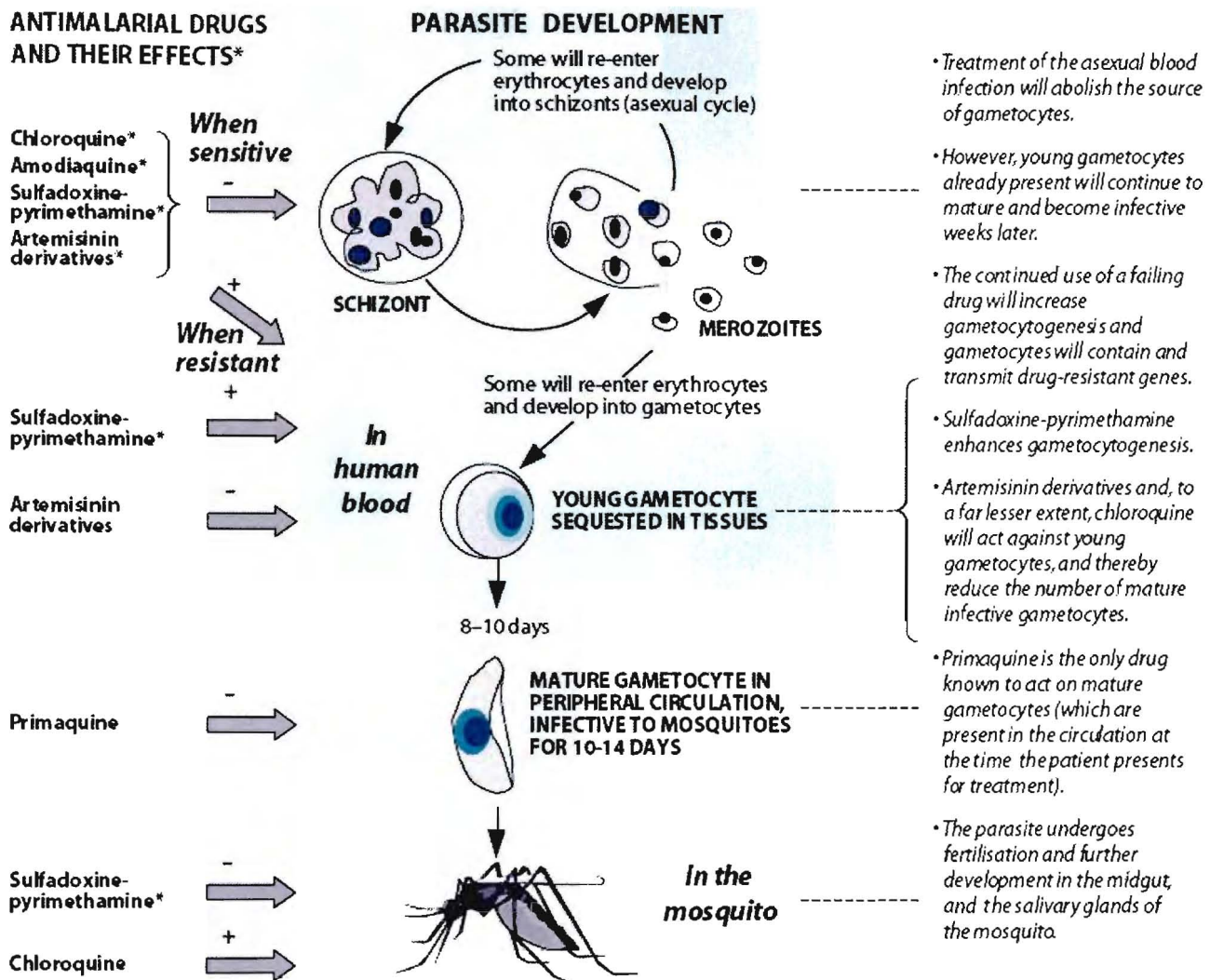
Infection	Drug	Adult dosage	Paediatric dosage
Chloroquine-resistant <i>P. falciparum</i>:			
Oral drug of choice	Mefloquine	250 mg once/week, initiated 1 – 2 weeks before exposure to malaria and continued for 4 weeks after last possible exposure.	<u>5–19 kg</u> : 62.5 mg once/week <u>20–30 kg</u> : 125 mg once/week <u>31–45 kg</u> : 187.5 mg once/week <u>> 45 kg</u> : 250 mg once/week Initiated 1 – 2 weeks before exposure to malaria and continued for 4 weeks after last possible exposure.
	OR Doxycycline	100 mg daily, initiated 48 h before entering the area and continued for 4 weeks after leaving the area.	2 mg/kg daily (up to 100 mg/kg) initiated 48 h before entering the area and continued for 4 weeks after leaving the area; only for children > 8 years.
	OR Atovaquone-proguanil	One tablet daily (250 mg atovaquone and 100 mg proguanil) initiated 1 – 2 days before exposure to malaria	Considered safe for all age groups, although it is only registered in South Africa for adult use. Elsewhere it is registered for children > 10 kg.

		and continued for 7 days after leaving the area.	
Multidrug resistant <i>P. falciparum</i>:			
Oral drug of choice	Doxycycline	100 mg daily, initiated 48 h before entering the area and continued for 4 weeks after leaving the area.	2 mg/kg daily (up to 100 mg/kg) initiated 48 h before entering the area and continued for 4 weeks after leaving the area; only for children > 8 years.
All <i>Plasmodium</i> except chloroquine-resistant species:			
Oral drug of choice	Chloroquine phosphate	300 mg weekly, initiated 1 week prior to entering a malarious area and continued until 4 weeks after leaving.	5 mg/kg weekly, initiated 1 week prior to entering a malarious area and continued until 4 weeks after leaving.

1.5 Treatment of malaria

The objective of treating uncomplicated malaria is to cure the infection. This is important as it will help prevent progression to severe disease and prevent additional morbidity associated with treatment failure. Cure of infection means eradication from the body of infection that caused the illness. A second but equally important objective of treatment is to prevent the emergence and spread of resistance to anti-malarials. Where as the primary objective of anti-malarial treatment in severe malaria is to prevent death. Prevention of recrudescence and avoidance of minor adverse effects are secondary. In treating cerebral malaria, prevention of neurological deficit is also an important objective. In the treatment of severe malaria in pregnancy, saving the life of the mother is the primary objective (World Health Organization, 2006).

To counter the threat of resistance of *P. falciparum* to monotherapies, and to improve treatment outcome, combinations of anti-malarials are now recommended by the World Health Organization (WHO) for the treatment of *falciparum* malaria. Anti-malarial combination therapy is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and thus unrelated biochemical targets in the parasite. The concept is based on the potential of two or more simultaneously administered schizontocidal drugs with independent modes of action to improve the therapeutic efficacy and also to delay the development of resistance to the individual components of the combination. Drug combinations such as sulfadoxine-pyrimethamine rely on synergy between the two components. The drug targets in the malaria parasite are linked. These combinations are operationally considered as single products and treatment with them are not considered to be anti-malarial combination therapy (World Health Organization, 2006). Only patients who are ambulant, with normal mental state, adequate urine output and able to take oral medication (not vomiting repeatedly) can be considered as having uncomplicated malaria (Gibbon, 2005). Over the years various drugs have been developed and used in the treatment of malaria. Table 1.3 gives a summary of the anti-malarial drugs available in South Africa and table 1.4 gives a summary of the doses used to treat adults as well as children. Figure 1.3 shows a graphical presentation of the transmission of *P. falciparum* and the effects of the various anti-malarial drugs on the transmission.



* When parasites are sensitive to the drug unless otherwise stated. Positive and negative arrows indicate the effect of the drug, enhancement (+) and suppression (-) respectively, on the parasite stage or its development.

Figure 1.3: Transmission of *Plasmodium falciparum* and the effects of anti-malarial drugs (World Health Organization, 2006).

TABLE 1.3: AGENTS AVAILABLE FOR THE TREATMENT OF MALARIA IN SOUTH AFRICA

Drug	General information	Working mechanism on parasite life cycle	Drug of choice	Pharmacokinetics	Adverse effects and Special Prescriber's Points
Quinine	Quinine is an alkaloid derived from the bark of the <i>Cinchona</i> tree (World Health Organization, 2006).	Acts principally on the mature trophozoite stage development and does not prevent sequestration or further development of circulating ring stages of <i>P. falciparum</i> . It also kills the sexual stages of <i>P. ovale</i> , <i>P. vivax</i> and <i>P. malariae</i> , but not mature gametocytes of <i>P. falciparum</i> (World Health Organization, 2006).	Treatment of <i>P. falciparum</i> malaria or if the <i>Plasmodium</i> species is unknown or is mixed (Gibbon, 2005).	Distribution: Vd: Adults = 2-3.5 L/kg, <u>decreased</u> with congestive heart failure and malaria; <u>increased</u> with cirrhosis; crosses placenta and enters breast milk. Protein binding: <u>Newborns:</u> 60 % to 70 %. <u>Adults:</u> 80 % to 90 %. Metabolism: Extensively hepatic (50 % to 90 %) to inactive compounds. Bioavailability: Sulphate 80 %; Gluconate 70 %. Half-life elimination, plasma: <u>Children:</u> 2.5-6.7 hours; <u>Adults:</u> 6-8 hours; prolonged with the elderly. Excretion: Urine (15 % to 25 % as unchanged drug) (Porter,	May cause a complex of symptoms known as cinchonism, which is characterized by tinnitus, impaired high tone hearing, headache, nausea, dizziness and dysphoria and sometimes disturbed vision. More severe manifestations include vomiting, abdominal pain, diarrhoea and severe vertigo (Taylor & White, 2004). Should be used in combination with either doxycycline or clindamycin (Gibbon, 2005).

Artemether-lumefantrine and other Artesisinin – based combination therapies	<u>Artemether:</u> Artemether is the methyl ether of dihydroartemisin. It is more lipid soluble than artemesin or artesunate (World Health Organization, 2006). <u>Lumefantrine:</u> It belongs to the aryl aminoalcohol group of anti-malarials which includes quinine, mefloquine and holofantrine (World Health Organization, 2006).	<u>Artemether:</u> It is a potent and rapidly acting blood schizontocide and is active against all <i>Plasmodium</i> species. It has an unusually broad activity against asexual parasites, killing all stages from young rings to schizonts. In <i>P. falciparum</i> it also kills the gametocytes – including stage 4 gametocytes. It inhibits an essential calcium adenosine triphosphate, pfATPase 6 (Eckstein-Ludwig <i>et al.</i>, 2003).	Treatment of uncomplicated <i>P. falciparum</i> malaria and acute <i>P. vivax</i> malaria - currently registered in South Africa for patients under 65 kg, living in malaria-endemic areas, although the WHO recommends use in patients over 65 kg. (Gibbon, 2005). It is highly effective	2006). Bioavailability: Significantly increased with a high fat meal <u>Artemether:</u> Protein binding: >95 % Half-life elimination: ~ 1 hour (World Health Organization, 2006). Time to peak, plasma: 2 – 3 hours (Ezzet <i>et al.</i>, 1998). <u>Lumefantrine:</u> Absorption: Increases with 108% after a meal and is lower in patients with acute malaria than in convalescing patients. Time to peak, plasma: ~ 10 hours Half-life elimination: 3 days (World Health Organization, 2006).	The combination is an effective alternative to oral quinine. (Gibbon, 2005). <u>Artemether:</u> Mild gastrointestinal disturbances dizziness, tinnitus, neutropenia, elevated liver enzymes, bradycardia and prolongation of the QT interval. Type 1 hypersensitivity reactions (1 in 3000 patients) (Leonardi <i>et al.</i>, 2001). Neurotoxicity has been reported in animal studies with high doses of intramuscular artemether but has not sustained in humans (Van Vugt <i>et al.</i>, 2000). <u>Lumefantrine:</u> Well tolerated, side effects generally mild – nausea,
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		<u>Lumefantrine:</u> Has a similar working mechanism to the other aryl aminoalcohols (World Health Organization, 2006).	against multidrug-resistant <i>P. falciparum</i> (World Health Organization, 2006).		abdominal discomfort, headache and dizziness (World Health Organization, 2006).
Artesunate	Artesunate is the sodium salt of the hemisuccinate ester of artemisinin. It is soluble in water but has poor stability in aqueous solutions at neutral or acid pH (World Health Organization, 2006).	The same as artemether (World Health Organization, 2006).	It is not registered for use in South Africa but it is available from the manufacturing company (Gibbon, 2005).	Absorption: Very rapidly. (White, 1994) Time to peak, plasma: <u>Oral:</u> 1.5 hours, <u>intramuscular:</u> 0.5 hours (Batty <i>et al.</i> , 1998) and <u>rectal:</u> 2 hours (Illett <i>et al.</i> , 2002). Metabolism: Almost entirely converted to dihydroartemisin, the active metabolite (Navaratnam <i>et al.</i> , 2000). Half-life elimination: ~ 45 min (Newton <i>et al.</i> , 2000).	Mild gastrointestinal disturbances, dizziness, tinnitus, neutropenia, elevated liver enzymes, bradycardia and prolongation of the QT interval. Type 1 hypersensitivity reactions (1 in 3000 patients) (Leonardi <i>et al.</i> , 2001).
Sulfadoxine-pyrimethamine	<u>Sulfadoxine:</u> Sulfadoxine is a slowly eliminated sulfonamide	<u>Sulfadoxine:</u> Sulfonamides are competitive inhibitors	Used in Mpumalanga and Limpopo	<u>Sulfadoxine:</u> Absorption: Readily absorbed from gastrointestinal tract.	Do not use if patient has a sulfa allergy. (National Department of Health, 2003).

	that is a structural analogue and competitive antagonist of p-aminobenzoic acid (World Health Organization, 2006). <u>Pyrimethamine:</u> Pyrimethamine is a diaminopyrimide used in combination with a sulfonamide, usually sulfadoxine or dapsone (World Health Organization, 2006).	of dihydropteroate synthase (World Health Organization, 2006). <u>Pyrimethamine:</u> It exerts its anti-malarial activity by inhibiting plasmodial dihydrofolate reductase thus indirectly blocking the synthesis of nucleic acids in the malaria parasite. It is a slow-acting blood schizontocide and is also possibly active against pre-erythrocytic forms of the malaria parasite and inhibits sporozoite development in the	provinces only, ineffective in KwaZulu-Natal due to resistance (National Department of Health, 2003).	Time to peak, serum: 4 hours after oral dose Half-life elimination: 4 – 9 days Protein binding: 90 – 95 % Excretion: Urine (unchanged) (World Health Organization, 2006). <u>Pyrimethamine:</u> Onset of action: ~ 1 hour Absorption: Well absorbed Distribution: Widely, mainly in blood cells, kidneys, liver, lungs and spleen; crosses placenta and enters breast milk. Protein binding: 80 % to 87 % Metabolism: Hepatic Half-life elimination: 80 – 95 hours Time to peak, serum: 1.5 – 8 hours Excretion: Urine (20 % to 30% as unchanged drug) (Porter,	
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		mosquito vector (World Health Organization, 2006).		2006).	
Atovaquone-proguanil	<u>Atovaquone:</u> Atovaquone is a hydroxynaphtoquine antiparasitic drug active against all <i>Plasmodium</i> species (World Health Organization, 2006). <u>Proguanil:</u> Proguanil is a biguanide compound that is metabolized in the body via the polymorphic cytochrome P450 enzyme CYP2C19 to the active metabolite cycloguanil (Helsby <i>et al.</i>, 1990).	<u>Atovaquone:</u> Inhibits pre-erythrocytic development in the liver and oocyst development in the mosquito. It also interferes with cytochrome electron transport (World Health Organization, 2006). <u>Proguanil:</u> Proguanil has sporontocidal activity, rendering the gametocytes non-infective to the mosquito vector.	It is an effective alternative to oral quinine (Gibbon, 2005).	<u>Atovaquone:</u> Absorption: Significantly increased with a high fat meal Distribution: 3.5 L/kg Protein binding: >99 % Metabolism: Undergoes enterohepatic recirculation Bioavailability: Tablet: 23 %; Suspension: 47 % Half-life elimination: <u>adults:</u> 2 - 3 days <u>children:</u> 1 - 2 days Excretion: Faeces (94 % as unchanged drug) (Porter, 2006). <u>Proguanil:</u> Protein binding: 75 % Half-life elimination: 12 – 15 hours Metabolism: Hepatic (only	Gastric intolerance, mouth ulcers, stomatitis. Skin rash, hair loss, anaemia and neutropenia, hyponatraemia, elevated liver enzymes and amylase, headache, insomnia, fever and angioedema have been reported. If vomiting occurs within one hour of dosing repeat the dose. The daily dose should be taken with food/milk, and at the same time each day.

