

***IN VITRO* ANTIMALARIAL EFFICACY
ENHANCEMENT OF
SELECTED ANTIBIOTICS
WITH PHEROID™ TECHNOLOGY**

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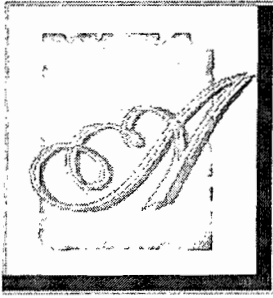
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*I hope that my achievements in life shall be these
~ that I will have fought for what was right and
fair, that I will have risked for that which mattered,
and that I will have given help to those who were
in need, that I will have left the earth a better place
for what I've done and who I've been.*

~ C. Hoppe ~



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Anèl van Niekerk

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ABSTRACT

***IN VITRO* ANTIMALARIAL EFFICACY ENHANCEMENT OF SELECTED ANTIBIOTICS WITH PHEROID™ TECHNOLOGY.**

The *Plasmodium falciparum* parasite, carried by *Anopheles* mosquitoes, is currently a global problem due to the rising incidence of resistance of the parasite to available antimalaria drugs. Resistance and difficult treatment groups, including pregnant woman and young children, are pressing for the development of new, safe and effective prophylactic and treatment antimalarials. Because of the extensive process of developing new drugs, researchers and health care professionals have turned to combination therapy where a fast acting antimalarial is combined with slower acting drugs, such as antibiotics.

The macrolide antibiotics, erythromycin and azithromycin, have been studied to a limited extent for their potential antimalarial effect. Certain advantages, such as their safety profile (especially that of azithromycin) in pregnancy and administration to young children, motivates continual research into the advancement of the effect these drugs exude on malaria. Drug delivery systems contribute to the efficacy of medicines, conquering several difficulties of treatment with oral medication. Pheroid™ technology is a patented drug delivery system, mainly consisting of plant and essential fatty acids, and has been demonstrated to entrap, carry and deliver pharmacologically active compounds and other useful molecules.

This study compared the *in vitro* effects of the macrolide antibiotics on the growth of a chloroquine-resistant strain (RSA 11) of *Plasmodium falciparum* to the effects of the macrolides entrapped in Pheroid™ vesicles on the same strain over and extended observation period of 144 hours. ELISA assays were conducted by analysing the HRP II (histidine-rich protein) levels on a pre-coated microtitre plate. The effects of the type of formulation, concentration and time were compared.

The *in vitro* difference between erythromycin alone and entrapped in Pheroid™ vesicles were found to be statistically significant ($P = 0.000000$) while the effects of both formulations did not seem to be concentration dependant ($P = 0.628424$). Prolonged exposure was also statistically meaningful ($P = 0.008268$), though it seems that exposure need not exceed 96 hours. The type of formulation, in the case of azithromycin (azithromycin alone vs. azithromycin entrapped in Pheroid™ vesicles), proved statistically significant ($P = 0.002572$), while neither formulation seemed concentration dependant ($P = 0.427731$). Prolonged exposure was found to be statistically insignificant for azithromycin ($P = 0.221941$).

Keywords: *Plasmodium falciparum*, malaria, Pheroid™ technology, erythromycin, azithromycin, RSA 11, ELISA assay, HRP II



UITTREKSEL

IN VITRO ANTIMALARIA EFFEKTIVITEITS VERBETERING VAN GESELEKTEERDE ANTIBIOTIKUMS MET PHEROID™ TECHNOLOGIE.

Die *Plasmodium falciparum* parasiet, oorgedra deur *Anopheles* muskiete, is tans 'n wêreldwye probleem weens die verhoogde insidensie van weerstandbiedendheid van die parasiet teen beskikbare antimalaria middels. Weerstandbiedendheid en moeilik behandelbare groepe, soos swanger vrouens en jong kinders, dryf die behoefte vir die ontwikkeling van nuwe, veilige en effektiewe profilaktiese en behandelings middels. Die ontwikkeling van nuwe geneesmiddels is 'n tydsame proses, en daarom het navorsers en gesondheidsorgwerkers die gebruik van kombinasie terapie geïmplementeer, waar vinnig werkende antimalaria middels met stadig werkende geneesmiddels soos antibiotikas gekombineer word.

Die makrolied antibiotikas, eritromisien en azitromisien, is voorheen tot 'n mindere mate ondersoek vir die potensieële effek wat die middels op malaria parasiete uitoefen. Sekere voordele, soos hul veiligheids profiel (veral azitromisien) vir toediening tydens swangerskap en in jong kinders, dien as motivering vir verdere navorsing in die bevordering van die middels se effek op malaria. Geneesmiddel afleweringstelsels dra by tot die effektiwiteit van geneesmiddels en oorkom menig probleme wat ondervind word met orale dosering. Pheroid™ tegnologie is 'n gepatenteerde geneesmiddelafleweringstelsel wat hoofsaaklik uit plant en essensiële vetsure bestaan. Die stelsel het bewys dat dit farmakologies aktiewe verbindings en ander bruikbare molekules kan vasvang, oordra en versprei.

Hierdie studie het die *in vitro* effek van die makrolied antibiotikas op die groei van 'n chloroquine-weerstandbiedende vorm (RSA 11) met die effek van die antibiotikas vasgevang in Pheroid™ vesikels oor 'n uitgebreide waarnemings tydperk van 144 uur met mekaar vergelyk.

ELISA analise is uitgevoer deur die HRP II (histidien-ryke proteïen) vlakke op voorafbedekte mikro-plate te analiseer. Die effek van die tipe formulering, konsentrasie en tyd was vergelyk. Die *in vitro* verskil tussen eritromisien alleen en vasgevang in Pheroid™ vesikels, was statisties betekenisvol ($P = 0.000000$) terwyl die effek van beide formulerings nie geblyk het om konsentrasie afhanklik te wees nie ($P = 0.628424$). Verlengde blootstelling was ook statisties betekenisvol ($P = 0.008268$) maar dit wil voorkom of blootstellingstyd nie nodig het om 96 uur te oorskrei nie. Die tipe formulering, in azitromisien se geval (azitromisien alleen teenoor azithromisien vasgevang in Pheroid™ vesikels), was statisties betekenisvol ($P = 0.002572$), terwyl nie een van die twee formulerings gebleik het om konsentrasie afhanklik te wees nie ($P = 0.427731$). Verlengde blootstelling was nie van statistiese betekenis vir azitromisien nie ($P = 0.221941$).

Sleutelwoorde: *Plasmodium falciparum*, malaria, Pheroid™ tegnologie, eritromisien, azitromisien, RSA 11, ELISA assay, HRP II



INTRODUCTION AND AIM OF STUDY

The term malaria is derived from the Italian ‘mal’aria’ which means ‘bad air’. The disease was earlier associated with swampy areas (Tuteja, 2007). Presently, malaria remains, together with tuberculosis and AIDS, one of three major communicable diseases (Lewison & Srivastava, 2008). In Africa, this killer disease has been ranked as the second highest contributor to the Disability Adjusted Life Year (DALY). The DALY is an estimation of a disease’s direct cause of mortality and morbidity (Snow *et al.*, 2003; Snow *et al.*, 2004). Four species of *Plasmodium* is responsible for the spread of malaria, of which *P. falciparum* is the most dangerous (Tracy & Webster, 2001). Annually, millions of people succumb to malaria and roughly half a billion people are afflicted by it (CDC, 2007). Susceptible groups, resembling young children, pregnant woman, and nonimmune individuals, are at high risk of developing severe malaria and also constitute a high percentage of the mortality rate (Lagerberg, 2008; Tracy & Webster, 2001).

Efforts to eradicate and control this infectious disease have repeatedly faltered, partly due to the rising incidence and spread of drug resistant *P. falciparum* parasites (Ekland & Fidock, 2008). Resistance is dependable upon the spontaneous mutation or gene amplification (genetic change) in a malaria parasite which interferes with the parasite’s susceptibility to an antimalarial drug (Chiyaka *et al.*, 2008). Drug pharmacokinetics (Hastings *et al.*, 2002), over-usage of antimalarials (Simpson *et al.*, 2000), cross-resistance (Iyer *et al.*, 2001), and inadequate treatment by means of inapt prescribing patterns, incorrect administration, meager absorbance and noncompliance (Simpson *et al.*, 2000; White, 1999) have all been considered to aggravate the emergence of resistant parasites.

When studying this parasitic disease several factors ought to be considered. Malaria is perceived as a multi-factorial problem stretching over a large domain, including; medical, health, ecological, agricultural, social, political, and economic aspects (Temel, 2005).

Therefore, when implementing strategies to control and minimise the morbid effect of malaria on communities, information from various fields of work need to be scrutinised and evaluated in order to implement optimal control measures (AAAS, 1991). Regarding the medical and public health research, results should aim to provide information critical for prevention, early diagnosis and treatment of malaria (Temel, 2005).

Some antibiotics have been studied for their antimalarial properties and are generally combined with antimalarials in an effort to overcome the global challenge of drug-resistant *P. falciparum* (Nakornchai & Konthiang, 2006). The slow onset of antimalarial action of antibiotics, excludes their use as single agents (Ohrt *et al.*, 2002). Antibiotics, like azithromycin, seem to exude better activity with prolonged exposure to *Plasmodium* parasites (Yeo & Rieckman, 1994). Treatment optimisation is dependant upon continuous research and development of safe and effective medicinal interventions. The relative safety profile of the macrolide antibiotics, erythromycin and especially azithromycin in high risk groups, was one of the aspects taken into consideration in the decision to study these test compounds.

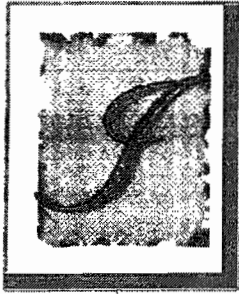
The Pheroid™ drug delivery system is a patented, colloidal system, comprising of a unique submicron emulsion type formulation consisting mainly of plant and essential fatty acids. The Pheroid™ has been proven to entrap drugs with high efficiency and deliver these with significant speed to target sites in the body. Furthermore, Pheroids™ are able to extravasate from the vascular system and are ideal for rapid release of active compounds. This characteristic of Pheroid™ technology may increase the antimalarial effect of slow acting antibiotics. The Pheroid™ also protects drugs from metabolism and inactivation in the plasma and other body fluids. The volume of distribution is decreased by this drug delivery system ultimately increasing the concentration at the target site, thereby achieving an enhanced but narrow therapeutic index (Grobler, 2004).

In this study the *in vitro* antimalarial effects of erythromycin and azithromycin, respectively, were compared to the effects of the test compounds entrapped in the Pheroid™ carrier system on a chloroquine resistant (RSA 11) *P. falciparum* strain. The effect of concentration and prolonged incubation periods were also investigated.

The specific objectives of this study were:

1. To develop an acceptable formulation of Pheroids™ together with the macrolide antibiotics, erythromycin and azithromycin, to apply in the *in vitro* drug efficacy assay (HRP II).
2. To evaluate the HRP II method in the applicability of *in vitro* drug efficacy assays with Pheroid™ technology.
3. To evaluate the *in vitro* efficacy of azithromycin and erythromycin alone and in combination with Pheroid™ vesicles against a chloroquine resistant strain (RSA 11) over an extended observation period of 144 hours.

Chapter 1 summarises malaria as infectious disease over viewing the problematic nature of the infection and in Chapter 2 the test compounds utilized in this study is discussed. Chapter 2 further elaborates on the advantages of Pheroid™ technology. Chapter 3 elucidates the materials purchased and methods applied in this study where after the results and discussion follows in Chapter 4.