		Cycloguanil inhibits plasmodial dihydrofolate reductase. It is possibly active against pre-erythrocytic forms of the parasite and is a slow blood schizonticide (World Health Organization, 2006).		partially). Excretion: Urine (less than 40 % unchanged) (Gibbon, 2005).	
Chloroquine	Chloroquine is a 4-aminoquinoline that has been used extensively for the treatment and prevention of malaria (World Health Organization, 2006).	Chloroquine interferes with parasite haem detoxification (Foley & Tilley, 1997).	Use is limited to the few remaining countries where chloroquine sensitive <i>P. falciparum</i> strains are encountered (Gibbon, 2005).	<u>Hydroxychloroquine:</u> Absorption: Complete Metabolism: Hepatic Half-life elimination: 32 – 50 days Excretion: Urine (as metabolites and unchanged drug); may be enhanced by urinary acidification (Porter, 2006).	Gastrointestinal effects are minimised by taking with food. In G6PD deficiency monitor for haemolysis. Six-monthly opthalmological examinations are recommended in all patients on long-term prophylaxis or therapy (Gibbon, 2005).

Table 1.4: DOSAGES FOR THE TREATMENT OF MALARIA FOR ADULTS AND PAEDIATRICS (Porter, 2006)

<u>INFECTION</u>	<u>DRUG</u>	<u>ADULT DOSAGE</u>	<u>PAEDIATRIC DOSAGE</u>
Chloroquine-resistant <i>P. falciparum</i>:			
Oral drugs of choice	Atovaquone-proguanil	4 adult tablets (250 mg atovaquone and 100 mg proguanil) daily x 3 days.	<u>< 5 kg</u> : not indicated <u>5 - 8 kg</u> : 2 paediatric tablets (62.5 mg atovaquone and 25 mg proguanil) once/day x 3 days <u>9 – 10 kg</u> : 3 paediatric tablets once/day x 3 days <u>11 -20 kg</u> : 1 adult tablet once/day x 3 days <u>21 -30 kg</u> : 2 adult tablets once/day x 3 days <u>30 - 40kg</u> : 3 adult tablets once/day x 3 days <u>> 40 kg</u> : 4 adult tablets once/day x 3 days
	OR Quinine sulphate plus Doxycycline or plus	650 mg q 8 h x 3-7 days 100 mg bid x 7 days	10 mg/kg q 8 h x 3-7 days 2 mg/kg bid x 7 days for children > 8 year

	Clindamycin or plus Sulfadoxine- Pyrimethamine	7 mg/kg tid x 7days 3 tablets on the last day of quinine	7 mg/kg tid x 7days <u>< 5 kg:</u> ¼ tablet once on last day of quinine <u>5 – 10 kg:</u> ½ tablet once on last day of quinine <u>11 - 20 kg:</u> 1 tablet once on last day of quinine <u>21 - 30 kg:</u> 1½ tablets once on last day of quinine <u>31 – 40 kg:</u> 2 tablets once on last day of quinine <u>> 40 kg:</u> 3 tablets once on last day of quinine
Alternatives	Mefloquine	750 mg followed 12 h later by 500 mg	15 mg/kg followed 12 h later by 10mg/kg
	OR Artesunate plus Mefloquine	4 mg/kg once/day x 3 days; 750 mg followed 12 h later by 500 mg	4 mg/kg once/day x 3 days; 15 mg/kg followed 12 h later by 10 mg/kg
All <i>Plasmodium</i> except chloroquine-resistant species:			
Oral drug of choice	Chloroquine phosphate	1 g (600 mg base), then 500 mg (300 mg base) 6 h later, then 500 mg (300 mg base) at 24 and 48 h.	10 mg base/kg (maximum 600 mg base), then 5 mg base/kg 6 h later. Then 5 mg/kg at 24 and 48 h.

All <i>Plasmodium</i>:			
Parental drug of choice	Quinidine gluconate	10 mg/kg loading dose (maximum 600 mg) in normal saline over 1-2 h, continuous infusion of 0.02 mg/kg/min until oral therapy can be started.	10 mg/kg loading dose (maximum 600 mg) in normal saline over 1-2 h, followed by continuous infusion of 0.02 mg/kg/min until oral therapy can be started.
	OR Quinidine dihydrochloride	20 mg/kg loading dose in 5 % dextrose over 4 h, followed by 10 mg/kg over 2-4h q 8 h (maximum 1800 mg/day) until oral therapy can be started.	20 mg/kg loading dose in 5 % dextrose over 4 h, followed by 10mg/kg over 2-4h q 8 h (maximum 1800 mg/day) until oral therapy can be started.
Alternative	Artemether	3.2 mg/kg IM, then 1.6 mg/kg once/day x 5-7 days.	3.2 mg/kg IM, then 1.6 mg/kg once/day x 5-7 days.

1.6 Pregnancy and malaria

Pregnant women in malaria-endemic areas do not always receive the necessary prevention and treatment they need, and this contributes to the extremely high numbers of maternal and infant deaths caused by malaria. In areas of low or unstable malaria transmission, women have no significant level of immunity and will develop clinical illness when parasitaemic. They are at risk of dying from severe malarial disease or from experiencing spontaneous abortion, premature delivery or stillbirth. In areas of high or moderate transmission, women are semi-immune, and most malarial infections although asymptomatic, can contribute to severe maternal anaemia and thus increased risk of maternal death. Malaria infection of the placenta

and malaria-caused maternal anaemia contribute to low birth weight, which results in higher infant mortality and impaired child development (World Health Organization, 2004).

The control approach to date, weekly chloroquine chemoprophylaxis, has been difficult to implement in terms of delivery and compliance and has been affected by increasing anti-malarial drug resistance. The limited number of safe and effective anti-malarial drugs during pregnancy and the weak collaboration between malaria control and reproductive health programmes have also limited the success of efforts to control malaria during pregnancy. (World Health Organization, 2004).

An effective framework for malaria prevention and control during pregnancy in areas of stable transmission has been identified and it recommends three interventions:

1. **Intermittent preventive treatment (IPT):** All pregnant women in areas of stable transmission should receive at least two doses of IPT after quickening. Currently the most effective drug for IPT is sulfadoxine-pyrimethamine because of its safety for use during pregnancy, efficacy in reproductive-age women and feasibility for use in programmes; it can be delivered as a single-dose treatment under the observation of a health worker.
2. **Insecticide-treated nets (ITN):** ITNs should be provided to pregnant women as early in pregnancy as possible. Their use should be encouraged for women throughout pregnancy and during the postpartum period.
3. **Case management of malaria illness and anaemia:** Effective case management of malaria illness for all pregnant women in malaria areas must be assured. Iron supplementation for anaemia should be given to pregnant women as part of routine antenatal care. Pregnant women should also be screened for anaemia, and those with moderate to severe anaemia should be managed according to national reproductive health guidelines (World Health Organization, 2004).

1.7 Drug resistance

Anti-malarial drug resistance has emerged as one of the greatest challenges facing malaria control today. Drug resistance has been implicated in the spread of malaria to new areas and the re-emergence of malaria in areas where the disease had been eradicated. Also population movement has introduced resistant parasites to areas previously free of drug resistance (Bloland, 2001).

Anti-malarial drug resistance has been defined as the “ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject. The drug in question must gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action” (Bruce-Chwatt *et al.*, 1986). Most researchers interpret this as referring only to persistence of parasites after treatment doses of an anti-malarial drug rather than prophylaxis failure, although the latter is a useful tool for early warning of the presence of drug resistance (Lobel & Campbell, 1986).

P. falciparum has developed resistance to nearly all anti-malarials in current use, although the geographical distribution to any single anti-malarial drug varies greatly. Chloroquine resistance is the most common and in South Africa resistance to sulfadoxine-pyrimethamine has also been reported (Murphy *et al.*, 1993; Looareesuwan *et al.*, 1997).

In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required. Over time resistance becomes established in the population and can be very stable, persisting long after specific drug pressure is removed (Thaithong, 1983).

Chloroquine (the cheapest and most cost effective way of treating malaria) is quickly becoming ineffective because of resistance (Anon, 2007c). As the malaria parasite digests haemoglobin, large amounts of a toxic by-product are formed. The parasite polymerizes this by-product in its food vacuole, producing non-toxic haemozoin. It is believed that resistance of *P. falciparum* to chloroquine is related to an increased

capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization (Foley & Tilley, 1997). This chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones (Krogstad *et al.*, 1987).

Antifolate combination drugs, such as sulfadoxine-pyrimethamine, act through sequential and synergistic blockade of 2 key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by dihydrofolate reductase (DHFR) while sulfonates and sulfonamides inhibit the step mediated by dihydropteroate synthase (DHPS) (Bruce-Chwatt *et al.*, 1986). Specific combinations of these mutations have been associated with varying degrees of resistance to antifolate combination drugs (Plowe *et al.*, 1998).

Many anti-malarial drugs in current usage are closely related chemically and development of resistance to one can facilitate development of resistance to others. Chloroquine and amodiaquine are both 4-aminquinolines and cross-resistance between these two drugs is well known (Basco, 1991; Hall *et al.*, 1975). The development of high levels of sulfadoxine-pyrimethamine resistance through continued accumulation of DHFR mutations may compromise the useful lifespan of newer antifolate combination drugs such as chlorproguanil-dapsone even before they are brought into use (Feikin *et al.*, 2000).

As mentioned above, to try and avoid drug resistance, drug combinations are being used. Although mismatched pharmacokinetics can also play a role in the development of resistance. The elimination half-life of pyrimethamine is between 80 and 100 hours, and is between 100 and 200 hours for sulfadoxine, leaving an extended period when the sulfa is “unprotected” by synergy with pyrimethamine (Watkins & Mosobo, 1993).

Programmatic influences on development of anti-malarial drug resistance include overall drug pressure, inadequate drug intake (poor compliance or inappropriate dosing regimens), pharmacokinetic and pharmacodynamic properties of the drug or drug combination and drug interactions (Wernsdorfer, 1994). Overall drug pressure, especially that exerted by programmes utilizing mass drug administration probably

has the greatest impact on development of drug resistance (Wernsdorfer, 1994; Payne, 1988).

The use of presumptive treatment for malaria has the potential for facilitating resistance by greatly increasing the number of people who are treated unnecessarily but will be exerting selective pressure on the circulating parasite population. In some areas and at some times of the year, the number of patients being treated unnecessarily for malaria can be very large (Olivar *et al.*, 1991).

A strategy to prevent drug resistance that has received much attention recently is the combination of anti-malarial drugs such as mefloquine, sulfadoxine-pyrimethamine or amodiaquine with an artemisinin derivative. Artemisinin drugs are highly efficacious, rapidly active, and have action against a broader range of parasite developmental stages. This action apparently yields **two notable results**:

Firstly, artemisinin compounds, used in combination with a longer acting anti-malarial, can rapidly reduce parasite densities to very low levels at a time when drug levels of the longer acting anti-malarial drug are still maximal. This greatly reduces both the likelihood of parasites surviving initial treatment and the likelihood that parasites will be exposed to suboptimal levels of the longer acting drug (White, 1999).

Secondly, the use of artemisinins has shown to reduce gametocytogenesis by 8- to 18-fold (Price, 1996). This reduces the likelihood that gametocytes carrying resistant genes are passed onwards and potentially may reduce malaria transmission rates. It should be noted that this argument contradicts a previously mentioned argument in that it promotes the use of a drug combination with grossly mismatched half-lives (White *et al.*, 1994).

Another strategy to combat drug resistance is to develop new therapeutic options (Surolia & Surolia, 2001). Investigations show that triclosan, an antibacterial agent found in mouthwashes, acne medicines and deodorants, also prevents the growth of *P. falciparum* (Beenson *et al.*, 2001). The target of triclosan in *Plasmodium* is enoyl-ACP reductase, a key regulatory enzyme for fatty acid synthesis found in bacteria and parasites. *Plasmodium* incorporates fatty acids differently than bacteria. *Plasmodium* species incorporate fatty acids in chain lengths of C-10, C-12 and C-14,

whereas bacteria produce chain lengths predominantly of C-14 and C-16. A curious feature of *Plasmodium* is the presence of a plastid, an organelle found in plants and algae. Plastid biosynthetic pathways are essential for parasite growth and are attractive therapeutic targets because of their fundamental differences from mammalian cells. One such pathway is type II fatty acid biosynthesis which has been well studied in bacteria and plant chloroplasts. Genes encoding plastid-localized enzymes of this pathway were recently identified in *P. falciparum* which block enzymes in the pathway in bacteria, inhibited growth of the parasite *in vitro* (Beeson *et al.*, 2001). Studies show that triclosan inhibited the growth of the intra-erythrocytic stages of *P. falciparum* *in vitro* and was similarly effective against chloroquine-sensitive and chloroquine-resistant parasites (Surolia & Surolia, 2001).

1.8 Conclusion

Malaria still remains a problem in over 90 countries with 1.62 billion people living in areas where malaria is increasing, with 300 - 500 million reported cases and 100 million deaths each year, 90 % of which is in Southern Africa. As with most things prophylaxis is better than a cure especially because of the rising problem of drug resistance. Drug resistance has been reported to at least three out of the four *Plasmodium* species that effect humans. In an effort to avoid further drug resistance, anti-malarials are used in combination therapies as well as searching for new effective and safe anti-malarials. Resistance to various new anti-malarials on the market like artemisinin and its derivatives have already been reported and fear of it spreading is high so it is used only in combination therapy. A better option could be the use of a drug delivery system that can bypass the method of current drug resistance. The Pheroid™ drug delivery system is such a drug delivery system. In chapter 2 the Pheroid™ drug delivery system will be discussed. When using this drug delivery system it is possible to start re-using drugs like chloroquine that are currently ineffective against malaria because of resistance. The Pheroid™ drug delivery system could thus bring new hope to the global fight against this deadly disease.

CHAPTER 2

Pheroid™ Technology and physico-chemical properties of anti-malarials used in this study

2.1 Introduction

The Pheroid™ (further referred to as Pheroid/s) drug delivery system is a novel, patented, colloidal delivery system, using Pheroid technology that consists mainly of modified essential fatty acids. Pheroid technology is based on what was previously called Emzaloid™ technology and it is able to enhance the absorption and/or efficacy of various categories of active ingredients and other compounds. It has been shown to result in major improvements in the control of size, charge and the hydrophilic – lipophilic characteristics of therapies when compared to other systems (Saunders *et al.*, 1999; Tzaneva *et al.*, 2003).

2.2 Structural Characteristics of Pheroids

Pheroids are unique and stable lipid-based submicron-and micron sized structures, uniformly distributed in a dispersion medium that may be adapted to the indication. The dispersed structures (dispersed phase) can be manipulated in terms of morphology, structure, size and function (Grobler *et al.*, 2007).

Pheroids consist of three phases, namely an aqueous phase consisting of sterile water, an oil phase consisting of fatty acids and a dispersed gas phase which is associated with the oil phase (Grobler *et al.*, 2007). With the omission of water and the addition of larger concentration of polyethylene glycol (PEG), pro-Pheroids can be formulated. The stable Pheroid vesicles will form once it encounters the fluid component that is found *in vivo*. This is a very important formulation tool for unstable molecules and a great advantage of the Pheroid system (Grobler, 2004).

The gas distributed in the oil phase is the volatile anaesthetic nitrous oxide (N₂O) which is a unique component of the drug delivery system. The association of N₂O with the dispersed phase has been shown to have at least three functions and contributes to the following:

1. Miscibility of the fatty acids in the dispersal medium;
2. Self-assembly process of the Pheroids and

3. Stability of the formed Pheroid (Grobler *et al.*, 2007).

As mentioned above, the oil phase consist of fatty acids, primarily of ethylated and pegylated poly unsaturated fatty acids, including the omega-3 and -6 fatty acids but excluding arachidonic acid. The fatty acids are in the cis-formation and therefore compatible with the orientation of the fatty acids in man (Grobler *et al.*, 2007).

Various types of Pheroids can be formulated, depending on the composition and method of manufacturing, to have a diameter of between 220 nm and 2 µm. The three main types of Pheroids are:

- Lipid-bilayer vesicles in both the nano- and micrometer size range,
- Microsponges and
- Depots or reservoirs that contain pro-Pheroids.

Parameters such as the required capacity (i.e. the amount and size of the active compound to be entrapped), the rate of delivery and the administration route are taken into account when deciding on the type and diameter of the Pheroids (Grobler *et al.*, 2007).

Pheroids are formed by a self-assembly process similar to that of low-energy emulsions and micro-emulsions, however no lyophilization or hydration of the lipid components is necessary. The combination of the fatty acids and the nitrous oxide provide an effective transportation model for hydrophilic and hydrophobic drugs. It was noted in controlled experiments that the stability and efficacy of various formulations had dramatically decreased if the essential fatty acids or the nitrous oxide was absent from the formulation (Grobler *et al.*, 2007).

2.3 The Pheroid drug delivery system compared to other lipid based delivery systems

When the Pheroid drug delivery system is compared to other lipid based delivery systems, substantial differences come to light. Table 2.1 gives a summary of the similarities, differences and main advantages of the Pheroid drug delivery system (Grobler, 2004).

Table 2.1 Comparison between the Pheroid drug delivery system and other lipid based delivery systems (Grobler, 2004)

Pheroid drug delivery system	Other delivery systems
Consists mainly of essential fatty acids, a natural and essential ingredient of the body and thus do not elicit immune responses in the human body as shown by cytokine studies.	Generally contain a proportion of substances foreign to the body, e.g. artificial polymers and has been shown to elicit immune responses.
Pheroids can be optimised for the active compound and indication of the drug. By being manipulated in terms of size, charge, lipid composition and membrane packing. The desired type(s) can be repeatably obtained.	Problems with the degree of liposomal systems, liposomal types and sizes have been described. Filtration mechanisms have been introduced to limit large size variations.
Pheroids is polyphilic and so drugs that have different solubility's as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
Entrapment of active compounds in Pheroids reduces the volume of distribution and consequently the concentration at the target site is increased. An enhanced but narrow therapeutic index can be achieved, with a decrease in aspecific toxicity.	A reduction in the volume of distribution and an enhanced concentration of active compounds in tumours have been shown by liposomes encapsulating small molecule chemotherapeutic agents.
All <i>in vitro</i> studies conducted showed that drug resistance was reduced or eliminated. Theories are still under investigation but a possible mechanism of action is that the Pheroids allow the release of active compounds beyond the membrane zone and drug efflux pumps found in drug resistant organisms.	Some delivery systems are prone to drug resistance or adverse immune responses. The compositions in the system generally prohibit active compounds to be released beyond the drug efflux pumps developed by the drug-resistant organisms.
Bioavailability is enhanced due to Pheroids being able to inhibit the drug	Liposomal systems containing this feature have not been described.

efflux mechanism in the intestinal lumen.	
Pheroids enhances the absorption as well as the bioavailability of oral, topical and buccal administration of active compounds in all products tested so far.	Some delivery systems have been shown to enhance absorption where others decrease absorption.
Microsponge type Pheroids are ideal for combination therapies, as one drug is entrapped in the interior volume and the other in the sponge spaces. The geographical separation of active compounds into different spaces minimizes interaction between compounds or drug interactions.	For most delivery systems combination treatments are problematic.

2.4 Pharmaceutical applicability of the Pheroid drug delivery system

Considering the uniqueness of this drug delivery system a variety of in-depth studies were conducted to better our understanding of it. The efficacy enhancement of oral and parenteral therapies were tested with therapeutic compounds and preventative vaccines. The results obtained confirmed the following abilities of the delivery system:

- Decreased time to onset of drug action;
- The increased delivery of active compounds;
- Reduction of the minimum inhibitory concentration of active compounds;
- The increase in therapeutic efficacy;
- Reducing cytotoxicity;
- Gene entrapment and transferral to cell nuclei and
- The reduction and suggested elimination of drug resistance (Grobler, 2004; Langley, 2007).

2.5 Possible use of fatty acids in the treatment of *Plasmodium falciparum* malaria

As mentioned in par. 2.2, Pheroids contain a number of fatty acids in the oil phase. Certain long chain polyunsaturated fatty acids derived from essential fatty acids have shown to be toxic to *Plasmodium falciparum* both *in vitro* and *in vivo* studies (Arun Kumar & Das, 1999).

Studies showed that fatty acids with 18 carbon atoms, irrespective of the number and position of the double bonds, failed to enhance the neutrophil – mediated killing of the parasite. In contrast the neutrophil-mediated parasite killing action was increased with poly-enoic fatty acids with 20 carbon atoms. Optimum parasite killing was seen with arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid. By increasing the carbon chain length to C28 and longer, resulted, interestingly in a decrease in activity. The addition of the antioxidant, Vitamin E, also significantly reduced the ability of the fatty acids to enhance the killing of the parasites by the neutrophils (Kumaratilake *et al.*, 1997). The anti-malarial activity of the fatty acids is concentration dependant (Kumaratilake *et al.*, 1992).

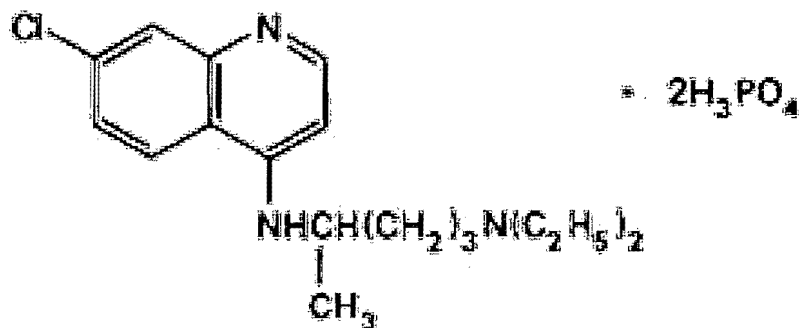
Fish oil exhibits a much lower inhibition of the parasite growth in comparison to docosahexaenoic acid possibly due to the much larger molecule size (Kumaratilake *et al.*, 1992).

Nitric oxide has shown to also contain anti-malarial properties (Rockett, 1991; Anur Kumar & Das, 1999). However, the Pheroid drug delivery system contains nitrous oxide and not nitric oxide and so the anti-malarial properties of nitric oxide will not be discussed.

2.6 Physical and chemical properties of anti-malarials

There are currently numerous medications used for the treatment and prophylaxis of malaria as discussed in chapter one. During this study the focus was only on chloroquine phosphate and mefloquine hydrochloride (HCl). Pheroid and pro-Pheroid formulations containing these two active pharmaceutical ingredients were manufactured. The information regarding the chemical and physical properties of the two anti-malarial drugs were found in the International Pharmacopoeia 2006, unless otherwise stipulated.

2.6.1 Chloroquine phosphate



Molecular formula: C₁₈H₂₆ClN₃•2H₃PO₄

Relative molecular mass: 515.9

Chemical name: 7-Chloro-4-[[4-(diethyl amino)-1-methylbutyl]amino]quinoline phosphate (1:2); *N*⁴-(7-chloro-4-quinoliny)-*N*¹,*N*¹-diethyl-1,4-pentanediamine phosphate (1:2).

Description: A white or almost white, crystalline powder: odourless or almost odourless. Chloroquine phosphate has a bitter taste; it is slowly discoloured by light.

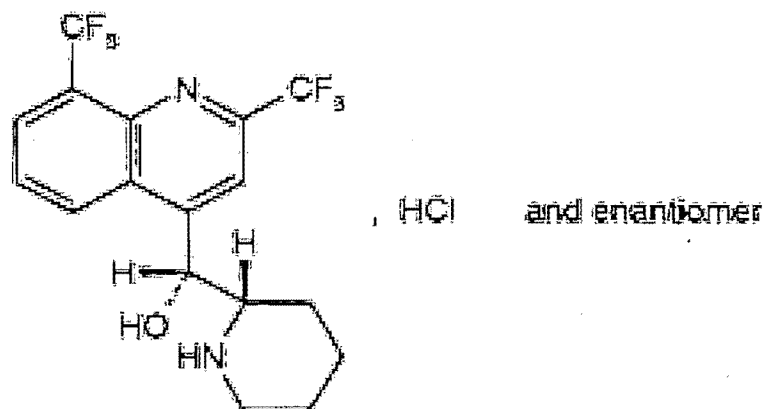
Solubility: Chloroquine phosphate is freely soluble in water; practically insoluble in alcohol, chloroform and ether (Hong, 1976).

Polymorphism and melting temperatures: Chloroquine phosphate may exist in 2 polymorphic forms differing in melting temperature, one of which melts at about 194°C, the other at about 215°C; mixtures of the 2 forms melt between 194°C and 215°C.

pH value: pH of a 0.10 g/ml solution, 3.5-4.5.

UV absorbance: Chloroquine phosphate dissolved in 0.01 N HCl shows maximum absorbance at 222 nm, 328 nm and 343 nm (Hong, 1976).

2.6.2 Mefloquine hydrochloride



Molecular formula: C₁₇H₁₆F₆N₂O, HCl

Relative molecular mass: 414.8

Chemical name: DL-*erythro*-α-2-piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol monohydrochloride; (*R*^{*}, *S*^{*})-(±)-α-2-piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol monohydrochloride.

Description: A white to slightly yellow, odourless crystalline powder.

Solubility: Mefloquine HCl is soluble in ethyl acetate and ethanol; slightly soluble in water and hydrochloric acid (0.1 M) and sparingly soluble in dimethylsulfoxide and methylene chloride.

Melting temperature: Mefloquine HCl melts at about 260 °C, with decomposition.

UV absorbance: When dissolved in methanol mefloquine HCl shows a maximum UV absorbance at 222 nm, 283 nm, 304 nm and 318 nm (Merck & Co., Inc., 2006).

2.7 Formulation with Pheroid and pro-Pheroid

When deciding on the appropriate concentrations of drugs to use in the formulations of the Pheroid/actives and pro-Pheroid/actives the dosages of current anti-malarials were used according to the South African Medicines Formulary (SAMF) (Gibbon, 2005). The dosage for an adult on treatment with chloroquine base is 1.5 g over a three day period administrated as follows: initially 600 mg, followed by 300 mg 6-8 hours later with 300 mg once daily on the second and third days. Chloroquine base

150 mg is approximately equivalent to chloroquine phosphate 250 mg. When treating a patient with mefloquine base the dosage is as follows: 25 mg/kg in 2-3 divided doses 6-8 hours apart. With the loading dose being 750 mg, then 500 mg after 6-8 hours and 250 mg after a further 6-8 hours (Gibbon, 2005). Mefloquine HCl 165 mg is approximately equivalent to 150 mg mefloquine base (Merck & Co., Inc., 2006). According to *in vitro* studies done chloroquine phosphate entrapped in Pheroid vesicles proved to be more effective than chloroquine phosphate entrapped in microsponges. Whereas mefloquine HCl entrapped in Pheroid microsponges proved to be more effective than mefloquine HCl entrapped in Pheroid vesicles (Langley, 2007). A decision was made to work at a concentration of 15 mg/ml for chloroquine phosphate in Pheroid vesicles, chloroquine phosphate in pro-Pheroid and mefloquine HCl in pro-Pheroid and 25 mg/ml for mefloquine HCl in Pheroid microsponges. It should be noted that chloroquine phosphate is light sensitive and the necessary precautions should be taken.

2.8 Conclusion

The Pheroid drug delivery system is a unique and highly effective system as it increases the amount of drug at the target site; it increases bioavailability and drug absorption and most important, in the case of malaria, it decreases drug resistance. It shows great potential for use with current anti-malarials. The anti-malarials used in this study is chloroquine phosphate and mefloquine HCl. It is important to note the respective solubilities of these anti-malarials as it will influence the type of Pheroid used. Chloroquine phosphate should be more suitable in Pheroid vesicles or microsponges since it is water soluble. Mefloquine HCl on the other hand should be more suitable in a pro-Pheroid formulation because it is only slightly water soluble. Also, it should be mentioned that although the possible anti-malarial properties of fatty acids are mentioned in par 2.5, the Pheroid drug delivery system may contain elements that are unfavourable to the anti-malarial activity of the fatty acids such as vitamin E.

CHAPTER 3

Validation of HPLC assay methods for chloroquine phosphate and mefloquine hydrochloride

3.1 Definition of validation parameters

3.1.1 Specificity

Specificity is the ability of the method to discriminate between the intended analyte and other components that may be present in the sample. Other components may include impurities, degradants or excipients (Dong, 2006).

3.1.2 Precision (Repeatability)

Precision is the measure of the degree of repeatability of an analytical method under normal operational circumstances (Swartz & Krull, 1997). Repeatability is a measure of the ability of the method to generate similar results for multiple preparations of the same homogeneous sample by using the same instrument in a short time duration (Dong, 2006).

3.1.3 Linearity and Range

Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range. Range is the comprehensive interval between the upper and the lower levels of the analyte that have been demonstrated to be determined with precision, accuracy and linearity using the method (Swartz & Krull, 1997).

3.2 Validation test procedures and acceptance criteria

3.2.1 Specificity

Method:

Solvents used:

- Chloroquine phosphate – water
- Mefloquine HCl - mobile phase
- Pheroid and pro-Pheroid - methanol.

1. Prepare a standard solution in Pheroid, pro-Pheroid and the appropriate solvent.
2. Analyse by means of HPLC.
3. Also analyse by means of HPLC a sample of the Pheroid, pro-Pheroid and the appropriate solvent. as procedural blanks.
4. Examine the chromatograms to determine whether any interfering peaks can be detected.

Acceptance criteria:

The method is proven to be specific when no reagents or contaminants interfere with peak analysed.

3.2.2 Precision (Repeatability)

Method:

1. Prepare respectively, 1 standard sample covering the top, middle and low concentration values within the range.
2. Analyse in triplicate.
3. Calculate the % relative standard deviation using the following equation:

$$\%RSD = \frac{Std\ Deviation}{Mean} \times 100$$

Acceptance criteria :

The % RSD $\leq$ 2.

3.2.3 Linearity and Range

Method:

1. Prepare a standard stock solution.
2. Make suitable dilutions of the stock solution to obtain 5 standards with concentrations covering the range $\pm$ 50-150 % of the target concentration.

Acceptance criteria :

The linear regression analysis should yield a regression coefficient (R squared) of not less than 0.98.

3.3 Method for chloroquine phosphate**3.3.1 Origin of method**

The method was based on a method by Deng *et al.* (2006) entitled *Sensitive fluorescence HPLC assay for AQ-13, a candidate aminoquinoline antimalarial, that also detects chloroquine and N-dealkylated metabolites.*

3.3.2 Summary

In Table 3.1 a summary of the validation results are given.

Table 3.1: Summary of validation results for the chloroquine phosphate method

Parameter	Acceptance criteria	Results
Specificity	Non-interference from contaminants and reagents should be demonstrated by running a procedural blank (injecting the solvent)	Complies
Precision (Repeatability)	The percentage RSD ≤ 2 .	Complies Chloroquine: (0.0476 mg/ml), %RSD = 0.39 (0.071 mg/ml), %RSD = 0.05 (0.1429 mg/ml), %RSD = 0.13
Linearity and Range	The method is linear over a range of 50-150 % of the expected sample concentration (0.06mg/ml). $R^2 \geq 0.98$	Complies Chloroquine is linear in the range 0.0476 – 0.1429 mg/ml. $R^2 = 0.999984$

3.3.3 Chromatographic conditions

Analytical instrument: Shimadzu Prominence series HPLC equipped with a LC-20AD quaternary pump, SIL-20AC auto sampler, SPD-M20A diode array detector and Shimadzu LC Solution data acquisition and analysis software.

Column: Phenomenex Luna C-18 (250 mm x 4.6 mm, 5 μ m).

Mobile phase: Equal amounts of acetonitrile and 0.02 M Borate buffer pH 9.00 $\pm$ 0.05. PH adjusted with 10 M NaOH and phosphoric acid.

Flow rate: 1.50 ml/min

Injection volume: 20 μ l

Detection: 340 nm

Retention time: About 12.5 min

Solvent: Milli-Q water

3.3.4 Standard solution preparations

3.3.4.1 Standard solution in Pheroid

1. Accurately weigh about 12 mg chloroquine reference standard into a 100 ml amber volumetric flask.
2. Dissolve in about 15 ml of Pheroid.
3. Shake well for about 15 minutes and make up to volume with Pheroid.
4. Pipette 5 ml of Pheroid solution into a 10 ml amber volumetric flask and make up to volume with methanol.
5. Analyse by means of HPLC.

3.3.4.2 Standard solution in pro-Pheroid

1. Accurately weigh about 15 mg chloroquine reference standard into a 10 ml amber volumetric flask.
2. Dissolve in about 5 ml of pro-Pheroid.
3. Shake well for about 15 minutes and make up to volume with pro-Pheroid.
4. Pipette 2 ml of pro-Pheroid solution into a 10 ml amber volumetric flask add about 5 ml water.

5. Shake well for about 15 minutes and make up to volume with water.
6. Pipette 2 ml of solution into a 10 ml amber volumetric flask and add about 5 ml of methanol.
7. Shake well for about 15 minutes and make up to volume with methanol.
8. Analyse by means of HPLC.

3.3.4.3 Standard solution in water

1. Accurately weigh about 15 mg Chloroquine reference standard into a 250 ml amber volumetric flask.
2. Dissolve in about 15 ml water.
3. Shake well for about 15 minutes and make up to volume with water.
4. Analyse by means of HPLC.

3.3.5 Validation results

3.3.5.1 Specificity

Methanol, water, Pheroid and pro-Pheroid were injected as blanks for this method as seen in Fig 3.1 – 3.4. Figures 3.5 – 3.7 show chromatograms of the chloroquine phosphate in mobile phase, in Pheroids and in pro-Pheroid.