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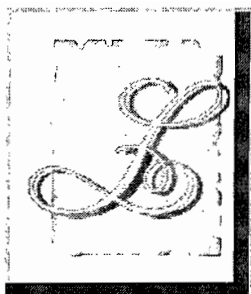
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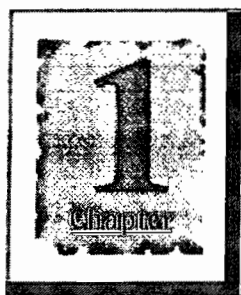


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MALARIA

1.1 INTRODUCTION

Malaria, an infectious disease caused by obligate intracellular protozoan parasites of the genus *Plasmodium*, is presently one of the foremost causes of global mortality and morbidity (Tracy & Webster, 2001; Tuteja, 2007). In humans, four species are known to cause malaria, namely; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* (Pasvol, 2005; Tuteja, 2007). All of the above mentioned parasites have the ability to cause serious illness, though *P. falciparum* remains the mediator of rigorous, potentially terminal malaria (Rosenthal, 2004; Tuteja, 2007). In 1880, a French army surgeon Charles Louis Alphonse Laveran was the first to observe parasites in the blood of a patient infected with malaria. He was granted the Nobel Prize for his discovery in 1907. At the time, Laveran had believed that there were only one causative species of malaria; *Oscillaria malariae*. However, in 1890, the Italian investigators Giovanni Batista Grassi and Raimondo Filetti introduced the names *P. vivax* and *P. malariae* for two of the malaria parasites concerning humans. An American, William H. Welch, named the malignant tertian malaria parasite *P. falciparum* in 1897 (CDC, 2004a). The vector of this parasite is the *Anopheles* mosquito (Lewison & Srivastava, 2008). Dr. Ronald Ross, a British officer in the Indian Medical Service, established that malaria could be transmitted from infected humans to mosquitoes in 1897 and was awarded the Nobel Prize in 1902 (CDC, 2004a; Tuteja, 2007). Finally, the fourth human malaria parasite was described in 1922 by John William Watson Stephens (CDC, 2004a). Chapter 1 will briefly discuss malaria as an infectious disease, summarising key points such as the spread of the infection, risk groups, causative agents, treatments and associated problems like drug- and insecticide-resistance.

1.2 EPIDEMIOLOGY

One definition of the term epidemiology is depicted in Dorland's Illustrated Medical Dictionary, (1988) as: 'the science concerned with the study of the factors determining and influencing the frequency and distribution of disease, injury, and other health-related events and their causes in a defined human population for the purpose of establishing programs to prevent and control their development and spread'. The following sections will give an overview of the epidemiological factors that play an important role in malaria.

1.2.1 Statistics

To emphasize the complexity of malaria it is essential to enumerate the effects these parasitic infections convey on the population as well as on the economy. Estimates are derived from the statistics made available by the World Health Organization (WHO) in the malaria report of 2008, data from Centers for Disease Control and Prevention (CDC) and the Medical Research Council of South Africa.

1.2.1.1 Malaria cases and mortalities

According to WHO, (2008) estimates there were 230 million cases of *P. falciparum* infections worldwide during 2006. Malaria claimed an estimated 881 000 lives globally of which 90 % were in the African Region. Data suggests that only 1 in 5 malaria deaths were reported internationally in 2006, thus concluding that these estimates were expected to be much higher. An estimated 85 % of mortalities due to *P. falciparum* infection occurred in young children under the age of five. CDC, (2007) reported that malaria was the fourth cause of death in children in developing countries, in 2002, subsequent to birth complications, lower respiratory infections and diarrheal diseases, consequently accountable for 10.7 % of children's deaths in developing countries. South Africa experienced a malaria epidemic in 2000 with a reported 64 622 malaria cases. Newer statistics indicate that there had been a 15 % decrease in the number of reported malaria cases during January - December 2003, although the number of malaria deaths displayed a 21 % increase when compared to the same period in 2002 (DOH, 2003b). Nevertheless, the number of reported malaria cases have declined steadily since the epidemic in 2000, but malaria remains a threatening disease even in countries where a measure of control through interventions like combination drug therapy have led to a decrease in the number of malaria cases.

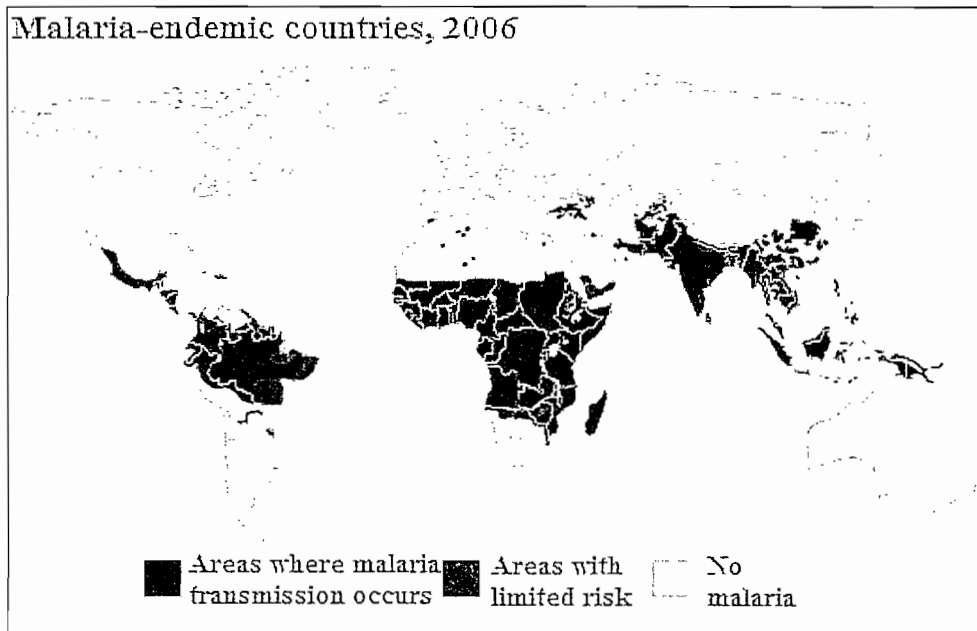


FIGURE 1.1: Global regions where malaria was prevalent during 2006 (MVIPath, 2010).

In South Africa, malaria is mainly confined to the northern and eastern border areas with roughly 4.3 million individuals at risk of developing the disease (Blumberg & Frean, 2007). Malaria transmission is mostly seasonal in South Africa with an onset in October. Transmission season usually ends in May with malaria cases peaking between January and February (DOH, 2003b). Nearly every South African, including occupants of seasonal malaria transmission areas, is non-immune and is, as a result, at risk to develop severe malaria. A conclusion can be drawn that despite successes in malaria control since the epidemic in 2000, many challenges remain. Vector control through indoor residual spraying, case management, disease surveillance, epidemic preparedness and response, and public awareness are the key strategies implemented in the control of malaria in South Africa (Blumberg & Frean, 2007).

1.2.3 Climate

Various climatic factors add to the incidence of malaria in a specific region. During seasons with high rainfall, water build-up can create breeding sites where the mosquitoes deposit their eggs and larvae and pupae develop into adulthood. After 9 - 12 days, adult mosquitoes emerge and temperature, humidity, and rainfall will ultimately contribute to their survival. Temperature is especially important for *P. falciparum* cannot complete its growth cycle in the

Anopheles mosquito at temperatures below 20°C. The duration of the extrinsic cycle is shortened during warmer temperatures accordingly increasing the chances of transmission. Warmer temperatures also lead to human behavior such as sleeping without bed-nets or sleeping outside, thereby increasing the risk of infection (CDC, 2004b).

1.2.4 *Anopheles* mosquitoes

The intensity of malaria transmission will be dependent on which species of *Anopheles* mosquitoes are present in an area at a given time. *Anopheles* species may vary in selected behavioral traits, consequently impacting their abilities to act as malaria vectors. The females of some species favor a blood meal from humans (“antropophilic”) while others prefer animals (zoophilic). Some species bite indoors (“endophagic”) and others outdoors (“exophagic”). The antropophilic, endophagic species will encounter humans more frequently and will therefore be more effective malaria vectors (CDC, 2004b). Of the approximately 422 species of *Anopheles* mosquitoes, 60 are known to cause malaria (Service, 1993; Tuteja, 2007). The main mosquito vector of malaria in sub-Saharan Africa is *Anopheles gambiae*. In malaria-endemic regions, insecticide treated nets (ITNs) saturated in pyrethroid insecticides are commonly in use as a protective measure in vector control, but emergence of pyrethroid resistant mosquitoes has notably reduced their efficacy. A potential alternative, which could be safely developed as a new class of ITNs, is an acetylcholine esterase (AChE) inhibitor (Carlier *et al.*, 2008).

1.2.5 Control

Eradicating efforts need to be constantly revised and adjusted for their successes and failures may lead to the implementation of control strategies to manage malaria. In the past 50 years the WHO has led many a global effort to control this infectious disease (Olumese, 2005). The roll back malaria initiative was launched in 1998 and aimed to halve the malaria mortality by 2010 and again by 2015. According to the WHO Expert Committee on Malaria Control: twentieth report (as quoted by Olumese, 2005) the programme is based on a strategy of:

- early diagnosis and prompt antimalarial intervention,
- vector control,
- intermittent preventive therapy in pregnancy (IPT) (see section 1.4), and
- prevention and control of malaria epidemics.

Seasonal transmission, an effective national malaria control programme and the well-developed scientific, economic and health infrastructure contribute to the control of and decline in malaria cases in South Africa (Blumberg & Frean, 2007).

1.3 *PLASMODIUM FALCIPARUM*

Responsible for the most dangerous form of malaria, *P. falciparum* infection can produce extreme parasitaemia, sequestration of infected erythrocytes in the peripheral microvasculature, hypoglycemia, hemolysis, and shock with multiorgan failure. If detected early and treated appropriately infection generally responds within 48 hours; conversely, inadequate treatment may result in *recrudescence* of infection when parasites multiply persistently in the blood (Tracy & Webster, 2001). The following sections describe the life cycle of *P. falciparum* and the pathogenesis leading to the clinical symptoms.

1.3.1 Life cycle

The asexual stage of the parasite develops in the human liver and the sexual stage continues in the mosquito (Wolmarans, 2004), as demonstrated in Figure 1.2. Immediate schizogony is triggered once *P. falciparum* is injected into a human host (Tuteja, 2007).

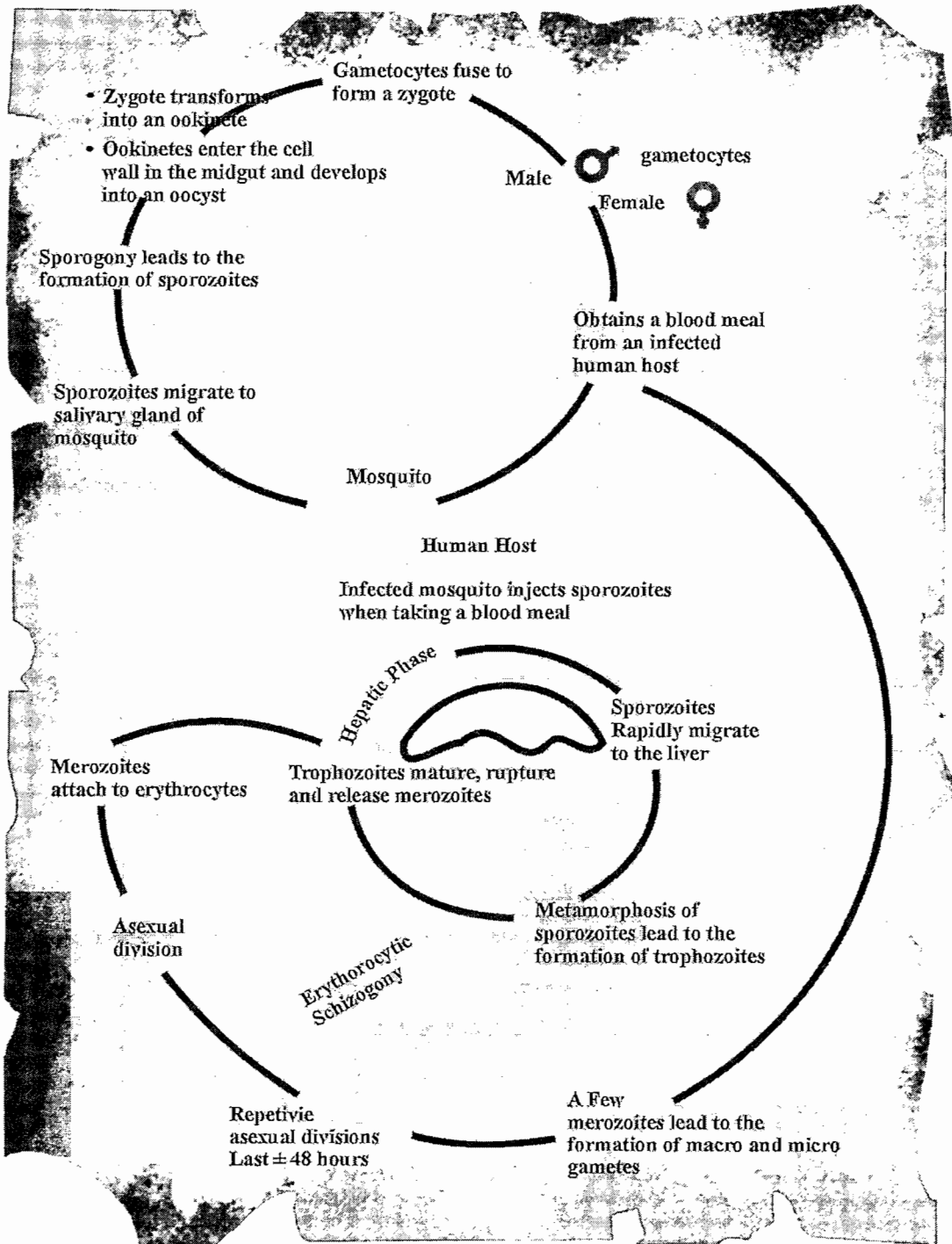


FIGURE 1.2: Life cycle of the *P. falciparum* parasite
(Adapted from Tuteja, 2007; Wolmarans, 2004).

1.3.1.1 Tissue schizogony (hepatic phase)

Mobile, infective sporozoites from the salivary gland of the *Anopheles* mosquito are injected into the human host once the vector obtains a blood meal (Tuteja, 2007; Wolmarans, 2004).

Within one to two days after invasion, these sporozoites reach the hepatocytes where they undergo metamorphosis and change into a trophozoite or tissue schizont (Tracy & Webster, 2001; Wolmarans, 2004). Pre-erythrocytic schizogony takes place when these trophozoites mature and give rise to tens of thousands of merozoites, each capable of invading a red blood cell once released from the liver (Tuteja, 2007; Wolmarans, 2004). The time taken to complete tissue schizogony in the case of *P. falciparum*, is 8 - 25 days (Tuteja, 2007).

1.3.1.2 Erythrocytic schizogony

Specific receptors are involved during the attachment of the merozoites to the erythrocyte membrane (Tuteja, 2007; Wolmarans, 2004). Subsequent to attachment, asexual division is initiated and the parasite develops through different phases in the erythrocyte. The early trophozoite is frequently referred to as the 'ring form' (Tuteja, 2007). Multiple rounds of nuclear division lead to the formation of schizonts which, upon reaching maturity, contain around 20 merozoites each. After lysis of the erythrocyte these merozoites are released to invade further uninfected red blood cells (Tuteja, 2007). During lysis of the erythrocyte, metabolic waste of the parasite and hemozoin is also released. Hemozoin is the end product of hemoglobin digestion by the parasite. The released metabolic waste is one of the features responsible for the clinical manifestations of malaria (Wolmarans, 2004). Following repetitive asexual divisions, lasting approximately 48 hours in the case of *P. falciparum*, some merozoites once again invade erythrocytes and lead to the formation of macro- and microgametocytes (Tuteja, 2007; Wolmarans, 2004).

1.3.1.3 Sexual phase of *Plasmodium falciparum* in the mosquito

When obtaining a blood meal from an infected human host, a female *Anopheles* mosquito may ingest male and female gametocytes into its midgut. After combining, the gametes undergo fertilization and form a zygote (Ashley *et al.*, 2005; Tuteja, 2007). The zygote finally transforms into an ookinete, which enters the wall of a cell in the midgut and ultimately develops into an oocyst. Within the oocyst, sporogony leads to the formation of multiple sporozoites which migrate to the salivary gland for onward transmission when the oocyst bursts. The mosquito remains infective for 1 - 2 months and when biting a susceptible host sets the *Plasmodium* life cycle into motion (Tuteja, 2007).

1.3.2 Pathogenesis

There are mainly two features contributing to the clinical manifestations of malaria, namely; host inflammatory response associated with episodes of fever and chills, and anemia, which results from the demolishment of red blood cells (Wolmarans, 2004). The average incubation period is 10 - 14 days with symptoms occurring within 6 weeks after departure from a malaria endemic region (Pasvol, 2005). A few days before a malaria attack a patient will present with flu-like symptoms including malaise, headache, loss of appetite and light fever. A typical malaria attack commences with chills, which upon activation of the hypothalamus result in intense fever often accompanied by nausea and vomiting (Wolmarans, 2004). An important feature of malaria is the volatile increase in cytokines (particularly TNF- α) (Pasvol, 2005). *P. falciparum* infection can lead to cerebral malaria, which occurs in the presence of TNF- α (Wolmarans, 2004). Severe manifestations can rapidly cause fatalities (Pasvol, 2005).

1.4 RISK OF MALARIA DURING PREGNANCY

Important risk groups raising concern due to limiting treatment options is pregnant woman and children under the age of five (WHO, 2007). The complexity of medicinal interventions in these risks groups render them especially vulnerable to infection with *P. falciparum*. These groups are also more susceptible to develop severe malaria (Luxemburger *et al.*, 1997; Gibbon, 2005). A Study conducted in rural Gambia, concluded that pregnant woman attracted twice the number of *A. gambiae* mosquitoes than non-pregnant woman. Physiological and behavioral changes that occur during pregnancy could contribute to the increased attractiveness (Lindsay *et al.*, 2000). Young children generally need to be admitted to hospital for observed treatment, due to a lack of pediatric antimalarial formulations (Ashley *et al.*, 2005). Pregnant woman, or woman attempting to get pregnant, should always be strongly discouraged to visit malaria endemic areas, mainly because they are more at risk to succumb to severe malaria. Furthermore, should they survive an episode of malaria in pregnancy and go on to deliver, the adverse effects on the infant are likely to be permanent (McGready *et al.*, 2004). Spontaneous abortion, preterm delivery, low birth weight, stillbirth, congenital infection and maternal death can occur when infected with malaria during pregnancy (Lagerberg, 2008). Even though malaria prophylaxis is available, it is important to remember that these drugs are in no case 100 % effective and pose a potential iatrogenic side-effect risk (McGready *et al.*, 2004). Furthermore, physiological changes (increased intravascular volume, delayed gastric emptying time, elevated oestrogen and cortisol levels

and increased body fat content) that occur during pregnancy may alter the pharmacokinetics of antimalarial drugs, thereby influencing their efficacy (Na-bachang *et al.*, 2005). The following interventions are recommended by the WHO, (2004) to limit exposure and to manage the disease:

- Intermittent preventative treatment.

The goal of this initiative is to promote the use of antimalarial drugs given in standard treatment dosages at predefined intervals after the first movement of the fetus is noted. Sulfadoxine-pyrimethamine is currently considered the most effective and safe in areas with stable *P. falciparum* transmission and where resistance against this drug is low. The WHO recommends the use of chloroquine in uncomplicated cases of malaria where chloroquine-sensitive strains are prevalent and sulfadoxine-pyrimethamine in chloroquine-resistant areas.

Quinine is the preferred drug in cases where both of the previously mentioned drugs are rendered ineffective and during the first trimester of pregnancy in patients presenting with severe malaria.

- Insecticide treated nets (ITN).

Woman should be urged to obtain an ITN as soon as pregnancy is suspected. Their use and effectiveness as protective measure against mosquito bites ought to be emphasized, as they contribute to the prevention of malaria.

- Effective case management of associated malaria and anemia.

The WHO recommends the use of iron supplementation during pregnancy. Patients should also be screened for malaria and treated accordingly if moderate or severe anemia is diagnosed.

1.5 DIAGNOSIS

A decrease in mortality and morbidity, in addition to decreased resistance to and proper use of antimalarials, is dependent upon a prompt and accurate diagnosis of malaria. In cases where malaria treatment is delayed or if ineffective medications are given, uncomplicated malaria can rapidly develop to cerebral malaria and other severe manifestations of the infection (Lagerberg, 2008). Malaria must always be considered in a febrile patient presenting with myalgia and ought to be considered a medical emergency (CDC, 2007; Pasvol, 2005). There are three components contributing to an optimal diagnostic outcome:

- taking an ample travel history, keeping in mind that malaria transmission can sometimes occur in non-endemic regions,
- clinical sharpness and knowledge, and
- laboratory tests (Blumberg & Frea, 2007).

1.5.1 Clinical diagnosis

The initial symptoms of malaria, including fever, chills, headaches, muscle pains, nausea and vomiting, are often not as striking as symptoms and clinical findings associated with severe malaria (confusion, coma, neurologic focal signs, severe anemia, and respiratory difficulties). The first symptoms of malaria are also prominent in other common viral infections like the flu. A laboratory test thus needs to confirm the clinical suspicion of malaria to justify the use of antimalarial therapy (CDC, 2007).

1.5.2 Blood films

A fast and reliable method of detecting malaria is by making thin and thick blood smears for microscopic assessment. When examining these blood films, accurate speciation of the parasite and parasite density can be determined. In cases where chemoprophylaxis has been taken, initial blood films could present negative and should therefore be repeated when clinical suspicion is high. In general, films taken daily for 3 consecutive days (off antimalarial therapy) are an appropriate measure, though this may have to be lengthened in cases where symptoms persist (Pasvol, 2005). Microscopy still remains the ‘gold standard’ as far as diagnostic tools are concerned since it is a rapid method and does not require the use of expensive equipment (Sing, 1997). A disadvantage of this method, however, is the fact that it requires skill and expertise which is not always readily available (Blumberg & Frea, 2007). The method is also considered particularly labour intensive, especially where numerous samples need to be screened in a short period of time (Sing, 1997). (Refer to Figure 3.3 in Chapter 3 for a description of the method for creating a thin blood smear.)

1.5.3 Alternate methods

Several diagnostic methods, including DNA hybridization methods and polymerase chain reaction-based assays, have been developed in an effort to overcome some of the difficulties encountered during the use of microscopy (Sing, 1997). A method known as the quantitative buffy coat method stains parasite nuclear material by taking blood into a small capillary tube

containing a float and a fluorescent dye: acridine orange. Sensitivity of detection is increased, but cost and inability to accurately speciate the parasite limits the use of this method. A relatively easy but expensive test is the antigen-capture test. A monoclonal antibody is used to bind histidine-rich protein II of *P. falciparum*. Furthermore, a polymerase chain reaction analysis is valuable in accurate species diagnosis, mixed infection and the detection of low-level parasitemia. All of these methods are limited in terms of cost, time and the fact that they can be non-quantitative or nonspecific (Pasvol, 2005). Rapid diagnostic tests are sensitive and specific for *P. falciparum*. Advantages include: readily available results, tests are less liable to the theoretical risk of being falsely negative due to parasite sequestration, and the visibility of the results to the healthcare professional and patient may result in better prescription practices. Malaria is extremely over-diagnosed and microscopy results often ignored in areas of low or moderate malaria transmission. A randomised trial, conducted in Tanzania, found that the deployment of rapid diagnostic tests with standard training made no difference in the over-diagnosis of malaria in patients presenting with fever (Reyburn *et al.*, 2007).