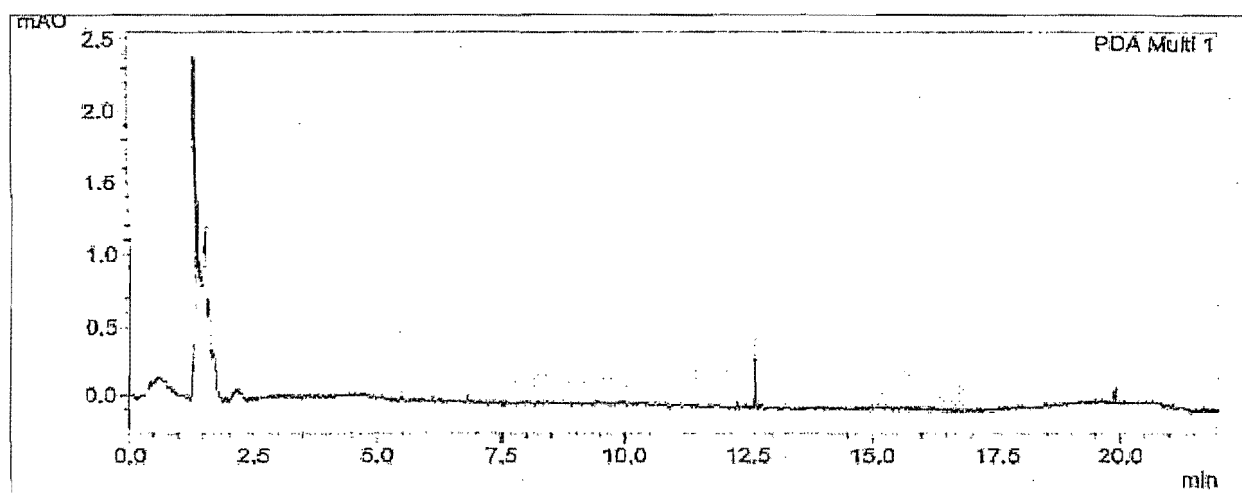


Figure 3.1: Chromatogram of methanol (solvent) for the chloroquine phosphate method.

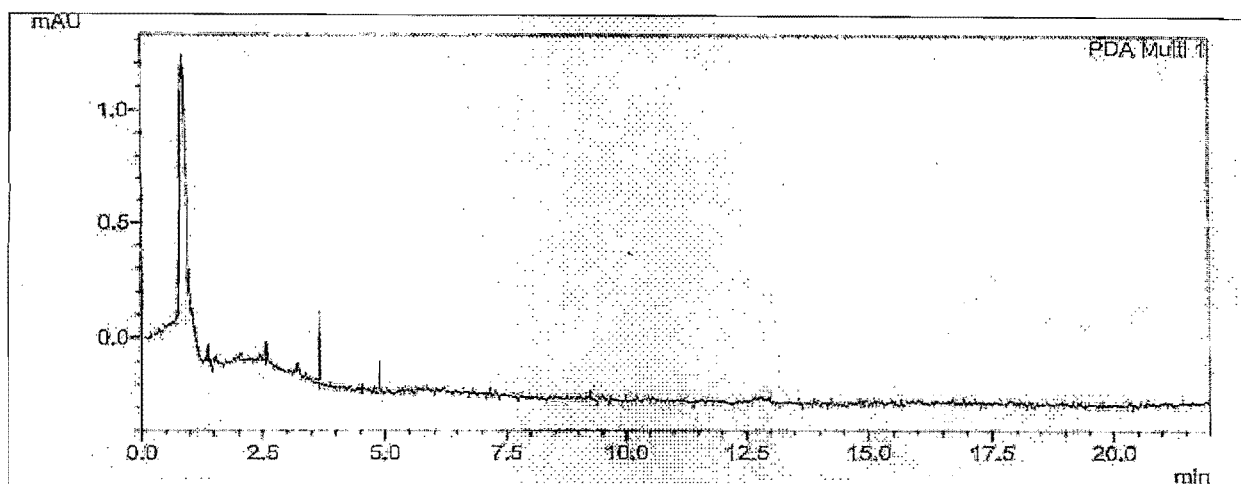


Figure 3.2: Chromatogram of water (solvent) for the chloroquine phosphate method.

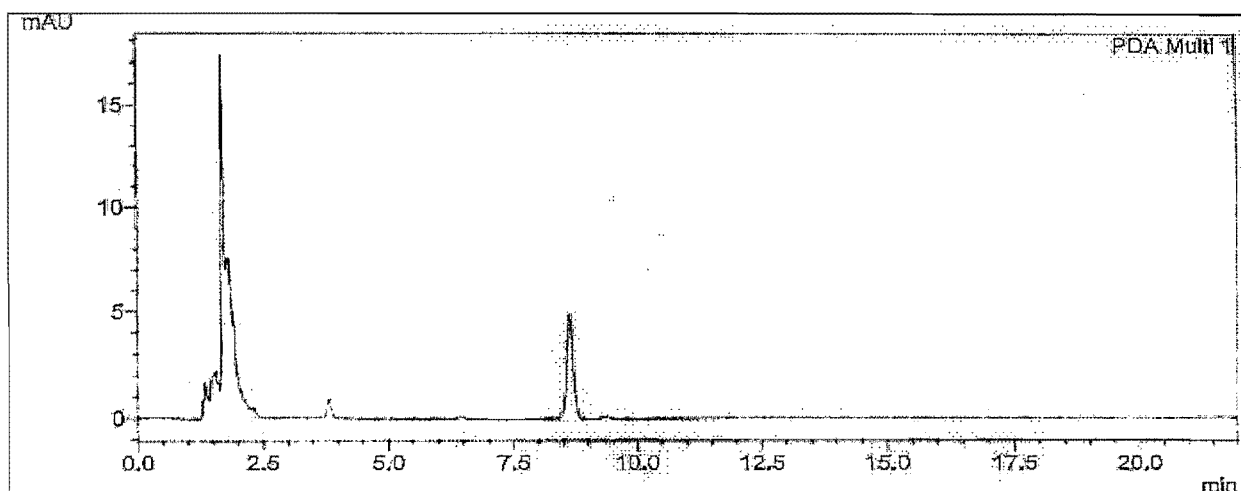


Figure 3.3: Chromatogram of Pheroid for the chloroquine phosphate method.

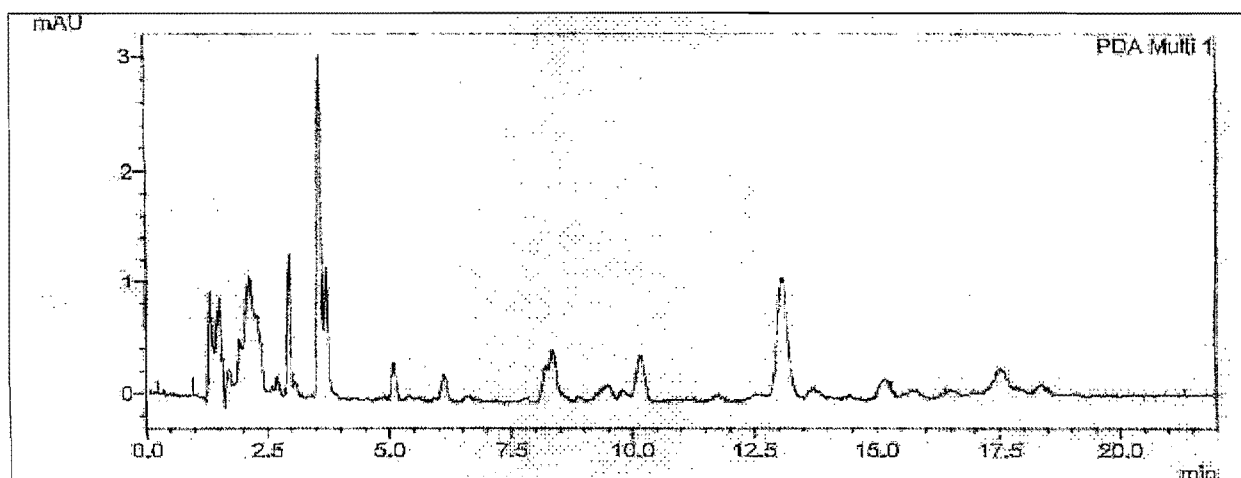


Figure 3.4: Chromatogram of pro-Pheroid for the chloroquine phosphate method.

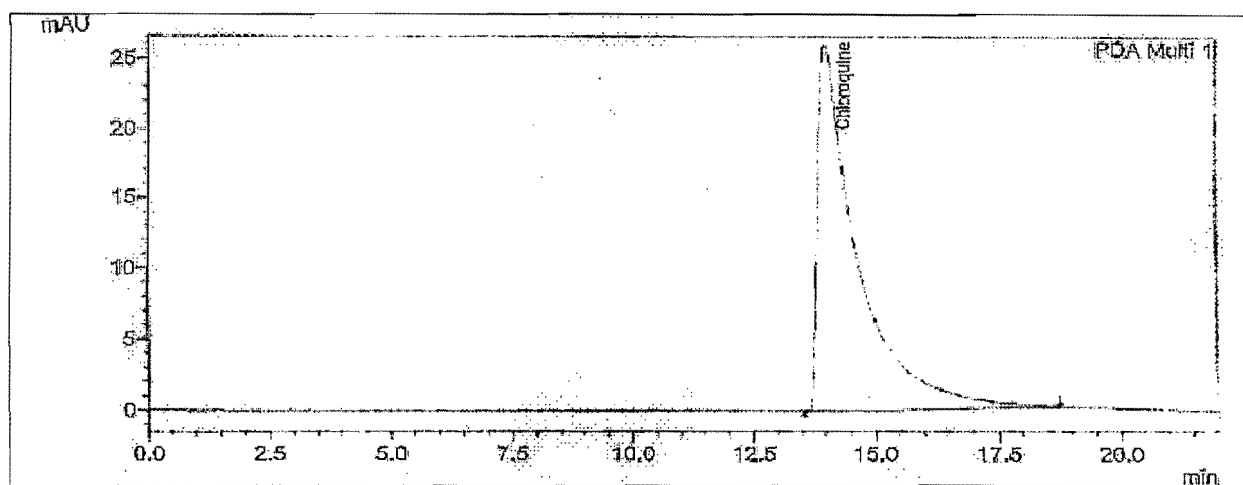


Figure 3.5: Chromatogram of chloroquine phosphate in water.

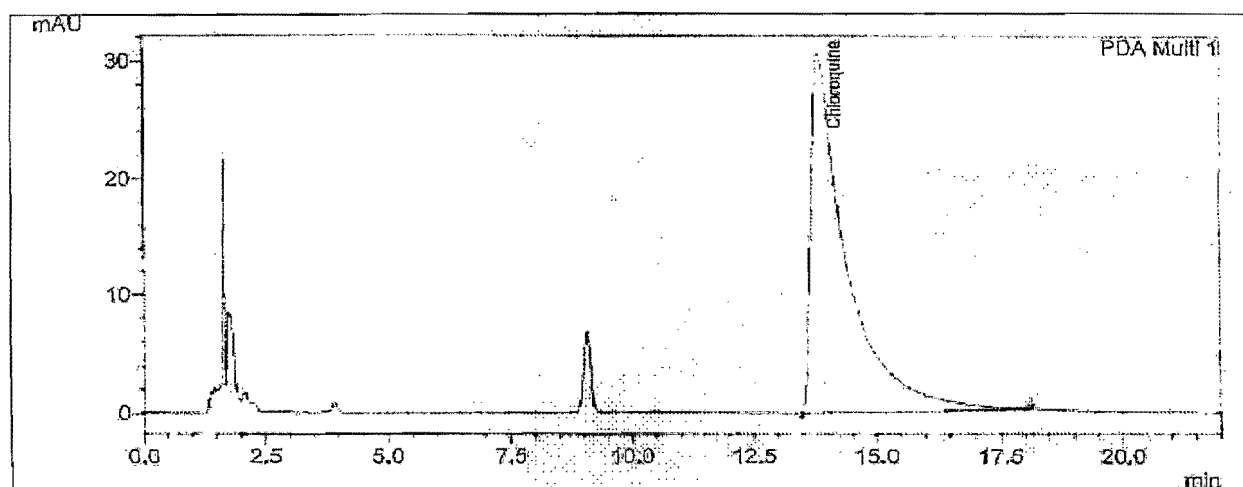


Figure 3.6: Chromatogram of chloroquine phosphate in Pheroid.

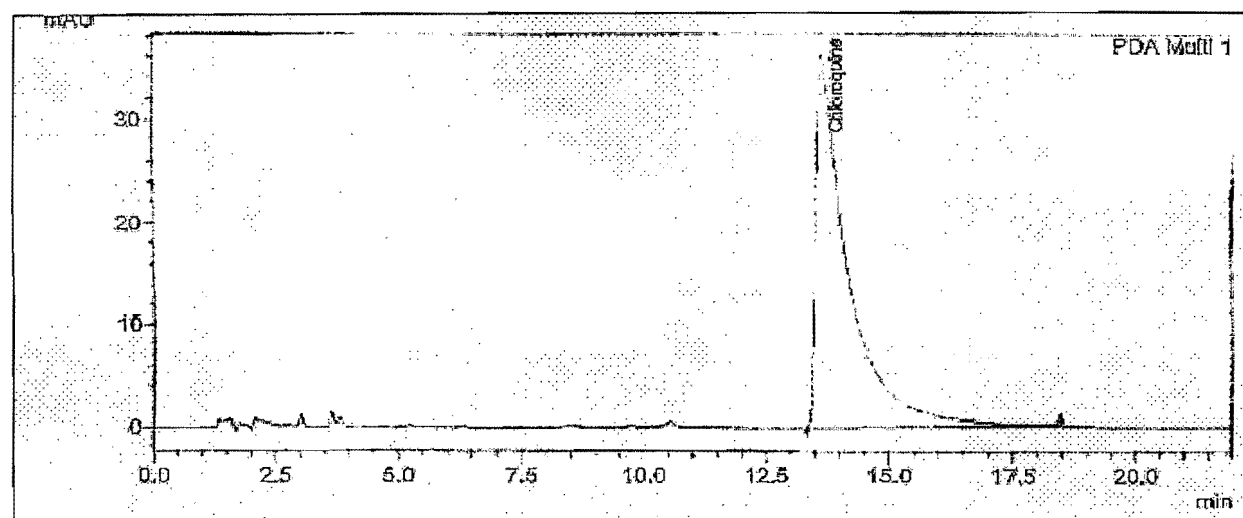


Figure 3.7: Chromatogram of chloroquine phosphate in pro-Pheroid.

3.3.5.2 Precision (Repeatability)

Table 3.2: Results for chloroquine phosphate to determine Intra-day precision

mg/ml	Area			Mean area	SD	% RSD
0.0476	685456	680980	685632	684022.67	685456	0.39
0.0714	1061001	1060195	1061184	1060793.33	1061001	0.05
0.1429	2198339	2192708	2195496	2195514.33	2198339	0.13

Intra-day precision was satisfactory with % RSD values between 0.05 – 0.39.

3.3.5.3 Linearity and Range

Table 3.3: Peak area, % RSD and concentration for chloroquine phosphate

mg/ml	Area			Mean Area	% RSD
0.0476	685456	680980	685632	684022.67	0.39
0.0595	873349	878702	882770	878273.67	0.54
0.0714	1061001	1060195	1061184	1060793.33	0.05
0.0953	1437113	1444145	1440185	1440481	0.24
0.1429	2198339	2192708	2195496	2195514.33	0.13

Table 3.4: Regression parameters of chloroquine phosphate

R squared	0.999984
Intercept	-69195.6997
Slope	15849172.75

The method is linear from 0.0476 – 0.1429 mg/ml (50-150 % of expected sample concentration) with a $R^2 = 0.999984$ (Figure 3.8). Values lower than 0.0476 mg/ml proved to be too low and the method was not linear for the lower concentrations.