1.6 TREATMENT

Oral therapy is considered adequate in the treatment of uncomplicated malaria, whereas severe manifestations of the infection require hospitalisation and parenteral therapy (Ashley *et al.*, 2005). The use of protective measures against mosquito bites, including insect repellants, insecticides, bednets, long-sleeved clothing, refraining from the outdoors between dusk and dawn, and restricting visits to malaria endemic regions to the dry season if possible, should always be implemented as added defense as no chemoprophylactic regime is entirely reliable (DOH, 2003a; Rosenthal, 2004; Gibbon, 2005). The WHO recommends the use of antimalarial combination therapy which is based on the principle of simultaneously administering two or more blood schizontozides with different sites of action. This allows for a more effective therapeutic outcome while concurrently lowering resistance (WHO, 2006).

1.6.1 Antimalarial drug therapy

The most commonly used antimalarials, as listed in Table 1.1, derives from five classes of compound: the quinolones and arylaminoalcohols, the antifols, the artemisinin derivatives, the hydroxynaphthaquinones, and antibacterial agents (Ashley *et al.*, 2006).

TABLE 1.1: Major antimalarial drugs and their uses (Rosenthal, 2004).

DRUG	USE
Chloroquine	Treatment and chemoprophylaxis of infection with sensitive parasites
Amodiaquine	Treatment of infection with some chloroquine-resistant <i>P.falciparum</i> strains
Quinine	Oral treatment of infections with chloroquine-resistant <i>P.falciparum</i>
Quinidine	Intravenous therapy of severe infections with <i>P.falciparum</i>
Mefloquine	Chemoprophylaxis and treatment of infections with <i>P.falciparum</i>
Primaquine	Radical cure and terminal prophylaxis of infections with <i>P.vivax</i> and <i>P.ovale</i>
Sulfadoxine-pyrimethamine (Fansidar)	Treatment of infections with some chloroquine-resistant <i>P.falciparum</i>
Proguanil	Chemoprophylaxis (with chloroquine)
Doxycycline	Treatment (with quinine) of infections with <i>P.falciparum</i> ; chemoprophylaxis
Halofantrine	Treatment of infections with some chloroquine-resistant <i>P.falciparum</i>
Lumefantrine	Treatment of <i>P.falciparum</i> in fixed combination with arthemeter (Coartem)
Artemisinins	Treatment of infection with multidrug-resistant <i>P.falciparum</i>
Atovaquone-proguanil (Malarone)	Treatment and prophylaxis of <i>P.falciparum</i> infection

The majority of antimalarial drugs were developed due to their action against asexual erythrocytic forms of malarial parasites. Drugs in this category include chloroquine, quinine, quinidine, mefloquine, atovaquone, and the artemisinin compounds. Proguanil, pyrimethamine, sulfonamides, sulfones and antimalarial antibiotics are slower acting and less effective. The only drug applied in the eradication of latent tissue forms, responsible for relapses of *P. vivax* and *P. ovale* infections, is primaquine. Monotherapy no longer controls infection with *P. falciparum* (Tracy & Webster, 2001).

1.6.2 Chloroquine

Discovered in 1934 by Hans Andersag, this compound was initially known as Recochin. Chloroquine would only be recognised as being a safe and effective antimalarial 12 years later (CDC, 2004a). Today, chloroquine is no longer recommended in areas of high malaria transmission, including South Africa, due to the development of resistance (DOH, 2003a), but it is still considered first-line therapy in cases of sensitive strains of *P. falciparum* and other causative parasite species of human malaria (Rosenthal, 2004). The exact mechanism of action of this blood schizonticide is not yet fully understood, though it is believed that chloroquine concentrates in the food vacuoles of the parasite thereby leading to toxin buildup of free heme by preventing polymerisation. Heme is the breakdown product of hemoglobin and is ultimately polymerised into hemozoin (Rosenthal, 2004). Chloroquine is tremendously safe when taken in proper doses. Toxicity includes effects on the cardiovascular- (hypotension, vasodilation, suppressed myocardial function, cardiac arrhythmias and eventual cardiac arrest) and central nervous - systems (confusion, convulsions and coma) (Tracy & Webster, 2001).

1.6.3 Amodiaquine

This drug is very similar to chloroquine and it is probable that it shares the same mechanism of action. Amodiaquine has been widely used, in the treatment of malaria, due to its cost-effectiveness, limited toxicity and effectiveness against chloroquine resistant *P. falciparum* strains in some areas. The main toxicities of amodiaquine include agranulocytosis, aplastic anemia, and hepatotoxicity. It is not recommended as prophylactic agent as long term use can possibly increase toxicity (Rosenthal, 2004).

1.6.4 Quinine and Quinidine

Early during the 17th Century, the bark from the Cinchona tree was renowned for its use as treatment in fevers (CDC, 2004a). In 1820, the alkaloid quinine was extracted from the bark and implemented as antimalarial. This rapid acting blood schizonticide remains the drug of choice in the treatment of severe malaria. It is not typically used in chemoprophylaxis due to its toxicity. Quinine is commonly combined with a second drug (usually doxycycline) to shorten its duration of use and limit toxicity (Rosenthal, 2004). When administered in excessive dosages, toxicity includes cinchonism, hypoglycemia, and hypotension (Tracy & Webster, 2001).

1.6.5 Mefloquine

The Malaria Research Program, established in 1963 by the Walter Reed Institute for Medical Research, aimed to develop new antimalarials in an attempt to fight emerging strains of drug-resistant *P. falciparum*, hence the development of mefloquine (Tracy & Webster, 2001). This blood schizonticide, active against the asexual stages of *P. falciparum* and *P. vivax*, is used as prophylactic treatment of chloroquine-resistant *P. falciparum*. In South Africa, mefloquine is not registered for treatment of malaria, but is indicated as a prophylactic agent against chloroquine-resistant *P. falciparum*. Mefloquine is not recommended as chemoprophylaxis in travellers requiring fine motor-coordination like piloting an aircraft, diving or mountaineering as dizziness and vertigo can occur as hazardous adverse effects. Furthermore, prophylaxis must not exceed 1 year and if administered for an extended period of time, intermittent liver function and ophthalmic tests should be performed (Gibbon, 2005).

1.6.6 Primaquine

Ehrlich discovered the weak plasmodicidal activity of methylene blue in 1914. This discovery was later exploited to develop the 8-aminoquinoline antimalarials. Primaquine was one of three 8-aminoquinolines selected during World War II because of their potency and limited toxicity (Tracy & Webster, 2001). Primaquine acts as a tissue schizonticide against the liver stages of *P. vivax* and *P. ovale*. This antimalarial is inactive when used as a single agent and is only used in combination with a blood schizonticide (Katzung *et al.*, 2002). Toxicity due to large dosages includes epigastric distress, mild-to-moderate abdominal distress, mild anemia, cyanosis and leukocytosis (Tracy & Webster, 2001).

1.6.7 Sulfadoxine-pyrimethamine (Fansidar)

Sulfadoxine and pyrimethamine are both antifolate drugs, acting as blood schizonticides against *P. falciparum*. When these two drugs are used in combination a synergistic effect is obtained through the chronological blockade of two steps in the synthesis of folic acid (Katzung *et al.*, 2002). It is indicated for the treatment of uncomplicated *P. falciparum* malaria, preferably in combination with artesunate. It is not recommended for prophylaxis. Serious adverse effects are mostly related to the sulphonamide component responsible for potentially fatal skin reactions such as erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis (Gibbon, 2005).

1.6.8 Proguanil and Atovaquone

Proguanil, a dihydrofolate reductase inhibitor, is used in a set dose combination with atovaquone, a cytochrome-b inhibitor, equally impeding *Plasmodial* pyrimidine synthesis. In South Africa this combination is registered solely as prophylactic treatment in adults (Gibbon, 2005).

1.6.9 Doxycycline

Various antibiotics have been shown to possess relative antimalarial activity. Their exact mechanism of action against the malaria parasite remains unclear and because of their slow onset of action they should not be administered as a single agent in the treatment of malaria infection (Rosenthal, 2004). Doxycycline, a bacteriostatic tetracycline, is active against erythrocytic schizonts of all parasites responsible for malaria in humans but not against the liver stages (Gibbon, 2005; Rosenthal, 2004). It is usually administered in combination with quinidine or quinine (Rosenthal, 2004).

1.6.10 Halofantrine and Lumefantrine

Because of its cardiac toxicity, Halofantrine should not be used as prophylactic agent and should therefore be considered second-line therapy in the treatment of uncomplicated malaria. This 9-phenanthrenemethanol derivative acts as blood schizonticide against all of the *Plasmodium* species causing malaria in humans (Gibbon, 2005; Rosenthal, 2004). Lumefantrine, analogue to Halofantrine is usually administered in combination with Artemeter as Coartem (Rosenthal, 2004).

1.6.11 Artemisinin and derivatives

The Qinghao plant (*Artemisia annua L*), also known as the sweet wormwood, was first described by Ge Hong of the East Yin Dynasty as being an effective antipyretic and has been used for this purpose for over 2000 years in China (CDC, 2007; Gkrania-Klotsas & Lever, 2007; Rosenthal, 2004). The active ingredient, known as artemisinin, was isolated in 1971 and the two most important analogs synthesized from artemisinin are artesunate and arthemeter (CDC, 2007; Rosenthal, 2004). The artemisinin compounds are the most rapidly acting, effective, and safe drugs for the treatment of severe malaria (Newton & White, 1999) and produce less toxicity than the antimalarial alkaloids (Tracy & Webster, 2001). Rapid

clearance of parasitemia and quick resolution of symptoms are two apparent advantages of artemisinin and its derivatives (WHO, 2006). However, a major constraint of these antimalarials is their cost which is approximately 10 times that of current antimalarial drugs (Reyburn *et al*, 2007).

1.7 RESISTANCE TO ANTIMALARIAL DRUGS

In Tropical areas, antimalarials together with antipyretics are of the most frequently used drugs. Their misuse largely contributes to the rise of resistance (White, 2004). ‘Presumptive treatment’ has been implemented in highly endemic regions where the frequency of asymptomatic infections and lack of resources can lead to incorrect management. With this method a patient presenting with fever, without an apparent cause, is treated for malaria based on clinical suspicion without laboratory verification. Because of the severity of the disease the implementation of this method can be potentially life-saving, though a misdiagnosis can lead to the unnecessary use of antimalarials. This adds to problems like additional expenses and the increase of resistant parasites (CDC, 2007). Cross-resistance contributes to the decline in the effectiveness of available antimalarials. When the parasite gains resistance to a specific drug, it would most likely become resistant to other drugs belonging to the same chemical group. Former first-line antimalarial chloroquine, is now completely ineffective in areas with resistant *P. falciparum*. This was the recommended drug for use in pregnant woman, and had other apparent advantages, like its cost-effectiveness and its extensive availability (White, 2004). Sulfadoxine-pyremthamine resistance has also limited its use (Eckland & Fidock, 2008; White, 2004). Clinical studies conducted in the search for drug resistant parasites are limited by various factors, including cost, logistics, patient compliance, patient variability concerning drug metabolism and absorption, nutritional status of patients, overall health, occurrence of re-infection, degree of pre-existing immunity and the genetic complexity of the resistant phenotype. The ultimate measure of resistance is the rate of clinical failures (Eckland & Fidock, 2008). Combination therapy has been implemented as standard care as to minimise the spread of resistance (CDC, 2007; White, 2004). Using two drugs with different sites of action lowers the possibility of the parasite to develop resistance. The main obstacles in the implementation of combination therapy remain incomplete coverage and inadequate treatment. Artemisinin derivatives have proved to be effective alternatives when used in combination with antimalarials. They are rapid acting, highly efficient and well-tolerated. The development of a vaccine has been highly anticipated, but research and clinical studies may still continue for more than a decade (White, 2004).

Though a great deal is done to reduce resistance, the development of safe and effective treatment alternatives remain a much needed challenge.

1.8 IN VITRO ASSAYS FOR THE EFFICACY OF ANTIMALARIAL DRUGS AGAINST MALARIA PARASITES

In vitro drug-sensitivity tests involve the measurement of the effect of drugs on the increase and development of malaria parasites. Antimalarial drugs fundamentally restrain parasite growth, a parameter that can easily be measured *in vitro* (Noedl *et al.*, 2003). Since host-related factors such as drug failure or host immunity are excluded, *in vitro* testing can offer a more objective approach to inherent drug sensitivity than *in vivo* testing (Schuster, 2002). The first assay to determine the *in vitro* sensitivity of malaria parasites to antimalarial drugs was completed by Bass in 1922 (Basco, 2007). With the surfacing of drug-resistant *P. falciparum* roughly 40 years later, Rieckmann developed the “macrotest” or “macrotechnique” based on Bass’ method (Rieckman *et al.*, 1968; Rieckman, 1971). Currently, all assays originate from an *in vitro* culture technique developed by Trager & Jensen, (1976). Methods were scrutinised and compared in terms of advantages and drawbacks. Figure 1.3 demonstrates all methods taken into consideration. A short summary of each method will follow the illustration.

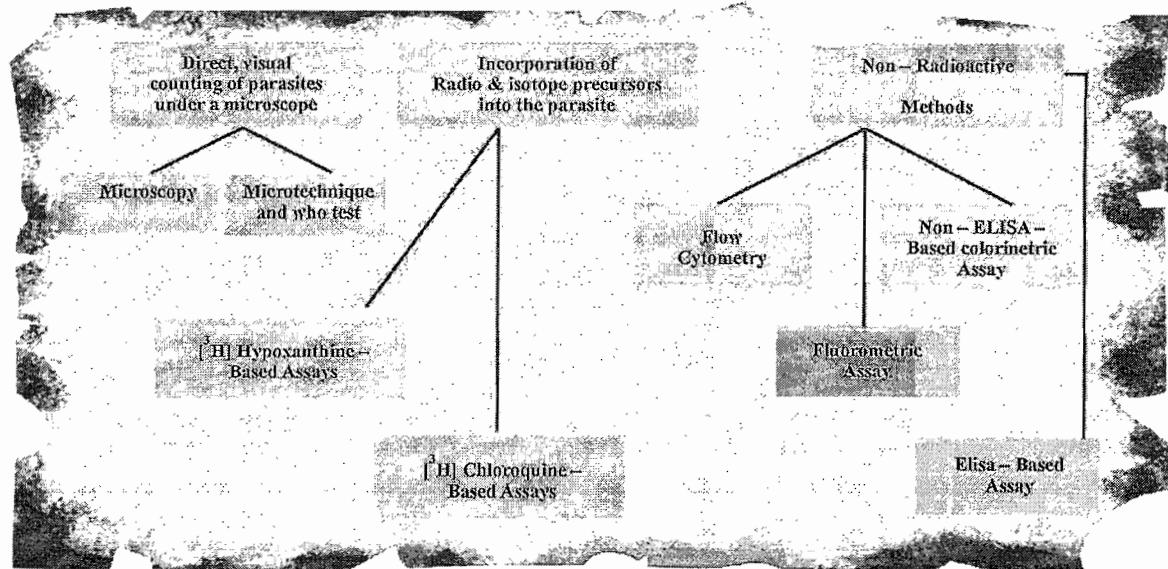


FIGURE 1.3: *In vitro* assays for the sensitivity of human malaria parasites to drugs (Adapted from: Basco, 2007).

1.8.1 Microtechnique and WHO test

Rieckmann *et al.* (1978) developed the *in vitro* microtechnique by infecting owl monkeys (*Aotus trivirgatus*) with pre-adapted *P. falciparum* strains. Soon after infection had occurred, numerous capillary blood samples were taken (50 µl total volume per experiment, 17 - 58 % erythrocyte volume fraction, 940 - 106 000 asexual parasites per µl). Microtitre plates were prepared by pre-coating the plate with six to seven concentrations of chloroquine diphosphate (chloroquine-sensitive strains: 0.1 - 2.5 ng, chloroquine resistant strain: 2.5 - 25 ng). Each assay contained two drug-free control wells. Whole capillary blood (5 µl) was added to 50 µl of buffered RPMI 1640 medium and incubated in a candle jar at 38 - 39°C for 24 - 30 h. Immediately after incubation thick blood smears were prepared from each well, and the amount of schizonts was counted against 500 leukocytes. *In vitro* response was articulated as the percentage of the number of schizonts with each drug concentration, measured up against the number of schizonts in drug-free controls. This technique has demonstrated two key advantages i.e. the implementation of simplified *in vitro* procedures and the small amount of capillary blood needed. The macro- and microtest assays of the WHO, were designed as part of the global monitoring programme to support the description of the epidemiology of drug-resistant malaria, and are not intended for drug screening or individual diagnosis. The microtest of the WHO was designed by Wernsdorfer, fundamentally implying Rieckmann's microtechnique (Basco, 2007).

1.8.2 Radioisotope methods

Various radiolabelled precursors, including precursors of nucleic acids (purines and pyrimidines), proteins (amino acids) and phospholipids (polar head groups) have been used to support parasite maturation. In malaria-parasite-containing cultures uninfected, mature human erythrocytes and platelets do not synthesize DNA, RNA, proteins or membranes, and human leukocytes do not increase and have a propensity to disintegrate over a few days. The amalgamation of radioactive precursors is an indirect measure of the metabolic activity of parasites, in spite of the low background level in uninfected red blood cells. [³H]hypoxanthine acts as preferred purine base for radiolabelling parasite DNA and RNA for isotope microtests and its incorporation is directly comparative to the number of *P. falciparum* infected erythrocytes under the customary conditions of *in vitro* drug sensitivity assays during the 42-h incubation period (Basco, 2007).

1.8.2.1 [³H] Hypoxanthine-based assays

Presently considered the ‘gold standard’ for *in vitro* drug sensitivity assays and one of the most regularly used assay methods in well-resourced laboratories these assays are swift, sensitive and precise in determining the effects of drugs on parasite growth. Cultures are routinely maintained under optimal conditions before performance of assays. Maximum parasitemia are sustained at 2 %. When performing drug assays, cultures are diluted in complete RPMI 1640 medium with uninfected erythrocytes to a final erythrocyte volume fraction of 1.5 % and an initial parasitemia of 0.25 - 0.5 %. Dual serial dilutions of drug solutions in complete RPMI 1640 medium are prepared in replica in 96-well plates. This allows assessment of seven drug concentrations over a 64-fold range. Radioactive assay has become the reference method for drug sensitivity testing in advanced countries, but is not yet considered standard method in most malaria-endemic countries (Basco, 2007).

1.8.2.2 [³H] Chloroquine-based assays

This rapid *in vitro* test, specifically designed for chloroquine-resistance in *P. falciparum* was first reported by Gluzman *et al.* (1990). Its theory is based on two observations, the first being the appearance of chloroquine-resistant parasites to actively expel chloroquine while chloroquine-sensitive parasites accumulate the drug (Verdier *et al.*, 1985; Krogstad *et al.*, 1987), and secondly, the addition of calcium channel blockers, tricyclic antidepressants, tricyclic antihistamines and phenothiazines restrain chloroquine efflux in chloroquine-

resistant *P. falciparum* strains, but not in chloroquine-sensitive strains (Martin *et al.*, 1987; Bitonti *et al.*, 1988; Basco & Le Bras, 1990, 1991, 1994). Despite the rationality and simplicity of the concept on which these assays are based, it can fail to distinguish between chloroquine-sensitive and chloroquine-resistant clinical isolates, when compared with the response in the [³H] hypoxanthine-based assay (Bickii *et al.*, 1998).

1.8.3 Non-radioactive methods

1.8.3.1 Flow cytometry

FACS, the abbreviation for fluorescence-activated cell sorter, is an *in vitro* method which involves the direct exposure of human malaria parasites to drugs in culture plates. This method can be used to identify and calculate malaria-infected erythrocytes and, in the presence of a DNA-targeted fluorescent dye, distinguish the asexual developmental phase on the basis of the amount of DNA in individual host erythrocytes. Because uninfected erythrocytes have no DNA content, they are consequently not fluorescent, while fluorescence intensity increases proportionally as trophozoites develop into schizonts. Raw data obtained from flow cytometric analysis are scientifically presented in histograms, showing the number of cell counts against escalating intensity of fluorescence. Flow cytometry is fast, precise, highly sensitive, highly DNA-specific, objective, automated and non-radioactive. It is, nevertheless, expensive, requires a highly skilled technician for maintenance and operation and is too complicated for routine application in the field (Basco, 2007).

1.8.3.2 Fluorometric assay

Because of the expensive nature of a FACS, alternative methods based on the principle of DNA labeling by means of fluorochromes have been developed. Drugs are tested in either 24- or 96-well culture plates without additional reagents. The erythrocytes are lysed at the end of incubation, by adding distilled water or saponin. After centrifugation, packed pellets are dissolved in guanidinium or sodium dodecyl sulfate solution and stained with DNA-binding fluorochromes (Basco, 2007).

1.8.3.3 Non-ELISA-based colorimetric assay

Lactate dehydrogenase (LDH) is synthesized by blood-stage malaria parasites as the terminal enzyme in their glycolytic (Embden-Meyerhoff) pathway, an anaerobic process. LDH

metabolizes pyruvate to lactic acid, regenerating NAD, which is necessary for production of ATP (Basco, 2007; Makler *et al.*, 1998). According to Vander Jagt *et al.*, as quoted by Basco, (2007), the presence of distinctive epitopes within the *Plasmodium* LDH (pLDH) protein and pLDH's unique enzymatic characteristics are two features that set pLDH apart from the human isotopes). pLDH possesses the ability to use an NAD analog 3-acetyl pyridine adenine dinucleotide (APAD), in the conversion of lactate to pyruvate (Makler & Hinrichs, 1993). The in vitro assays with LDH are performed in much the same way as the radioisotope method, except that [³H] hypoxanthine is omitted (Basco, 2007).

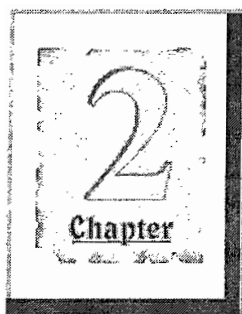
1.8.3.4 ELISA-based assays

HRP II, or histidine- and alanine- rich protein, is produced by both the asexual and early sexual stages of *P. falciparum* during growth and proliferation. The protein is exported through the erythrocyte cytoplasm and the surface membrane and ultimately accumulates in the extracellular plasma (Noedl, 2002; Uguen *et al.*, 1995). Inhibition of growth through anti-malarial drugs can simply be measured through an ELISA, by quantifying the HRP II levels of the parasite (Noedl, 2002). Various ELISA-based assays have been developed and other applications for these tests, such as bioassays or their use in vaccine development are also possible (Basco, 2007; Noedl *et al.*, 2003). This method has also been proven functional in the detection of the presence of parasites in cases of placental malaria (Leke *et al.*, 1999) a condition where malarial parasites accumulate and multiply within the intervillous spaces of the placenta (Bray & Sinden., 1979; McGregor, 1984). The ELISA assay, based on the detection and quantification of *P. falciparum* HRP II (Basco, 2007), was implemented in this study.