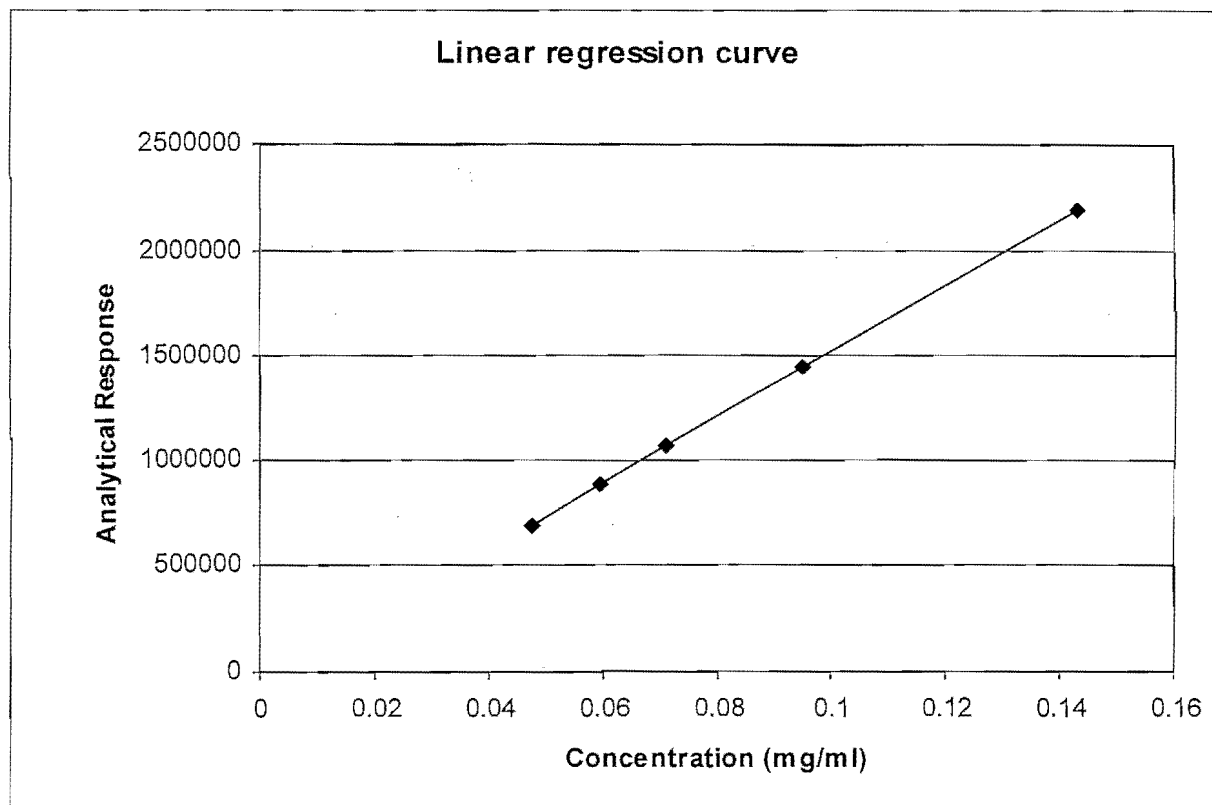


Figure 3.8: The linear regression curve for the chloroquine phosphate method.

3.4. Method for mefloquine hydrochloride

3.4.1 Origin of method

The method was based on a method by Bergqvist *et al.* (1991) entitled *Reversed-phase liquid chromatographic method for the simultaneous determination of the antimalarial drugs sulfadoxine, pyrimethamine, mefloquine and its major carboxylic metabolite in plasma.*

3.4.2 Summary

A summary of the validation results are given in Table 3.5

Table 3.5: Summary of validation results for the mefloquine HCl method

Parameter	Acceptance criteria	Results
Specificity	Non-interference from contaminants and reagents should be demonstrated by running a procedural blank (injecting the solvent).	Complies
Precision (Repeatability)	The percentage RSD ≤ 2 .	Complies Mefloquine: (0.0394 mg/ml), %RSD = 0.05 (0.1183 mg/ml), %RSD = 0.17 (0.1972 mg/ml), %RSD = 0.12
Linearity and Range	The method is linear over a range of 50-150 % of the expected sample concentration (0.12mg/ml). $R^2 \geq 0.98$	Complies Mefloquine is linear in the range 0.0394 – 0.1972 mg/ml. $R^2 = 0.9912$

3.4.3 Chromatographic conditions

Analytical instrument: Shimadzu Prominence series HPLC equipped with a LC-20AD quaternary pump, SIL-20AC auto sampler, SPD-M20A diode array detector and Shimadzu LC Solution data acquisition and analysis software.

Column: Phenomenex Luna C-18 (250 mm x 4.6 mm, 5 μ m).

Mobile phase: 48 % Acetonitrile and 52 % 0.1M Phosphate Buffer, pH 3.5 $\pm$ 0.05 adjusted with 1 M NaOH and phosphoric acid. 4 ml of 0.5 % Hexane Sulphonic Acid added for every 1000 ml of mobile phase.

Flow rate: 1.00 ml/min

Injection volume: 20 µl

Detection: 229 nm

Retention time: About 4.4 min

Solvent: Mobile phase

3.4.4 Standard solution preparations

3.4.4.1 Standard solution in Pheroid/pro-Pheroid

1. Accurately weigh about 50 mg mefloquine HCl reference standard into a 25 ml volumetric flask.
2. Dissolve in about 10 ml of Pheroid/pro-Pheroid.
3. Shake well for about 15 minutes and make up to volume with Pheroid/pro-Pheroid.
4. Pipette 3 ml of Pheroid/pro-Pheroid solution into a 50 ml volumetric flask and make up to volume with methanol.
5. Analyse by means of HPLC.

3.4.4.2 Standard solution in mobile phase

1. Accurately weigh about 12 mg mefloquine standard into a 100 ml volumetric flask.
2. Dissolve in about 15 ml mobile phase.
3. Shake well for about 15 minutes and make up to volume with mobile phase.
4. Analyse by means of HPLC.

3.4.5 Validation results

3.4.5.1 Specificity

As a blank for this method mobile phase, methanol, Pheroid and pro-Pheroid were injected; as chromatograms 3.9 – 3.12 show. Figures 3.13 - 3.15 show chromatograms of the mefloquine HCl in mobile phase, in Pheroid and in pro-Pheroid.

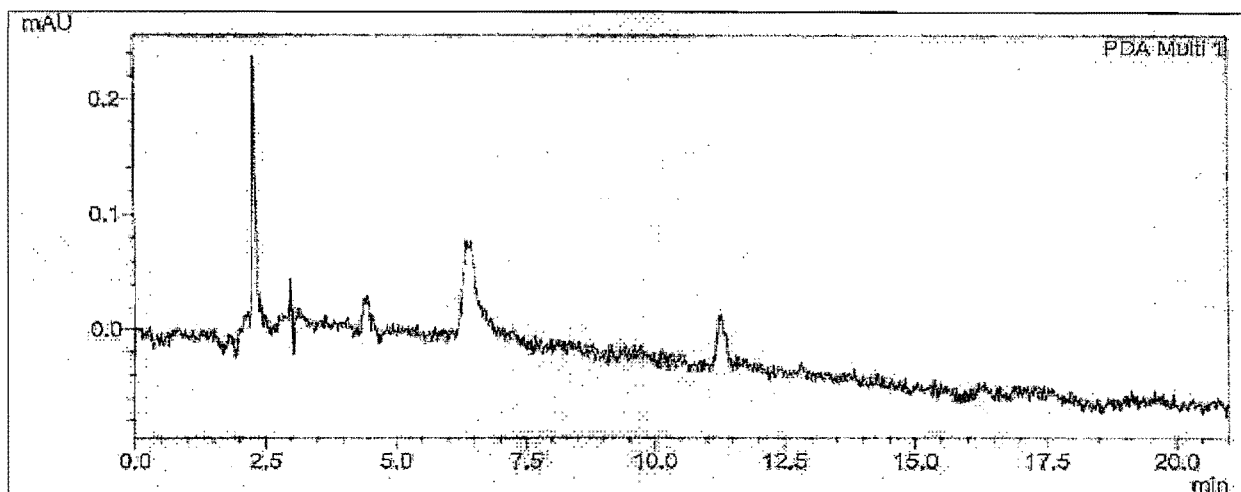


Figure 3.9: Chromatogram of mobile phase (solvent) for the mefloquine HCl method.

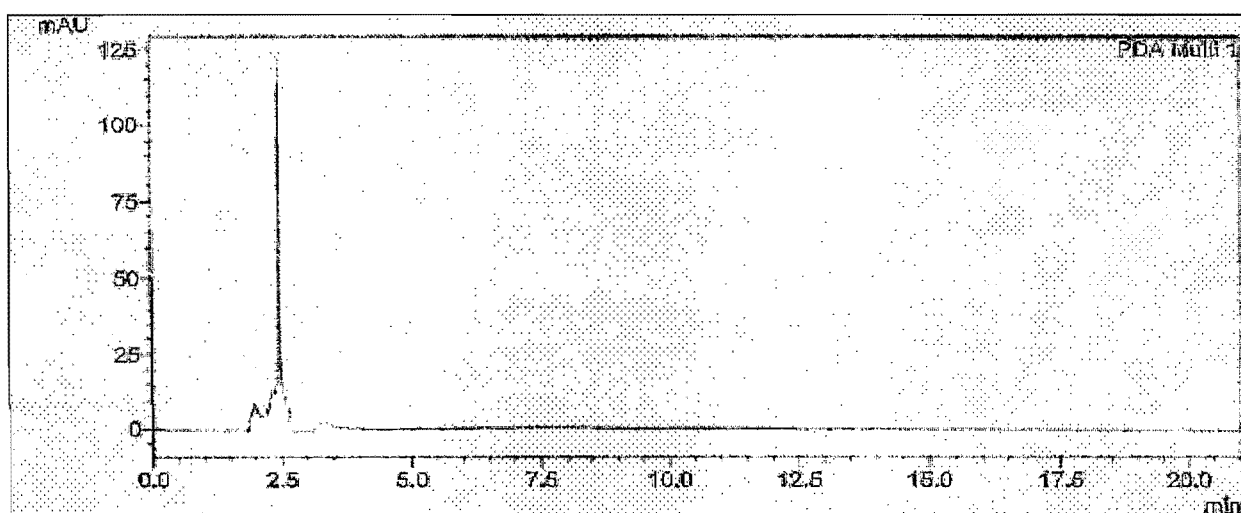


Figure 3.10: Chromatogram of methanol (solvent) for the mefloquine HCl method.

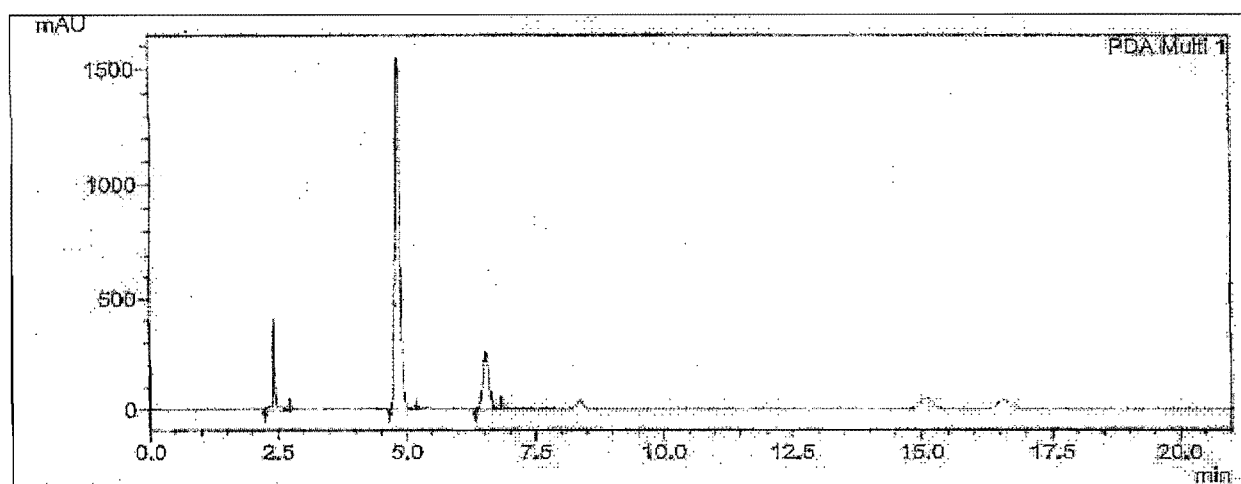


Figure 3.11: Chromatogram of Pheroid for the mefloquine HCl method.

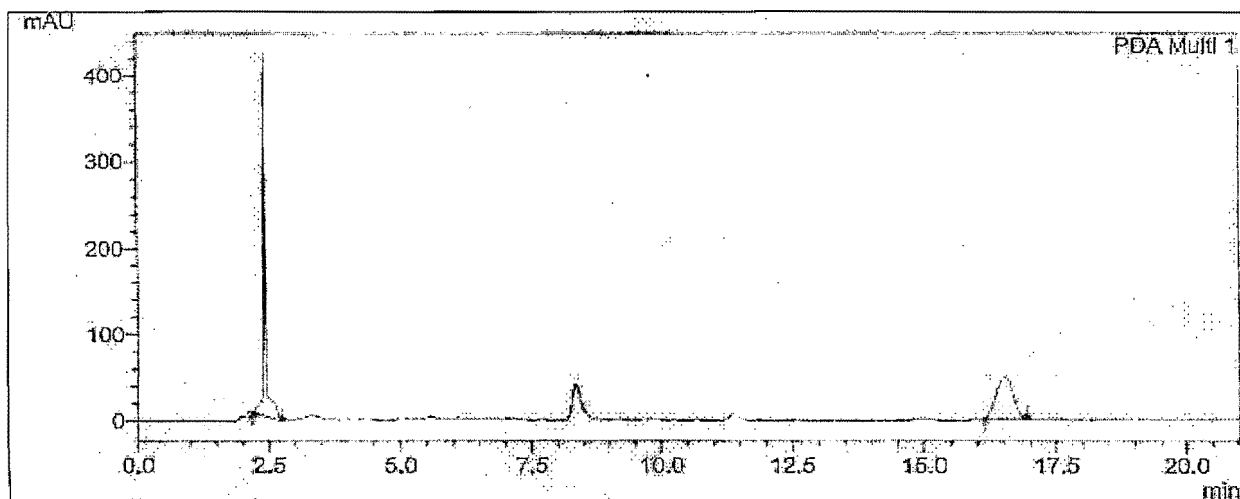


Figure 3.12: Chromatogram of pro-Pheroid for the mefloquine HCl method.

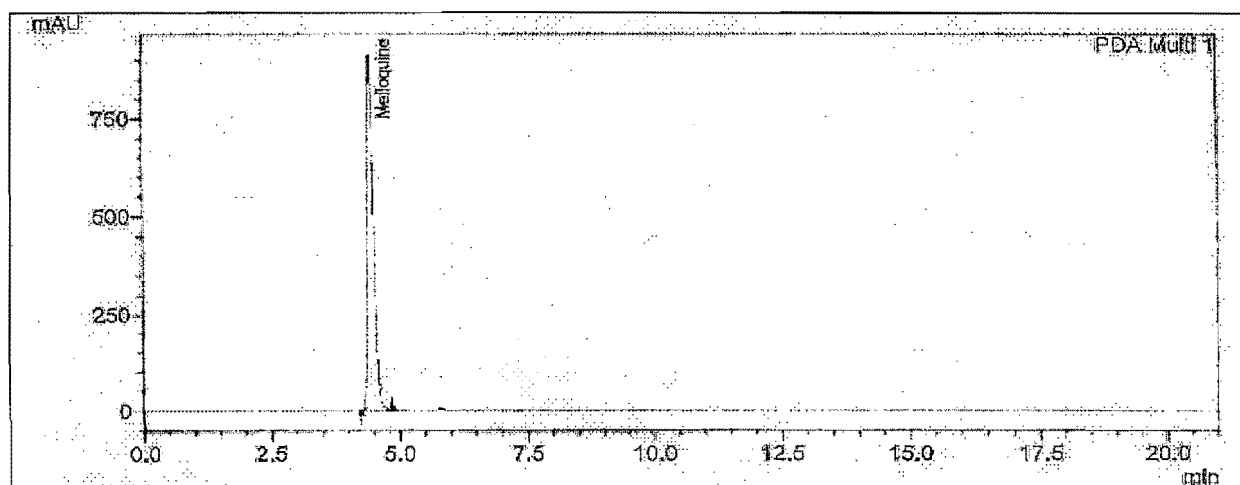


Figure 3.13: Chromatogram of mefloquine HCl in mobile phase.

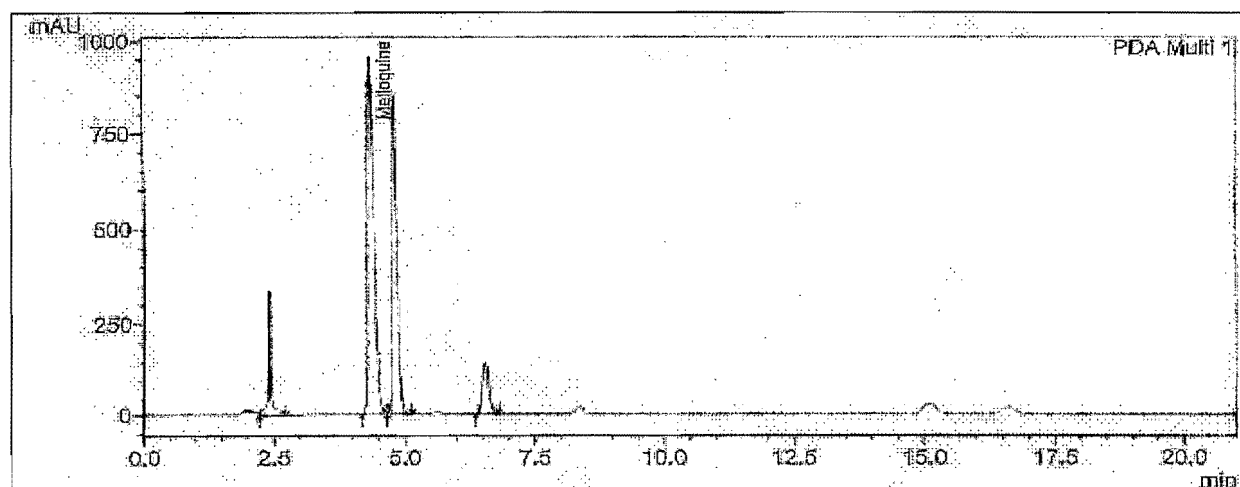


Figure 3.14: Chromatogram of mefloquine HCl in Pheroid.