1.9 CONCLUSION

Malaria is one of the deadliest diseases of modern time. Throughout history this infectious disease has had devastating effects on human lives. Ancient use of traditional and herbal remedies has contributed to the development of the antimalarials we know and use today, and research over time has led to a better understanding of the causative agent, vector, spread and control of this global killer disease. Malaria remains somewhat of a complex and deceptive disease and some areas remain a challenge. Pregnant woman and young children are severely afflicted by this infection and preventative, control and treatment options in these risk groups are still not ideal. Furthermore, the development of resistance of both the parasite and the

vector is a disturbing factor urgently pressing for the development of new treatment and preventative measures. Though it is evident that more needs to be done, only about 0.4% of world biomedical research is currently conducted on malaria (Lewison & Srivastava, 2008). When diagnosed early and correctly, malaria is a curable infection (CDC, 2006). Combination therapy has been implemented as standard care as monotherapy is no longer successful, due to resistance to available antimalarial drugs. *In vitro* drug sensitivity testing offers an objective approach to inherent drug sensitivity and various assays are available for the screening of samples to determine the efficacy of antimalarial drugs against malaria parasites. In the future, effective malaria control and eradication efforts will be dependant upon how well the scientific and public health communities can work together to lengthen the efficient life span of current control measures, while simultaneously developing new, alternative interventions to interrupt the complex life cycle of *Plasmodium* parasites. Both the *Plasmodium* parasite and its *Anopheles* vector offer a variety of targets for intervention and scientists have only just begun to harvest their genome sequences for a deeper understanding of host-parasite interactions. These findings may lead to new interventions on the road to eradicate malaria (Greenwood *et al.*, 2008).



MACROLIDE ANTIBIOTICS AND PHEROID™ TECHNOLOGY AS DRUG DELIVERY SYSTEM

2.1 INTRODUCTION

The emergence of *P. falciparum* resistance to antimalarial drugs, poses a grave health burden to the world's population (Wongsrichanalai, C. 2002; White N.J, 2004; Miller *et al.*, 2006). A method of overcoming this troublesome fact is by means of combination therapy, generally consisting of a rapid acting antimalarial drug and an agent with a longer half-life. It is imperative to combine drugs that possess compatible pharmacokinetics and pharmacodynamics, and which will not cause added toxicity or any adverse pharmacological interaction when in combination (White & Olliaro, 1996; Miller *et al.*, 2006). Many advances in parasitic infections have been made over time as far as the pharmacogenomics, etiology, and pathophysiology are concerned, though progress in therapeutic interventions has been somewhat poor. In spite of numerous research efforts, no effective vaccine against parasitic infections exists. While the present antimalarials are effective, most of them have been introduced over 50 years ago and are still not ideal in terms of tolerability, therapeutic regimen, duration of treatment, specificity, and patient compliance and the incidence of resistance is gradually decreasing their effectiveness (Date *et al.*, 2007). Antibiotics have been known for their antiplasmodial activity since 1940 (Menezes *et al.*, 1999) and their use in combination with antimalarial drugs can be implemented in the treatment of drug-resistant *Plasmodium* strains (Pradines *et al.*, 2001; Nakornchai & Konthiang, 2006; Puri & Singh, 2000). It is vital that drugs used in potential life-saving treatment of severe malaria are rapid acting and because of the slow onset of antimalarial action of antibiotics, like azithromycin, these agents can't be administered as a single agent in emergency treatment of malaria (Noedl *et al.*, 2001; Ohrt *et al.*, 2002). Antibiotics seem to display better antimalarial activity after prolonged exposure to *P. falciparum* *in vitro* (Pradines *et al.*, 2001; Yeo & Rieckmann, 1994). Pregnant woman and young children have proved to be especially difficult treatment groups and alternative, safe, and effective drugs are urgently needed (Nakornchai & Konthiang, 2006). In the past, pharmaceuticals were either administered orally or injected as

uncomplicated, rapid-acting chemical compounds. Technology and an expanded knowledge of the human body has led to the development of drug delivery systems that are able to target specific sites in the body, possessing the ability to control the rate and period of drug delivery (Vogelson, 2001). One such delivery system is the Pheroid™ drug delivery system. This patented technology is able to enhance the absorption of various categories of drugs (Grobler, 2004). The Pheroid™ drug delivery system holds significant advantages (which are mentioned in section 2.4.3) over other delivery systems. The Pheroid™ has been demonstrated to reduce or eliminate drug resistance *in vitro*. A promising benefit, especially regarding malaria, as resistance is currently one of the biggest problems faced. Due to the increased therapeutic efficacy of formulations of active compounds and Pheroids™, this study aimed to investigate whether the macrolide antibiotics' (erythromycin and azithromycin) antimalarial activity will be increased *in vitro*. Prolonged exposure was also investigated due to the slow antimalarial onset of these compounds. In Chapter 2 important aspects of discussion are:

- The macrolide antibiotics, erythromycin and azithromycin, in terms of their product information and their use in malaria. This study was conducted on erythromycin estolate and azithromycin dehydrate, which are generic substitutes of erythromycin and azithromycin, respectively.
- Drug delivery, in particular the Pheroid™ drug delivery system.
- The implementation of Pheroid™ technology in this study, motivated in terms of key advantages.

2.2 MACROLIDE ANTIBIOTICS - ERYTHROMYCIN AND AZITHROMYCIN

2.2.1 Macrolide antibiotics

Macrolides fundamentally consist of a macrocyclic lactone ring, which is where the groups' name originates from (Sood, 1999). Macrolide antibiotics have been used as antibacterial compounds for many years due to their versatility and broad spectrum of activity against gram-positive and gram-negative organisms (Chambers, 2004; Sood, 1999). The prototype of the macrolide antibiotics is erythromycin, which was isolated in 1952 from *Streptomyces erythreus* (Chambers, 2004). Newer macrolides include clarithromycin and azithromycin, semisynthetic derivatives of erythromycin. The advantages of clarithromycin and

azithromycin include a less frequent dosage regimen and both drugs seem to exude better tolerability (Chambers, 2004; Sood, 1999).

2.2.2 Erythromycin

The molecular formula of erythromycin is $C_{37}H_{67}NO_{13}$ (British Pharmacopoeia, 2010a). Erythromycin chemically consists of a macrolide ring (illustrated in Figure 2.1) and two sugars, desosamine and cladinose. In solution, erythromycin tends to be reasonably stable at 4°C but hastily becomes inactive at 20°C (Chambers, 2004). Erythromycin estolate is a generic substitute of erythromycin and was used for experimental purposes in this study. It is presented as a white, crystalline powder (British Pharmacopoeia, 2010b). This sulphate is practically insoluble in water but is freely soluble in organic solvents (British Pharmacopoeia, 2010b; Chambers, 2004). Erythromycin estolate ($C_{52}H_{97}NO_{18}S$) furthermore consists of large molecules possessing a molecular weight of 1056 g/mol (British Pharmacopoeia, 2010b).

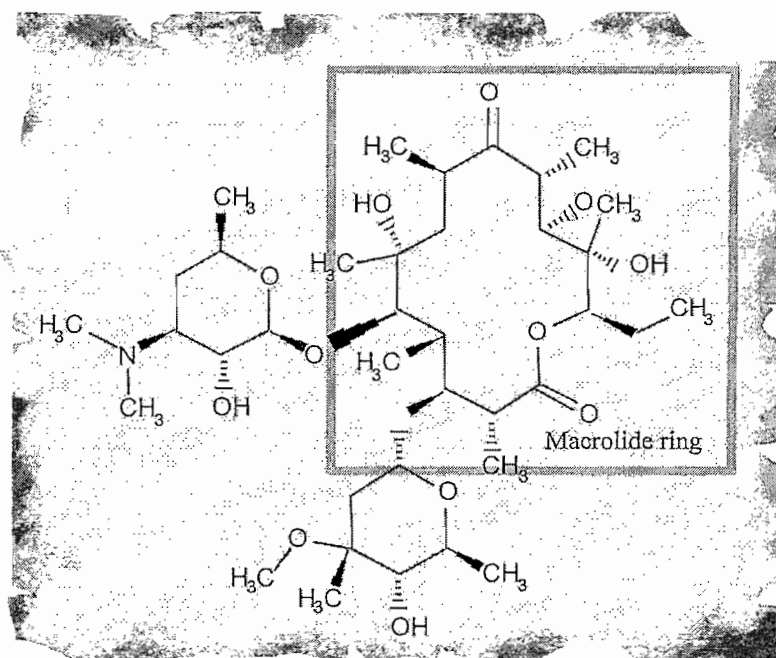


FIGURE 2.1: Chemical structure of erythromycin (PharmGKB, 2010).

2.2.2.1 Drug profile

Table 2.1 summarises the product information of erythromycin, highlighting the antimicrobial activity, pharmacokinetics, clinical uses and adverse reactions.

TABLE 2.1: Erythromycin product information

(Compiled from: Chambers, 2004; Gibbon, 2005; DOH, 2003a).

ANTIMICROBIAL ACTIVITY	PHARMACOKINETICS	CLINICAL USES	ADVERSE REACTIONS
<p>Gram positive: Pneumococci Streptococci Staphylococci Corynebacteria Mycoplasma Legionella <i>Chlamedia trachomatis</i> <i>C.psittaci</i> <i>C.pneumoniae</i> Helicobacter Listeria <i>Mycobacterium kansasii</i> <i>Mycobacterium scrofulaceum</i></p> <p>Gram negative: Neisseria species <i>Bordetella pertussis</i> <i>Bartonella henselae</i> <i>B. quintana</i> Some rickettsia species <i>Treponema pallidum</i> Campylobacter species <i>Haemophilus influenzae</i> to a lesser extent <i>Moraxella catarrhalis</i> <i>H. ducrei</i></p>	<p>Mechanism of action: Macrolides bind to the 50S ribosomal subunit of bacteria, inhibiting cell growth through the inhibition of protein synthesis by acting as bacteriostatic agents at low dosages and bactericidal agents at high dosages.</p> <p>Erythromycin base needs to be administered with enteric coating due to gastric acidity and the fact that the presence of food could compromise absorption.</p> <p>It is widely distributed and protein binding is high. $T_{1/2} = 1.5 - 2$ hours. It is partially metabolized in the liver, eliminated by hepatic concentration and excreted in the bile.</p> <p>Approximately 2-5% is excreted unchanged in the urine.</p>	<p>Alternative therapy in penicillin-allergic patients, Whooping cough, Legionnaire's disease, Mycoplasma and chlamydial infections, Moderate to severe acne, Gingivitis, Acute rheumatic Fever, Boil, abscess, Impetigo, Cellulites, Acute, moist or weeping eczema, Athlete's foot, Measles, Sexually transmitted diseases, Pneumonia, Non-gonococcal conjunctivitis, Otitis, Acute sinusitis, Bacterial tonsillitis.</p>	<p>(Contraindicated in porphyria. Category B drug in pregnancy. Cytochrome P450 inhibitor.)</p> <p>Most commonly gastrointestinal intolerance including abdominal pain, cramping nausea, vomiting and diarrhoea. Allergic skin reactions are rare. Hepatotoxicity may present as either benign elevation of serum transaminase or cholestatic hepatitis.</p>

2.2.2.2 Use in malaria

Erythromycin was the first macrolide to be introduced as a possible treatment and prophylactic agent in malaria (Noedl *et al.*, 2001). Robinson and Warhurst first observed the *in vitro* and *in vivo* antimalarial activity of erythromycin in 1972 and data suggests that further investigation into the antiplasmodial effect of this macrolide, could hold apparent advantages in the treatment of malaria (Gingras & Jensen, 1992; Gingras & Jensen, 1993; Menezes *et al.*, 1999). A previous study has demonstrated that erythromycin in combination with antimalarials like chloroquine, quinine, and mefloquine have respectively shown synergism, potentiation, and additive effects (Nakornchai & Konthiang, 2006). However, a study conducted during the eighties in eastern Thailand, where chloroquine-resistance is especially prevalent and where quinine-resistance is slowly rising, concluded that high dosages erythromycin administered together with standard dosages of chloroquine and quinine, respectively, had no remarkable effect on the cure rate of *falciparum* infection (Pang *et al.*, 1985). Results seem to vary, for a clinical study of Thai children infected with *P. falciparum* conducted by Chongsuphajaisiddhi *et al.* in 1983 demonstrated a cure rate of 80 % with combination treatment of erythromycin and quinine, in comparison to the 20 % cure rate found by Suntharasamai *et al.* in 1983 in treatment with quinine alone (Pang *et al.*, 1985).

2.2.3. Azithromycin

Azithromycin has the chemical name (2R,3S,4R,5R,8R, 10R,11R,12S,13S,14R)-13-[(2,6-dideoxy-3-C-methyl-3-O-methyl- α -L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)- β -D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one and possesses a molecular formula of $C_{38}H_{72}N_2O_{12}$ (Budavari, 2001). Azithromycin is commercially available as a dihydrate (Ghandi *et al.*, 2002). It is presented as a white crystalline powder and possesses a molecular weight of 785.0 g/mol (Budavari, 2001).

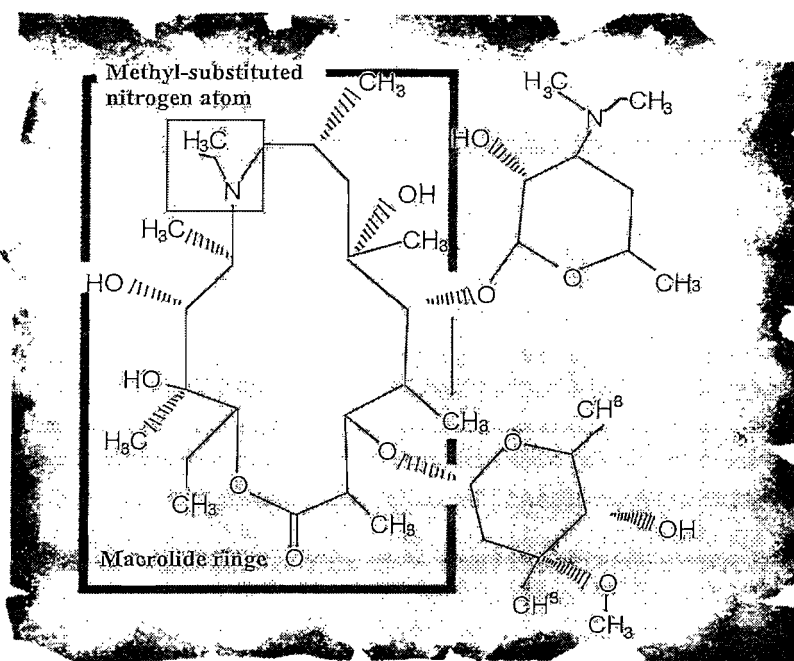


FIGURE 2.2: Chemical structure of azithromycin with the macrolide ring highlighted (DailyMed, 2009).

Azithromycin is a semisynthetic derivative of erythromycin and differs from the prototype by possessing a methyl-substituted nitrogen atom in the macrolide ring, as indicated in Figure 2.2. This substitution contributes to various advantages above erythromycin, including a broader spectrum of activity, more encouraging pharmacodynamics, and a longer elimination half-life (Sidhu *et al.*, 2006).

2.2.3.1 Drug profile

A summary of the product information of azithromycin is outlined in Table 2.2, listing the antimicrobial activity, clinical uses and adverse reactions.

TABLE 2.2: Summarized product information of azithromycin

(Compiled from Chambers, 2001; 2004).

ANTIMICROBIAL ACTIVITY	PHARMACOKINETICS	CLINICAL USES	ADVERSE REACTIONS
<p>Chlamydia <i>M. avium</i> <i>T. gondii</i> <i>Haemophilus ducreyi</i> <i>Moraxella catarrhalis</i> <i>Neisseria gonorrhoeae</i> <i>Mycoplasma pneumoniae</i></p> <p>Less active than erythromycin against staphylococci and streptococci but more active against <i>H. influenzae</i>.</p>	<p>Azithromycin is absorbed rapidly and distributed widely throughout the body (with the exception of the cerebrospinal fluid), when administered orally. Peak plasma drug concentrations after a loading dose of 500 mg is approximately 0.4 µg/ml.</p> <p>Azithromycin undergoes some hepatic metabolism, although biliary excretion is the major elimination route. 12 % is excreted unchanged in the urine. The elimination half-life is between 40 to 68 hours.</p>	<p>Chronic obstructive pulmonary disease, Bacterial sinusitis, Pneumonia, Pharyngitis/tonsillitis, Skin and skin structure infections, Urethritis and cervicitis, Genital ulcer disease, Otitis media.</p>	<p>Azithromycin possesses a 15-member (not 14-member) lactone ring, and does therefore not inhibit cytochrome P450 enzymes. Azithromycin consequently have less drug interactions than erythromycin.</p> <p>Side-effects include nausea, vomiting, diarrhea and abdominal pain.</p>

2.2.3.2 Use in malaria

Azithromycin is classified as the most potent macrolide antibiotic regarding malaria (Miller *et al.*, 2006). An *in vitro* study displayed a 10-fold greater potency of azithromycin over erythromycin against a chloroquine-resistant strain of *P. falciparum* (Gingras and Jensen, 1992). Azithromycin seems to illustrate better antimalarial activity by prolonging its exposure to malaria parasites (Yeo & Rieckman, 1994). Azithromycin has not been licensed for use as an antimalarial drug yet (Vallely *et al.*, 2007), but this macrolide antibiotic has demonstrated activity against *P. falciparum in vitro* (Ohrt *et al.*, 2002; Pradines *et al.*, 2001), in the murine malaria model (Anderson *et al.*, 1994) and in randomised controlled clinical trials (Taylor *et*

al., 2003, Anderson *et al.*, 1998, Krudsood *et al.*, 2000). Antibiotics act by binding to the 70S ribosome (consisting of the 50S and 30S subunits) and thereby inhibiting protein synthesis, finally leading to growth retardation (Sidhu *et al.*, 2006). Encountering malaria during pregnancy poses various challenges to health workers and researchers alike, as the disease creates multiple severe health risks for mother and unborn baby, and medicinal interventions are never 100 % effective and cause adverse effects of its own. Annually, thirty million pregnancies in sub-Saharan Africa are at risk of developing malaria. As an intervention in endemic areas the WHO urges the use of Intermittent Preventive Treatment of Malaria in Pregnancy (IPTp) mainly aiming to: (1) clear asymptomatic peripheral and placental parasitaemia and (2) provide intermittent chemoprophylaxis against malaria infection during pregnancy. Sulphadoxine-pyrimethamine is currently the treatment of choice in IPTp (Chico *et al.*, 2008). In early field malaria prophylactic trials, azithromycin exuded poor activity against *falciparum* malaria, excluding the possibility of developing this macrolide as a single agent (Ohrt *et al.*, 2002). Combining azithromycin and chloroquine showed synergism (Nakornchai & Konthiang, 2006, Chico *et al.*, 2008) and considerably improved clinical outcomes when compared with monotherapy (Dunne *et al.*, 2005) enabling this combination to be a possible alternative for the treatment of malaria during pregnancy. Chloroquine is not believed to be a strong teratogen, though lack of adequate data leads to a 'benefit versus risk' approach when prescribed in pregnancy (Wolfe & Cordero, 1985). Before the emergence of resistance, chloroquine was considered the safest antimalarial during pregnancy (Gibbon, 2005). Azithromycin can be used in young children (Zithromax product information, 1999; Pfizer Inc., New York) and is safe to use during pregnancy (Nakornchai & Konthiang, 2006; Gray *et al.*, 2001). Previous research suggests that the macrolides as a group are generally safe to use during pregnancy (Sarkar *et al.*, 2006). It is thus evident that azithromycin also holds potential to be further investigated and developed as an alternative antimalarial prophylactic and treatment agent in combination with existing antimalarials (Gingras & Jensen, 1992; Gingras & Jensen, 1993; Nakornchai & Konthiang, 2006).

2.3 DRUG DELIVERY SYSTEMS

Jain, (2008) defines a carrier system as: 'a formulation or device that enables the introduction of a therapeutic substance in the body and improves its efficacy and safety by controlling the rate, time and place of release of drugs in the body'.

2.3.1 Introduction

Drug delivery poses numerous problems, one of the major difficulties being solubility (Grobler, 2004). Furthermore, the potency and therapeutic effect of various drugs are restricted or reduced due to partial degradation before reaching their target. Modern day drug delivery systems therefore aim to integrally deliver medications to specific targets in the body, by means of a medium that is able to control the therapy's administration either through a physiological or chemical trigger (Vogelson, 2001). At present, there are various drug delivery systems available for formulation, including liposomes, nanotechnology, microparticles, bilosomes and polymers, to name a few. Date *et al.* (2007) recommends that the following factors need to be carefully assessed when developing drug delivery systems:

- Parasite/host cell interaction.
- Biological barriers that need to be overcome in order to reach the target organ, tissue or cells.
- Complete information about the receptors present on the cells infected by the parasite.
- Changes occurring in the infected cell after the invasion of parasite or with the progression of disease.
- Antigens or receptors present on the surface of the parasite.
- Pathophysiology of the disease.

2.3.2 Drug delivery in malaria

Because *Plasmodium* species are intra-erythrocytic parasites, there are several membranes that need to be crossed in order to reach most intraparasitic targets. These membranes include:

- the host red blood cell membrane,
- the parasitophorous vacuolar membrane,
- the parasite plasma membrane, and
- either a food vacuole membrane or a mitochondrial membrane, depending on the target of the drug (Biagini *et al.*, 2005).

In order to successfully design delivery systems the biological and pathophysiological aspects of the parasitic disease, and the biopharmaceutical aspects of the drugs available for the treatment, need to be carefully assessed (Date *et al.*, 2007). Existing antimalarial drugs have many side-effects, often affecting patients in such a way that they do not complete the

recommended treatment regimen as in the case of prophylactic treatment. Employing carrier systems are thus essential to advance the efficacy, specificity, tolerability and therapeutic index (Date *et al.*, 2007; Slabbert, 2008). It should, in addition, be versatile, cost-effective and allow oral administration of the drugs with particular intracellular targeting, ultimately leading to a shortened duration of treatment, maximum effect and reduced toxicity (Date *et al.*, 2007).

2.4 PHEROID™ TECHNOLOGY

The concept of a biomaterial active ingredient delivery system was developed by MeyerZall (Pty) Ltd. laboratories into a patented drug delivery system known as Pheroid™ technology (MeyerZall (Pty) Ltd., 2009). The University of the North-West of South Africa purchased the intellectual property on which the technology is based from the company in December 2003 (Grobler *et al.*, 2007).

2.4.1 Introduction

Pheroids™ are stable, polyphylic structures consisting mainly of plant and essential fatty acids. Multiple cell functions are dependent upon these fatty acids, though it needs to be ingested as it is not produced by the human body. Since studies suggest that the Western diet lacks adequate supply of these basic lipid molecules, Pheroid™ technology poses significant advantages over other drug delivery systems as this component of the system contribute to the maintenance of membrane integrity of cells, energy homeostasis, modulation of the immune system and a few regulatory aspects of programmed cell death. These therapeutic advantages as well as the ability of the structure to be manipulated in terms of morphology, structure, size and function add to the efficacy of this drug delivery system (Grobler, 2004).

2.4.2 Structural distinctiveness

The Pheroid™ drug delivery system is a colloidal system, containing Pheroids™ with a diameter of 200 nm - 2 µm in suspension (Grobler *et al.*, 2007). Various types of Pheroids™ can be formulated (Uys, 2006). A basic Pheroid™ holds a porous, vesicular structure ranging in size from 200 - 440 nm (Grobler *et al.*, 2007). The three main formulations of the Pheroid™ are:

- Pheroid™ vesicles, consisting of a lipid bilayer, ranging in size between 0.5 - 1.5 µm.

- Micro-sponges, 1.5 - 5 μm in size.
- Depots and reservoirs containing pro-Pheroids™ (Uys, 2006). The polymer polyethylene glycol (PEG) is a component frequently used in food and pharmaceutical products. It is fairly non reactive and non-toxic. All Pheroid™ systems contain a small part of PEG, though increased concentrations and bigger polymers have given rise to the development of pro-Pheroids™ (Grobler, 2004).