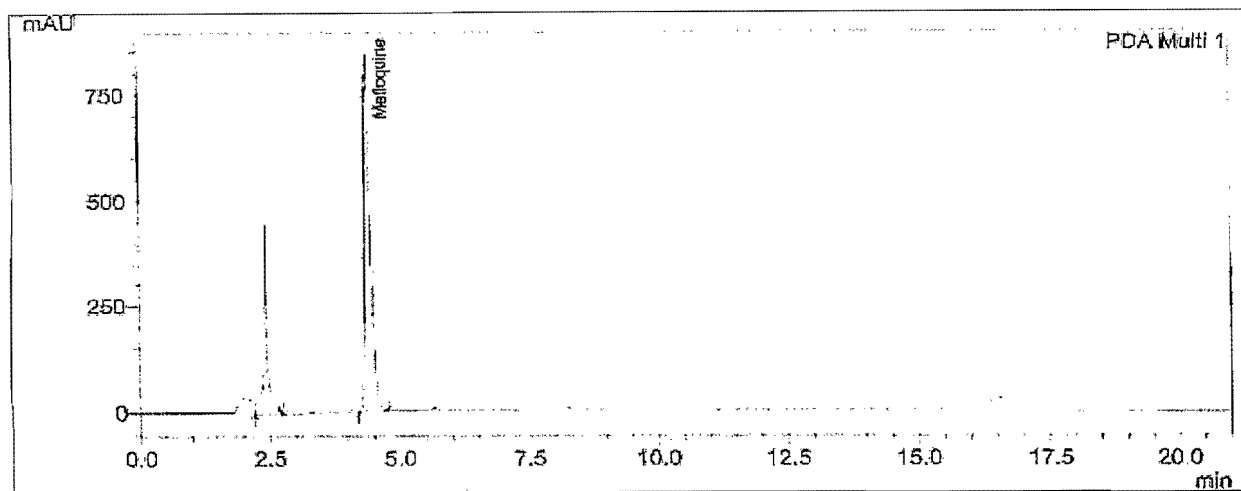


Figure 3.15: Chromatogram of mefloquine HCl in pro-Pheroid.

3.4.5.2 Precision (Repeatability)

Table 3.6: Results for mefloquine HCl to determine Intra-day precision

mg/ml	Area			Mean area	SD	% RSD
0.03944	2871149	2868911	2868602	2869554	1389.9	0.05
0.11833	7902492	7878840	7880211	7887181	13277.4	0.17
0.19722	11594439	11572446	11570256	11579047	13374.8	0.12

Intra-day precision was satisfactory with % RSD values between 0.05 – 0.17.

3.4.5.3 Linearity and Range

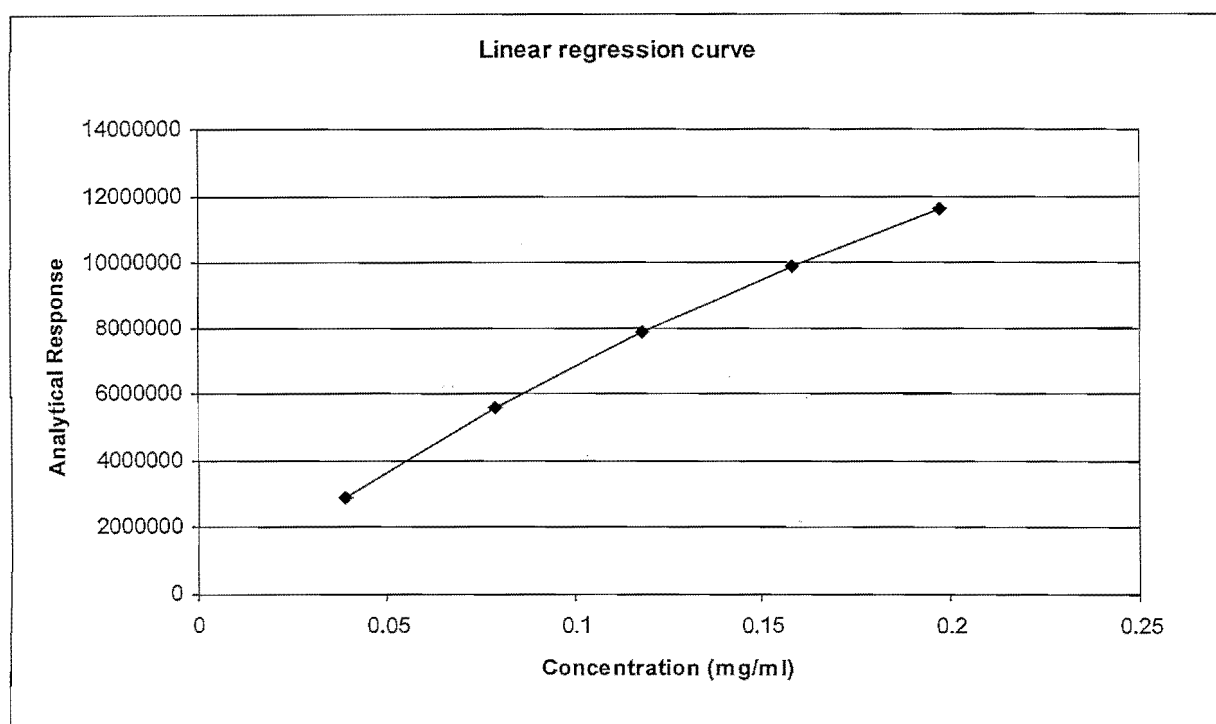
Table 3.7: Peak area, % RSD and concentration for mefloquine HCl

mg/ml	Area			Mean Area	% RSD
0.03944	2871149	2868911	2868602	2869554	0.05
0.07889	5567098	5553078	5572643	5564273	0.18
0.11833	7902492	7878840	7880211	7887181	0.17
0.15777	9908900	9900625	9889545	9899690	0.10
0.19722	11594439	11572446	11570256	11579047	0.12

Table 3.8: Regression parameters of mefloquine HCl

R squared	0.991791
Intercept	1033737.834
Slope	55152633.87

The mefloquine method complies to the specifications as it is linear from 0.0394 – 0.1972 mg/ml (50-150 % of expected sample concentration) with a $R^2 = 0.9912$ (figure 3.16).

**Figure 3.16: The linear regression curve for the mefloquine HCl method.**

3.5 Conclusion

Both methods performed well and should be suitable to analyse chloroquine phosphate and mefloquine HCl respectively in the Pheroid drug delivery system in products for stability testing.

CHAPTER 4

Stability Testing

4.1 Introduction

According to the International Conference of Harmonisation (ICH) Harmonised Tripartite Guideline (2003) stability testing is done to provide evidence on how a drug substance or product varies with time under the influence of a variety of environmental factors such as light, temperature and humidity. Stability testing is preformed to establish among other things; a shelf life for drug products and recommended storage conditions (ICH Harmonised Tripartite Guideline, 2003). Stability information generated, is used to ensure the quality, efficacy and safety of the formulated products (Grimm, 1993).

During stability testing the batches have to be representative of the product in terms of composition, manufacturing and packaging. The analytical methods used must be stability specific, validated and the apparatus used calibrated (Grimm, 1993). The testing should cover, as appropriate, the chemical, physical, microbiological and biological attributes (ICH Harmonised Tripartite Guideline, 2003).

Microbiological tests include antimicrobial effectiveness testing and microbial limit tests. Antimicrobial effectiveness must be demonstrated for multiple – dose, topical and oral dosage forms, as well as other dosage forms like ophthalmic, otic and nasal fluids. Antimicrobial preservatives are added to non-sterile dosage forms to protect them from microbiological growth or from micro-organisms that are introduced inadvertently during or subsequent to the manufacturing process (USP, 2007).

The storage conditions used during the stability testing must be selected with care. The storage conditions are determined by temperature and humidity. Both factors greatly affect the stability of a drug product (Grimm, 1993). The ICH guideline suggest that for accelerated stability studies, the storage conditions should be as follows: $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \text{ relative humidity (RH)} \pm 5\% \text{ RH}$ for a period of six months (ICH Harmonised Tripartite Guideline, 2003). However, the accelerated stability tests in this study were done over a three month period at the following conditions: 5°C ; $25^{\circ}\text{C} + 60\% \text{ RH}$; $30^{\circ}\text{C} + 65\% \text{ RH}$ and $40^{\circ}\text{C} + 75\% \text{ RH}$. The tests preformed on the products during the stability study were assays and antimicrobial effectiveness

testing. For the assay one sample was prepared and injected three times and methanol was used as solvent for the samples.

4.2 Assay

4.2.1 Method

The assays were performed according to the validated methods discussed in chapter 3.

4.2.2 Test specifications

The limits for the assay was predetermined as the initial value $\pm 5\%$.

4.2.3 Calculations

The following calculations were used to evaluate the results obtained:

$$\text{STD concentration} = \frac{\text{mass STD weighed (mg)} \times \text{potency (\%)}}{\text{Dilution factor}}$$

$$\text{Sample concentration} = \frac{\text{STD concentration} \times \text{sample area}}{\text{STD area}}$$

$$\text{Assay value} = \frac{\text{Sample concentration}}{\text{STD concentration}} \times 100$$

$$\% \text{ Recovery} = \frac{\text{Assay value}}{\text{Theoretical sample value}} \times 100$$

4.2.4 Assay results and discussion

4.2.4.1 Chloroquine phosphate in Pheroid vesicles

Table 4.1: Assay (% RSD) results for chloroquine phosphate in Pheroid vesicles

Storage conditions	Assay			
	Initial	Month 1	Month 2	Month 3
5°C	98.1 (0.13)	100.7 (0.15)	101.2 (0.32)	105.9 (0.61)
25°C + 60 RH		100.6 (0.74)	105.2 (0.67)	101.3 (0.88)
30°C+ 65 RH		102.0 (0.29)	103.1 (0.40)	97.8 (1.72)
40°C + 75 RH		99.1 (0.26)	95.9 (0.32)	105.6 (1.40)

The assay values of chloroquine phosphate in Pheroid vesicles are within the 5% range of the initial value and comply with the in-house specification.

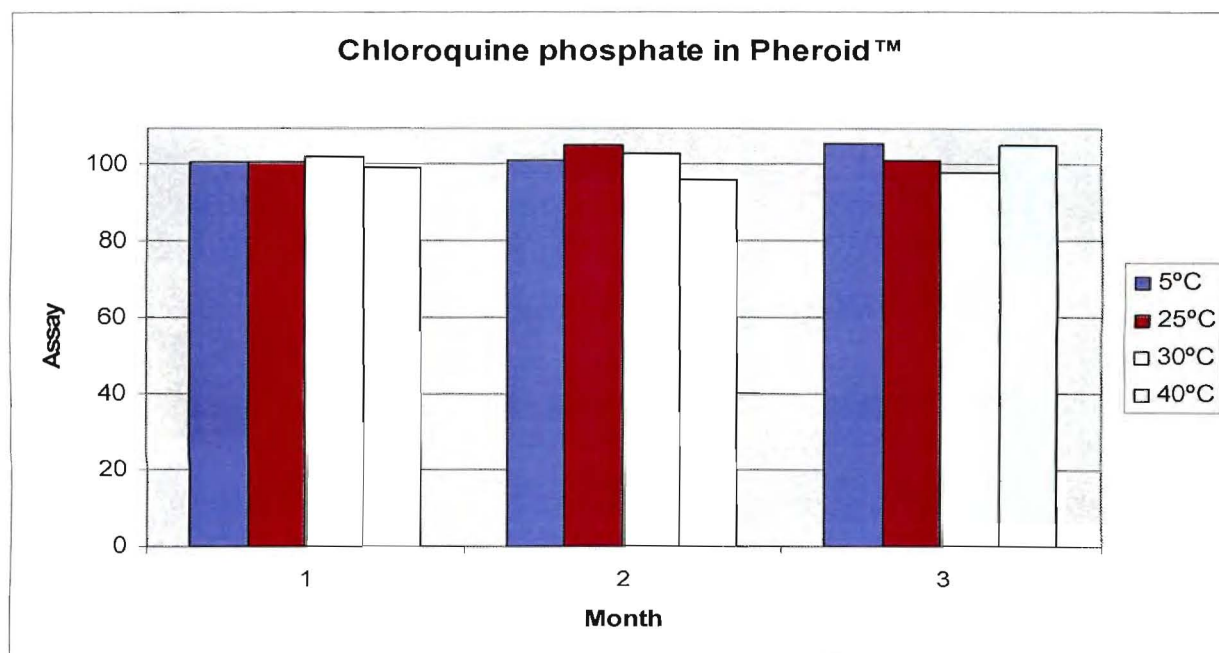


Figure 4.1: Graphic representation of the assay results of chloroquine phosphate in Pheroid vesicles.

4.2.4.2 Chloroquine phosphate in pro-Pheroid

Table 4.2: Assay (% RSD) results for chloroquine phosphate in pro-Pheroid

Storage conditions	Assay			
	Initial	Month 1	Month 2	Month 3
5°C	86.9 (0.31)	184.7 (0.66)	496.4 (0.37)	126.5 (1.42)
25°C + 60 RH		147.4 (1.45)	184.3 (1.22)	451.3 (0.40)
30°C+ 65 RH		116.1 (0.30)	147.8 (0.41)	143.1 (0.93)
40°C + 75 RH		271.8 (0.34)	162.9 (0.35)	129.7 (0.28)

The solubility of the chloroquine phosphate proved to be problematic for this formulation. As stated in chapter 2, chloroquine phosphate is freely soluble in water and practically insoluble in alcohol. When examining the final product it became clear that the chloroquine phosphate was suspended in the pro-Pheroid but it did not dissolve. It also appeared as though the chloroquine phosphate did not stay in suspension with the oil phase. A sediment formed quickly which was difficult to resuspend. When doing sample preparation it was necessary to vigorously shake and stir the suspension to try and resuspend the drug. Afterwards it was necessary to dilute some of the pro-Pheroid formulation in water to dissolve the chloroquine phosphate and make it possible to detect the chloroquine phosphate on the HPLC. After the dilution with water the product was further diluted with methanol and analysed on the HPLC.

The chloroquine phosphate in pro-Pheroid showed significant variation in results with the lowest value being 86.9 % and the highest 496.3 %. This may be attributed to sample inconsistency. A chromatogram of the 3 months at 25°C + 60 RH stability study is showed in figure 4.2.

The variation in results as well as the solubility problems leads to the conclusion that chloroquine phosphate should not be used in the pro-Pheroid system.

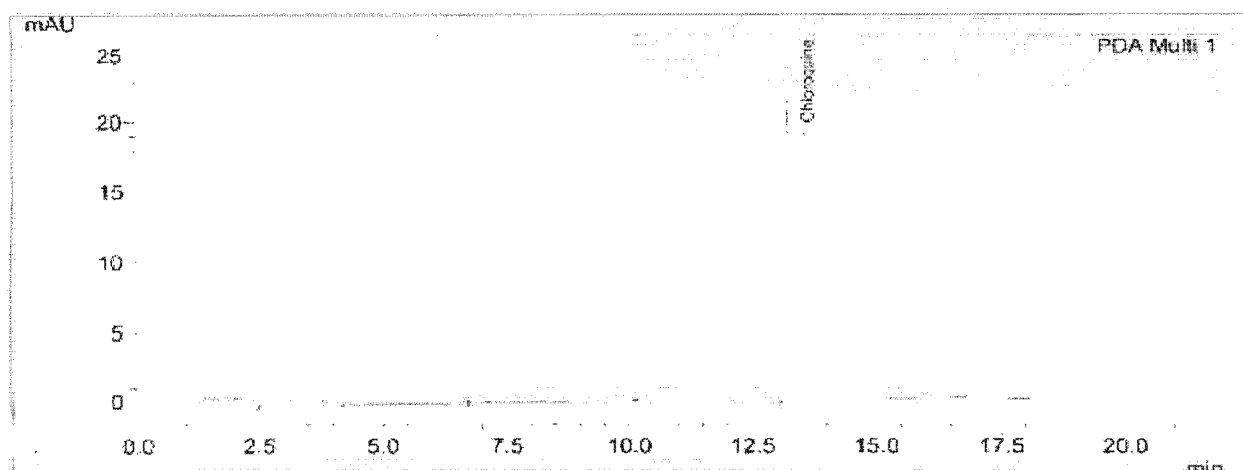


Figure 4.2: Chromatogram of chloroquine phosphate in Pheroid vesicles (3 months 25°C + 60 RH).

4.2.4.3 Mefloquine HCl in Pheroid microsponges

Table 4.3: Assay (% RSD) results for mefloquine HCl in Pheroid microsponges

Storage conditions	Assay			
	Initial	Month 1	Month 2	Month 3
5°C	42.6 (0.18)	19.6 (0.12)	-	-
25°C + 60 RH		23.8 (0.15)	53.6 (0.05)	24.2 (0.18)
30°C+ 65 RH		19.5 (0.19)	57.1 (0.16)	28.4 (0.41)
40°C + 75 RH		24.6 (0.05)	67.1 (0.06)	23.5 (0.31)

Low assay values for the initial, months 1 and 3 were obtained for mefloquine HCl in Pheroid. The assay values for month 2 at all the stability conditions, were higher than that obtained for the other stability intervals.

Stability at 5°C was completed although the results could not be used, since the study of the chromatograms showed peak interference from the Pheroid system that was not present during the first month of stability (Figure 4.3 - 4.5).

The influence of the Pheroid system on the drug recovery by means of HPLC could not have been predicted before the study was conducted. However, it is evident that the Pheroid system has a significant influence on the drug recovery and this should

be investigated further. Ideally the mefloquine HCl should not be used in the Pheroid microsponges.

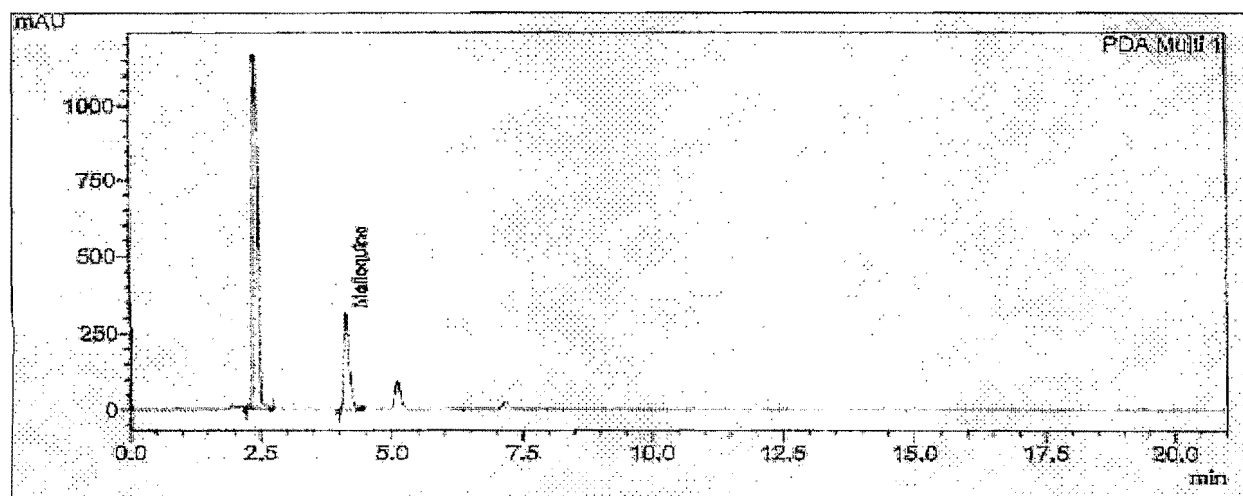


Figure 4.3: Chromatogram of mefloquine HCl in Pheroid microsponges (1 month 5°C).

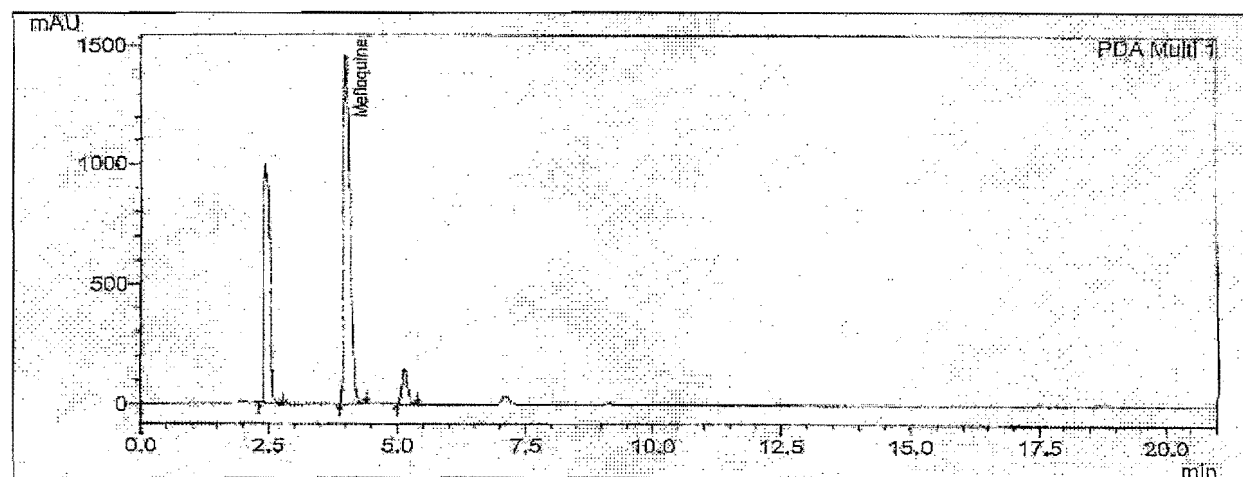


Figure 4.4: Chromatogram of mefloquine HCl in Pheroid microsponges (2 months 5°C).

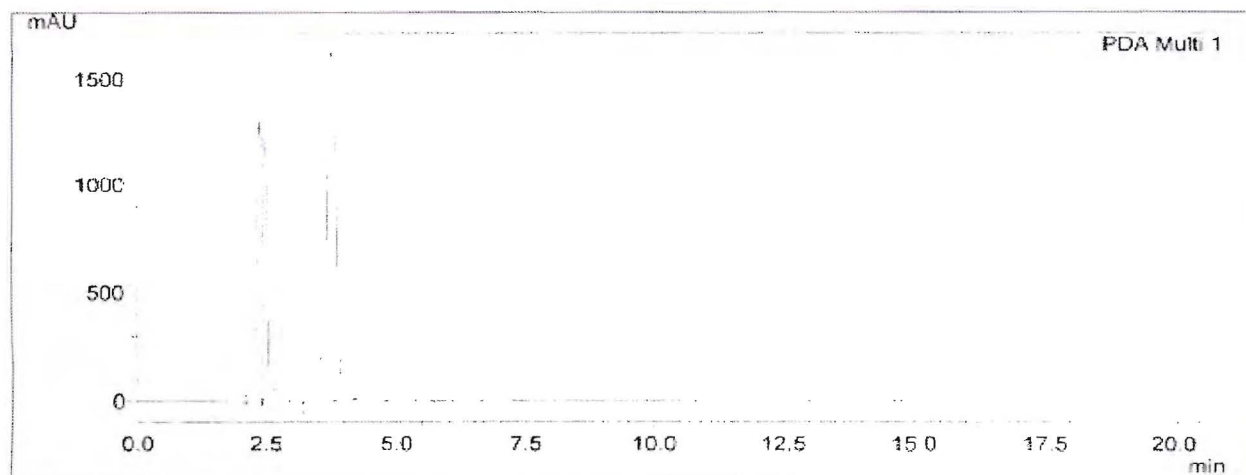


Figure 4.5: Chromatogram of mefloquine HCl in Pheroid microsponges (3 months 5°C).

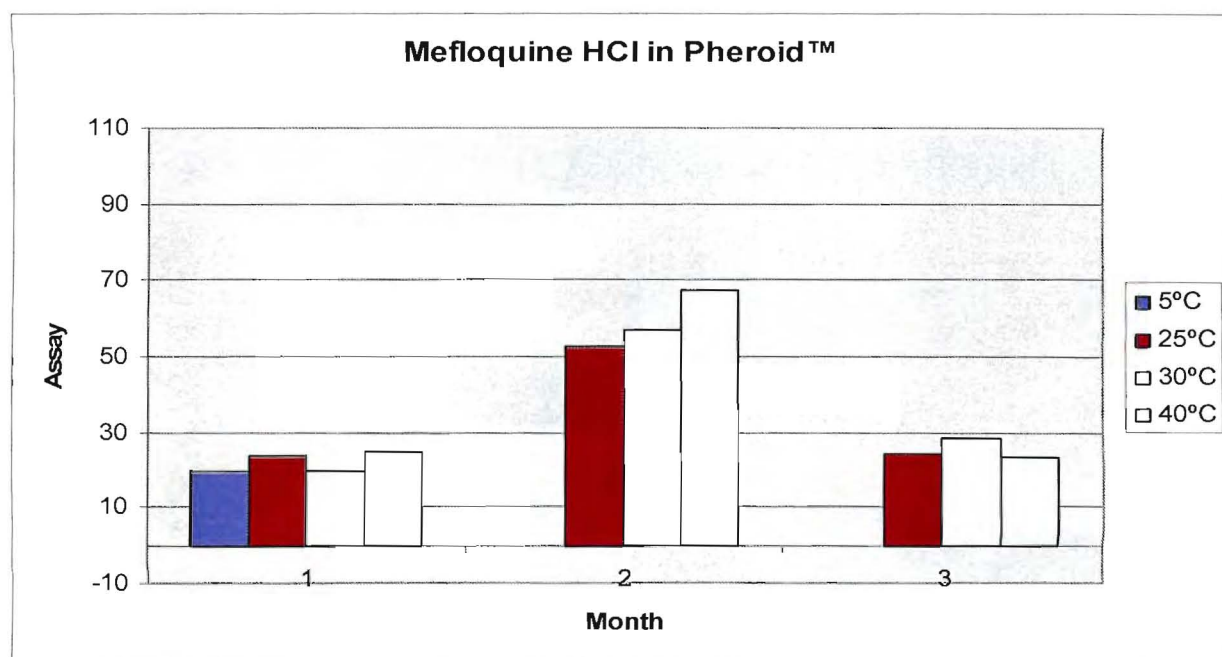


Figure 4.6: Graphic representation of the assay results of mefloquine HCl in Pheroid microsponges.

4.2.4.4 Mefloquine HCl in pro-Pheroid

Table 4.4: Assay (% RSD) results for mefloquine HCl in pro-Pheroid

Storage conditions	Assay			
	Initial	Month 1	Month 2	Month 3
5°C	90.4 (0.81)	143.5 (0.11)	132.6 (0.23)	145.1 (0.12)
25°C + 60 RH		126.3 (0.12)	132.9 (0.16)	150.5 (0.42)
30°C+ 65 RH		134.8 (0.15)	119.7 (0.07)	123.6 (0.23)
40°C + 75 RH		92.3 (0.17)	126.4 (0.14)	137.2 (0.47)

The mefloquine HCl as with chloroquine phosphate in pro-Pheroid did not stay in suspension with the oil phase. The sediment that formed was difficult to resuspend and vigorous shaking and stirring was needed for resuspension. However, some of the mefloquine HCl did dissolve in the pro-Pheroid system. Although the assay values for the mefloquine HCl in pro-Pheroid seem to be high, it remains mostly constant. A possible explanation for the high assay values is peak interference from the pro-Pheroid system. A more specific analytical method should be considered for future analysis.

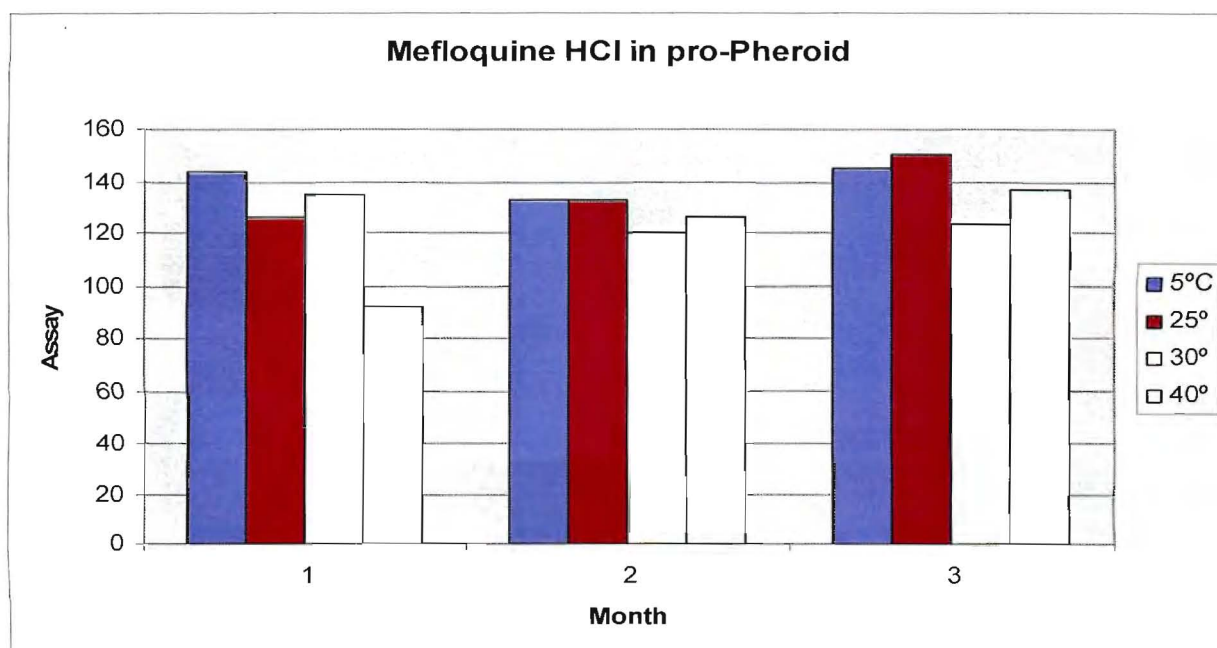


Figure 4.7: Graphic representation of the assay results of mefloquine HCl in pro-Pheroid.

4.3. Antimicrobial effectiveness testing

EnviroCare Laboratories performed the antimicrobial effectiveness testing according to the USP method. The results of the antimicrobial effectiveness tests are summarised in Annexure A. The antimicrobial preservatives used in the Pheroid formulations were methylparaben and propylparaben, where only propylparaben was used in the pro-Pheroid formulations. The following micro-organisms must be tested for (USP, 2007):

- *Staphylococcus aureus*
- *Pseudomonas aeruginosa*
- *Aspergillus niger*
- *Candida albicans*
- *Esscherichia coli*

The antimicrobial preservatives added to the chloroquine phosphate in Pheroid vesicles, the mefloquine HCl in Pheroid microsponges and the mefloquine HCl in pro-Pheroid formulations proved to be effective since they complied to the USP specifications. Those added to the chloroquine phosphate in pro-Pheroid proved to be effective for *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Esscherichia coli*. For the initial, month 1 25°C + 60 RH and month 3 25°C + 60 RH there was an increase from the initial calculated count at 14 to 28 days for *Aspergillus niger* and thus it did not comply with the USP specifications.

4.4 Conclusion

Instead of preparing samples in duplicate (or triplicate), one sample was prepared and injected 3 times. All conclusions based on assay values should be viewed with this in mind.

Accelerated stability studies, conducted on the four products formulated, indicate that only chloroquine phosphate in Pheroid complies with specifications. This product may be further developed for possible commercialisation.

It was found that chloroquine phosphate cannot be formulated with pro-Pheroid, because of solubility issues.

Results for mefloquine HCl in microsponges were inconclusive. Assay values for months 1 and 3 were low, whilst values for month 2 were much higher.

Despite the fact that mefloquine HCl in pro-Pheroid did not comply with assay specifications, the results obtained were consistent.

With the HPLC assay methods used, peak interference from the Pheroid system was significant. It would be advisable to further develop the methods to eliminate this problem. Questionable results may then be re-examined to distinguish between poor product performance and analytical issues.

The preservatives used showed good efficacy overall, with the exception of some *Aspergillus niger* growth in the formulation containing chloroquine phosphate in pro-Pheroid.

CHAPTER 5

Conclusion

Malaria is a disease that is caused by the *Plasmodium* parasite of which there are four species that effect humans, namely: *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae*. *P. falciparum* is the most common form found in Southern Africa including South Africa. Malaria occurs in 90 countries at present with 1.62 billion people living in areas where malaria is now increasing after having been previously reduced. Every year there are about 300 to 500 million cases of malaria making it one of the most common infectious diseases world wide with about 100 million deaths, of which 90% is in Africa, south of the Sahara. Young children, pregnant women and non-immune visitors to malaria-endemic areas are at greater risk of contracting this severe or fatal illness. In South Africa the most affected areas are KwaZulu-Natal, Limpopo, Mpumalanga and the Northern Province. There has been a sharp decline in the number of reported cases in South Africa during the period between 2006 and 2007. The South African health department has attributed this sharp decline to several interventions including:

- An increase in the indoor residual spraying of dichloro-diphenyl-trichloroethane (DDT) with the overall coverage of more than 80% and with the spraying being completed before the peak in malaria transmission;
- The use of artemisinin – based combination therapy;
- Advocacy with mass community mobilisation and training of health care workers in the malaria affected areas.

Resistance to anti-malarials has emerged as one of the biggest challenges facing malaria control today. *P. falciparum* has developed resistance to nearly all anti-malarials in current use, although the geographical distribution to any single anti-malarial drug varies greatly. Chloroquine resistance is the most common and in South Africa resistance to sulfadoxine-pyrimethamine has also been reported. To try and avoid drug resistance, drug combinations are being used as well as the search for new anti-malarials. Resistance to new anti-malarials is greatly feared, so a better option could be the use of a drug delivery system that is capable of bypassing the

current mode of resistance and thus expanding the lifetime of current anti-malarials. The Pheroid drug delivery system is such a drug delivery system.

Pheroids are unique and stable lipid-based submicron-and micron sized structures, uniformly distributed in a dispersion medium that may be adapted to the indication. Pheroids consist of three phases, namely an aqueous phase consisting of sterile water, an oil phase consisting of fatty acids and a dispersed gas phase which is associated with the oil phase. When comparing the Pheroid system with other drug delivery systems, the Pheroid system has a great number of advantages. In the case of malaria the most important advantage is the decrease of drug resistance.

The main objectives of this study were the development of HPLC methods for the analysis of the antimalarials in the Pheroid drug delivery system, the validation of the HPLC methods developed and the determination of drug stability of the formulated products during accelerated stability conditions.

Mefloquine HCl and chloroquine phosphate were each separately formulated in both the Pheroid and pro-Pheroid systems. HPLC analysis was done after each month.

Accelerated stability studies, conducted on the four products formulated, indicate that only chloroquine phosphate in Pheroid complies with specifications. This product may be further developed for possible commercialisation.

It was found that chloroquine phosphate cannot be formulated with pro-Pheroid, because of solubility issues.

Results for mefloquine HCl in Pheroid microsponges were inconclusive. Assay values for months 1 and 3 were low, whilst values for month 2 were much higher.

Despite the fact that mefloquine HCl in pro-Pheroid did not comply with assay specifications, the results obtained were consistent.

With the HPLC assay methods used, peak interference from the Pheroid system was significant. It would be advisable to further develop the methods to eliminate this problem. Questionable results may then be re-examined to distinguish between poor product performance and analytical issues.

The preservatives used showed good efficacy overall, with the exception of some *Aspergillus niger* growth in the formulation containing chloroquine phosphate in pro-Pheroid.

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ANNEXURE A

Results for the antimicrobial effectiveness testing

A1 Test specifications

The criteria for antimicrobial effectiveness according to the USP for oral products other than antacids are (USP, 2007):

- **Bacteria:** Not less than 1.0 log reduction from the initial count at 14 days, and no increase from 14 days' count at 28 days.
- **Yeast and Moulds:** No increase from the initial calculated count at 14 and 28 days.

The following micro-organisms must be tested for (USP, 2007):

- *Staphylococcus aureus*
- *Pseudomonas aeruginosa*
- *Aspergillus niger*
- *Candida albicans*
- *Escherichia coli*

A2 Growth characteristics and requirements:

1. Bacteria used:

- ***Staphylococcus aureus***

Family: Micrococcaceae (Prokaryote)

Morphology: Cocci

Temperature requirements: 6.5°C – 46°C (optimum of 37°C)

pH requirements: 4.2 – 9.3

S. aureus is a Gram + bacteria that is cocci shaped and is found in clusters. It causes abscesses, boils, endocarditis, food poisoning, impetigo, pericarditis, pneumococcal pneumonia and toxic shock syndrome (Ingraham & Ingraham, 2004).

- ***Pseudomonas aeruginosa***

Family: Pseudomonadaceae (Prokaryote)

Morphology: Bacillus

Temperature requirements: 4°C – 43°C (optimum of 37°C)

pH requirements: 6.6 – 7.0

This Gram - bacteria is rod shaped, has a blue green pigment and is most threatening to burn victims as it causes severe infections of the burned areas (Ingraham & Ingraham, 2004).

- ***Escherichia coli***

Family: Enterobacteriaceae

Morphology: Straight rod

Temperature requirements: 28°C – 37°C (optimum)

pH requirements: 6.0 – 7.0

E. coli is a Gram - bacteria that is rod shaped and causes diarrhoeal infections, meningitis and urinary tract infections (Ingraham & Ingraham, 2004).

2. Fungi used:

- ***Aspergillus niger***

Family: Deuteromycetes

Morphology: Black hyphae

Temperature requirements: 20°C – 28°C (optimum)

pH requirements: 4.4 – 4.6

Aspergillus is a fungus (or mould) that is very common in the environment and it causes a disease called Aspergillosis. There are many different kinds of aspergillosis and they vary in severity (Anon, 2008e).

- ***Candida albicans***

Family: Cryptococcus

Morphology: Cocci

Temperature requirements: 20°C – 35°C (optimum of 25°C)

pH requirements: 4.4 – 4.6

This fungi is an important cause of morbidity and mortality in immuno-compromised patients like HIV patients. It is one of the many organisms which live in the human mouth and gastrointestinal tract and under normal circumstances in 80 % of patients it has no harmful effects (Ingraham & Ingraham, 2004).

A3. Indication of growth

The following colour characteristics indicate the presence of the colonies on the TS agar plates:

- *Staphylococcus aureus*: White, rounded colonies.
- *Pseudomonas aeruginosa*: Light greenish, rounded colonies.
- *Escherichia coli*: Rigid, cream coloured colonies
- *Aspergillus niger*: Furry black colonies or mats
- *Candida albicans*: Small, white, slightly raised colonies.

A4 Antimicrobial effectiveness testing results

A4.1 Chloroquine phosphate in Pheroid vesicles

Table A1: Results of the antimicrobial effectiveness testing for chloroquine phosphate in Pheroid vesicles

Chloroquine in Pheroid Month 0		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	2.3	2
<i>Candida albicans</i>	>3	1.9

<i>Esscherichia coli</i>	>3	2.4
1 Month 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 30°C + 65 RH		

Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 40°C + 75 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 5°C		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 25°C + 60 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 30°C + 65 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 40°C + 75 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3

A4.2 Chloroquine phosphate in pro-Pheroid

Table A2: Results of the antimicrobial effectiveness testing for chloroquine phosphate in pro-Pheroid

Chloroquine in pro-Pheroid Month 0		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	1.8	>3
<i>Candida albicans</i>	>3	2.7
<i>Esscherichia coli</i>	>3	>3
1 Month 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	1.8	>3
<i>Candida albicans</i>	>3	2.7
<i>Esscherichia coli</i>	>3	>3
1 Month 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3

<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	2.6	2.6
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	2.6	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	2.6
<i>Candida albicans</i>	>3	2.4

<i>Esscherichia coli</i>	>3	>3
3 Months 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	2.4
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3

A4.3 Mefloquine HCl in Pheroid microsponges

Table A3: Results of the antimicrobial effectiveness testing for mefloquine HCl in Pheroid microsponges

Mefloquine HCl in Pheroid Month 0		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	2.2	2
<i>Candida albicans</i>	>3	2.2
<i>Esscherichia coli</i>	>3	2.4
1 Month 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 40°C + 75 RH		
Test organism	Log Reduction	

	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3

<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3

A4.4 Mefloquine HCl in pro-Pheroid

Table A4: Results of the antimicrobial effectiveness testing for mefloquine HCl in pro-Pheroid

Mefloquine HCl in Pheroid Month 0		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	2.1	2
<i>Candida albicans</i>	>3	2.2
<i>Esscherichia coli</i>	2.8	2.4
1 Month 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3

<i>Esscherichia coli</i>	>3	>3
1 Month 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
1 Month 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 5°C		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 25°C + 60 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 30°C + 65 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
2 Months 40°C + 75 RH		
Test organism	Log Reduction	
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 5°C		

Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 25°C + 60 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 30°C + 65 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3
3 Months 40°C + 75 RH		
Test organism		
Log Reduction		
	0h – 14 days	0h - 28 days
<i>Staphylococcus aureus</i>	>3	>3
<i>Pseudomonas</i>	>3	>3
<i>Aspergillus niger</i>	>3	>3
<i>Candida albicans</i>	>3	>3
<i>Esscherichia coli</i>	>3	>3

ANNEXURE B

**Poster presented at the 28th Annual Conference of the
Academy of Pharmaceutical Sciences of South Africa, Club
Mykonos Resort, Langebaan, Western Cape, South Africa,
2007.**



An in vitro evaluation of Pheroid™ microsponges for anti-malarial drug delivery

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Introduction

Malaria is a leading cause of death in Africa and other tropical and subtropical regions in the world. More than 300 million people are infected by the parasite and more than 1 million people are killed by the disease annually. Children and pregnant women is most at risk in the developing countries of Africa.^[1,2] The occurrence of resistance among parasites to commonly used antimalarial drugs (chloroquine) is causing a progressive increase in the severity and mortality of the disease. The Pheroid™ system is a patented drug delivery system which entraps drugs with high efficiency and delivers it to target sites in the body. It consists of a unique submicron emulsion type formulation. Because Pheroid™ possibly increase the drug concentration inside the cells, the optimal concentration of the Pheroid™ microsponges needs to be determined to maximize drug delivery.

Aims

The aim of this study was to determine an optimal Pheroid™microsponge formulation for use in *in vitro* drug studies. The effects of chloroquine in combination with different Pheroid™formulations were studied on *Plasmodium falciparum* in cell cultures.

Experimental Design

The chloroquine resistant RSA 11 strain were maintained in continuous culture using standard methods.^[3] Parasites were grown in RPMI 1640 culture medium supplemented with O⁺ erythrocytes, Albumax II and Gentamicin. Cultures used for this experiment contained sorbitol synchronized ring stages. Stocks of drug and Pheroid™microsponge formulation were prepared initially and diluted serially with specially formulated water to achieve the desired concentration range. The final chloroquine concentrations were 0nM, 50nM, 100nM and 200nM. The final Pheroid™ ratios were 1:5000, 1:2500, 1:1750, 1:1000 and 1:500. Each well of a 48 well plate was pre-dosed with 20µl of the appropriate Pheroid™-Drug solution. Parasite cultures were diluted with fresh uninfected erythrocytes and RPMI 1640 culture medium to achieve a starting parasitaemia of 1-3% and haematocrit of 3-5%. 180µl of the diluted parasite culture were added to each well. The parasites were incubated for one intraerythrocytic asexual replication (48 hours) at 37°C at an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. The percentage inhibition of parasite growth by chloroquine was determined by Giemsa staining of thin blood smears. Dry slides were viewed under a 60x oil objective of a Nikon TE300 microscope. A total of at least 1000 cells in ten different microscope fields were counted and the parasitaemia was calculated as a percentage.^[4,5,6]

Results

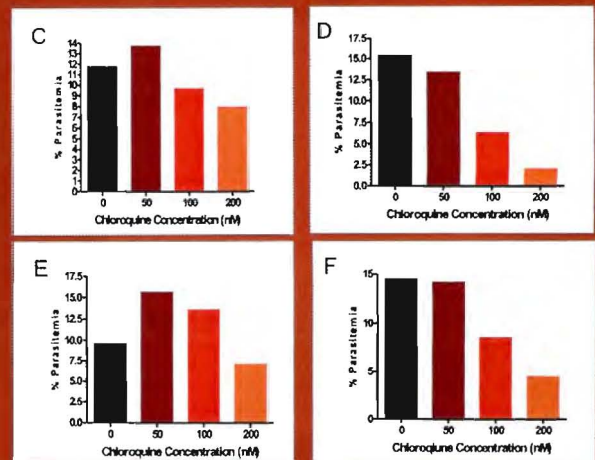
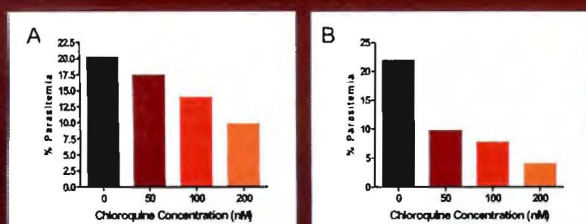


Figure 1: The % parasitemia at the different chloroquine concentration at (A) Control - No Pheroid™; (B) 1:5000 Pheroid™ ratio; (C) 1:2500 Pheroid™ ratio; (D) 1:1750 Pheroid™ ratio; (E) 1:1000 Pheroid™ ratio; and (F) 1:500 Pheroid™ ratio.

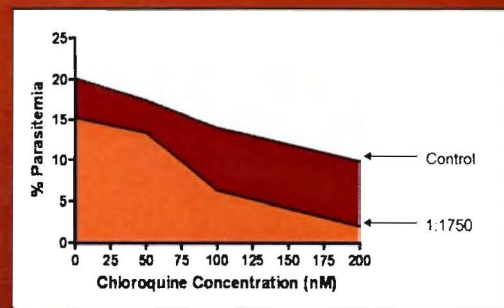


Figure 2: Optimal Pheroid™microsponge concentration.

Conclusion

The results of this study show that there is an overall decrease in the % parasitemia when the chloroquine concentration increased. The chloroquine in the Pheroid™ delivery system resulted in a remarkable decrease in the % parasitemia. There is a decrease in the parasitemia at all Pheroid™microsponge ratios in relation to the control. The 1:1000 and 1:500 ratio had a negative effect on the red blood cells. The 1:1000 ratio crenated the red blood cells and the 1:500 ratio causes red blood cell lysis. The 1:750 ratios gave a better reduction in the % parasitemia in relation to the control than the other Pheroid™ ratios. The optimal Pheroid™microsponge ratio, appeared to be the 1:1750 because it enhances the chloroquine effect the best without any observed negative effect on the red blood cells. The 1:1750 Pheroid™microsponge ratio maximize drug delivery and has the potential for further investigation.

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ANNEXURE C

**Poster presented at the 29th Annual Conference of the
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Rest Resort, Rustenburg, North West Province, 2008.**

Stability tests on anti-malarial drugs encapsulated in Pheroid™ and pro-Pheroid drug delivery systems

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INTRODUCTION

Malaria is one of the most serious and sometimes fatal diseases, caused by protozoan parasites. Approximately 300 million people worldwide are infected with malaria, with 90 % of about 100 million deaths, in Africa, south of the Sahara¹. The current treatment of malaria is restricted due to ever increasing drug resistance, cost of treatment and the availability of treatment². When looking at solutions for these problems, alternative drug delivery systems should be taken into serious consideration. Pheroid™ technology can be placed in this category and the stability of anti-malarial drugs in combination with the Pheroid™ drug delivery system was investigated.

BACKGROUND

The Pheroid™ drug delivery system consists of the interspersation of two liquid phases and a gas phase. The two liquid phases consist of an aqueous base and an oily base. Pro-Pheroid formation involves the process of gassing the oil phase with nitrous oxide, followed by mixing the drug into the oil phase. Stable Pheroid™ vesicles will form once the pro-Pheroid encounters the fluid component that is found *in vivo*. This is a important formulation tool for unstable molecules and a great advantage of the Pheroid™ system³.

OBJECTIVES

The aim of this project was to investigate the stability of the anti-malarial drugs, chloroquine phosphate and mefloquine hydrochloride, in Pheroid™ and pro-Pheroid formulations.

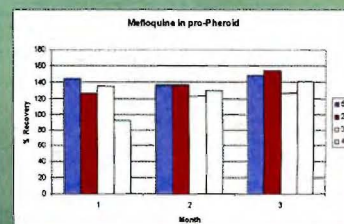
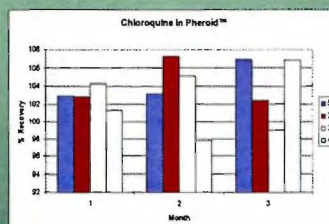
METHODS

Mefloquine HCl and chloroquine phosphate were each separately formulated in both the Pheroid™ and pro-Pheroid systems.

The four formulations were subjected to an accelerated stability study over a period of three months at the following conditions: 5°C, 25°C+60% RH, 30°C+65% RH and 40°C+75% RH. HPLC analysis was done after each month according to the respective assay methods. The methods were developed and validated in-house.

RESULTS AND DISCUSSION

The results showed that the type of Pheroid™ to be used depends on the solubility of the drug to be entrapped. Chloroquine phosphate in Pheroid™ vesicles proved to be stable over the three month period. Chloroquine phosphate is freely soluble in water and the highly variable results obtained with chloroquine phosphate in the pro-Pheroid (oil phase) indicates more of a solubility than a stability problem. Mefloquine HCl in pro-Pheroid seems to be more stable over the testing period than mefloquine HCl in Pheroid™ microsponges. Mefloquine HCl is only slightly soluble in water and this attributes to the low assay values obtained (29%) for the mefloquine HCl formulation in the Pheroid™ system.



These results showed that chloroquine phosphate in the Pheroid™ delivery system was stable and should be considered as a possible effective formulation for antimalarial treatment. A possible reason for the increase in concentration with the mefloquine HCl in pro-Pheroid is peak interference during analyses and this should be investigated further.

REFERENCES

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