The micrographs in Figure 2.3 offer an indication of active compounds entrapped in various Pheroid™ types. The images were obtained through confocal scanning laser microscopy by labeling the Pheroids™ with the fluorescent marker, Nile red.

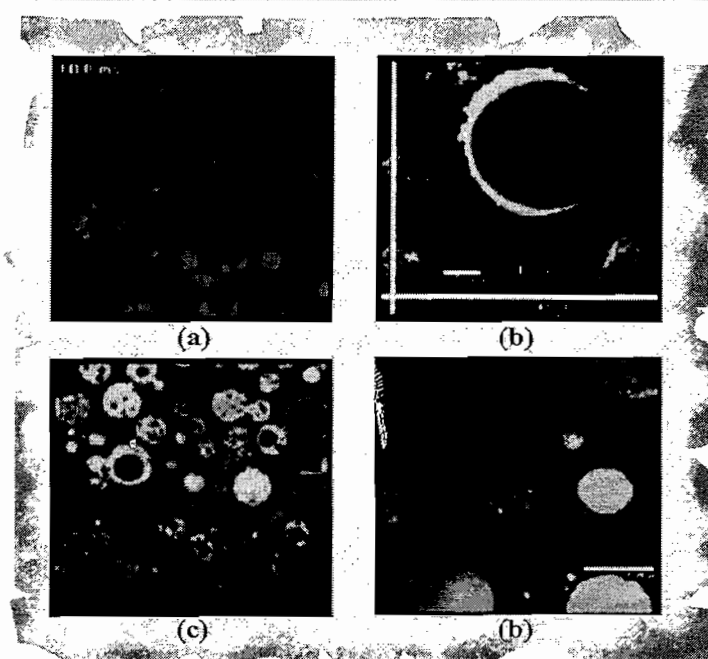


FIGURE 2.3: Confocal laser scanning micrographs. (a) Mixture of liposome-like bilayer vesicles and nanosponges (b) A Pheroid™ microsphere of the reservoir type (c) & (d) Colloids with three phases (Grobler *et al.*, 2007).

2.4.3 Advantages of the Pheroid™ drug delivery system

Pheroids™ have the ability to penetrate keratinized tissue, skin, intestinal lining, vascular system, fungi, bacteria, parasites and seem to have an impact on viral load. The components of the system can be manipulated in a definite manner contributing to high entrapment capabilities, haste rate of transport, delivery and stability, singling this safe, effective formulation out from other existing drug delivery systems. Though Pheroid™ technology is

similar in many ways to drug delivery systems like lipid-based delivery systems, with which it is often confused, there are also apparent differences and advantages, ultimately contributing to an enhancement of the therapeutic action (Grobler, 2004). Key advantages include:

- Decreased time to onset of action.
- Increased delivery of active compounds.
- Reduction of minimum inhibitory concentration.
- Increased therapeutic efficacy.
- Reduction in cytotoxicity.
- Certain drugs like peptides or proteins, often induces immunologic responses during treatment. When administered with the Pheroid™ drug delivery system, Pheroids™ appear to mask the compounds responsible for adverse intolerance reactions leading to the ability to heighten or lower dosages dependant on the health issue being addressed.
- Transdermal delivery.
- Ability to entrap and transfer genes to cell nuclei.
- Expression of proteins.
- Reduction and suggested elimination of drug resistance (Grobler, 2004).

2.4.4 Applicability of Pheroids™ in this study

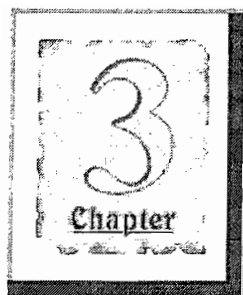
A study, comparing commercial erythromycin with erythromycin in formulation with Pheroids™, found that erythromycins' activity was increased in formulation (see Table 2.3). The various advantages of the Pheroid™ delivery system, the safety-profile of both erythromycin and azithromycin, and results from previous studies proving that both test compounds possess antimalarial activity, motivated the decision to combine Pheroid™ technology with the test compounds in the search for alternative, safe and effective antimalarials, especially concerning difficult treatment groups.

TABLE 2.3: Commercial erythromycin compared with erythromycin in formulation with Pheroids™ (Grobler, 2004).

ACTIVE AGENT	PHEROID™/COM	DOSE mg/5ml	S. AUREUS	B. EREUS
Erythromycin	Pheroid™	250	26.7	29.89
Erythromycin	Com	250	25.84	27.78

2.5 CONCLUSION

The macrolide antibiotics, erythromycin and azithromycin, both demonstrate activity against *P. falciparum*, *in vitro* and *in vivo*, and previous studies encourage further investigation into their possible antimalarial efficacy (Nakornchai & Konthiang, 2006, Noedl *et al.*, 2001, Puri & Singh, 2000, Miller *et al.*, 2006). Azithromycin, in particular, is safe for use in pregnant woman and young children. Because of the slow onset of antiplasmodial action of antibiotics, combination with rapid acting antimalarials may offer potential alternative treatment and prophylactic options. Prolonged exposure was also demonstrated to increase antimalarial activity. Drug delivery systems are necessary to overcome existing problems like toxicity and solubility. The Pheroid™ drug delivery system demonstrated various key advantages, and this study aimed to incorporate the antibiotics, erythromycin and azithromycin, into the Pheroid™ carrier system and to expose the formulation to a chloroquine-resistant strain of *Plasmodium falciparum* for a period of 144 hours. The unique composition of the Pheroid™ drug delivery system has been proven to enhance the efficacy of active compounds.



MATERIALS AND METHODS

3.1 INTRODUCTION

This study can be described as being tripartite. The first part of the study consisted of the formulation of Pheroids™ and incorporating the macrolide antibiotics, erythromycin and azithromycin, into the Pheroids™. The different formulations were characterised and the formulation for *in vitro* efficacy experiments was chosen. The second part of the study consisted of the evaluation of the HRP II ELISA method in the applicability of *in vitro* drug efficacy assays with Pheroid™ technology. The third part entailed the *in vitro* drug efficacy assays with the macrolide antibiotics alone and in combination with Pheroid™ technology. Chapter 3 will describe the instrumentation, methods and techniques used to obtain the results presented in the dissertation. The following topics will be addressed:

- the formulation of erythromycin and azithromycin in Pheroid™ vesicles,
- maintenance of *P. falciparum* (RSA 11) parasites *in vitro*,
- a detailed description of the HRP II assay for its applicability in this study, and
- the experimental procedure for the evaluation of the efficacy of erythromycin and azithromycin, respectively, on a chloroquine-resistant strain (RSA 11) in comparison with the efficacy of each test compound in combination with Pheroid™ vesicles.

3.2 FORMULATION OF PHEROIDS™

One of the objectives of this study was to accurately formulate erythromycin and azithromycin in combination with Pheroids™ for *in vitro* antimalarial efficacy assays. Pheroids™ are colloidal, submicron emulsion type systems essentially consisting of an oil-in-water (o/w) phase, and is presented as a liquid. A liquid drug delivery system is generally expected to be biocompatible, biodegradable, of fine and homogeneous particle size, have acceptable stability, be fit for targeting and be pharmaceutically presentable (Buszello & Müller, 2000). Carrier systems such as Pheroids™, primarily aim to control target specific

drug delivery. Due to the composition of the Pheroid™ it is sterically stabilized without the disadvantages of increased size or decreased elasticity, with steric stabilization referring to colloidal stability (Grobler, 2004). Because of its unique composition, Pheroids™ are able to entrap both water and lipid soluble drugs (Grobler, 2004) and can be formulated in various strengths, 4 % w/v and 8 % w/v in the case of vesicles, and 4.5 % w/v and 9 % w/v in the case of microsponges. The various strengths are an indication of the amount of the oil phase prepared in relation to the water phase. The water phase (sterile water gassed with nitrous oxide for 4 days) will be referred to as NW. Because of the possibility that Pheroids™ may increase the intracellular drug concentration it is essential to determine the optimal ratio of the Pheroid™ to NW, in order to maximise drug delivery. A higher ratio results in a higher Pheroid™ content in the formulation. A previous study concluded that the optimal Pheroid™ ratio, at different chloroquine phosphate concentrations against *P. falciparum* cultures, was 1:1750 (1 part Pheroid™: 1749 parts of NW). A ratio of 1:2500 also yielded acceptable results and this ratio allows for simplicity of calculation. Ratios of 1:500 and 1:1000 caused lysis of erythrocytes (Slabbert, 2008). Based on these findings, and for ease of calculation when considering the 10-fold dilution during experimental procedures, it was decided to implement Pheroid™ vesicles in a ratio of 1:250 in this study. Selection criteria for formulation approach are demonstrated in Figure 3.1.

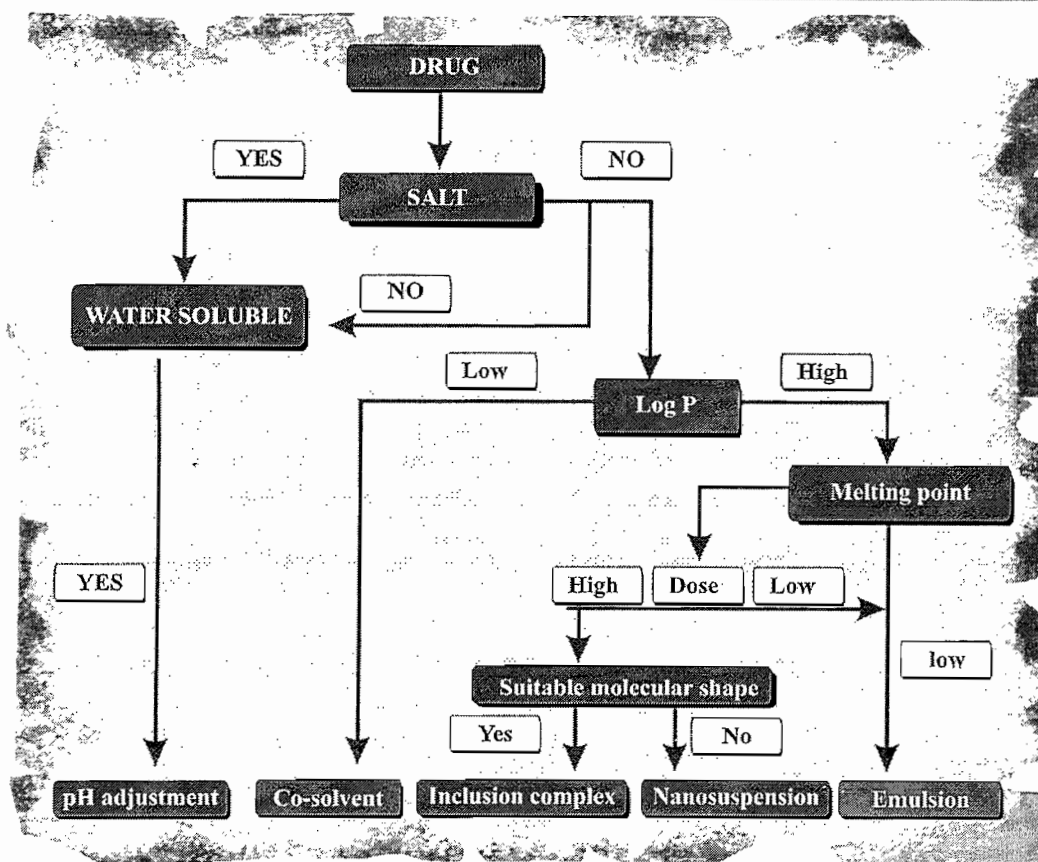


FIGURE 3.1: Selection criteria for formulation approach (Ravichandran, 2009).

3.2.1 Materials

The raw materials utilised in the oil phase of Pheroid™ vesicles are illustrated in Table 3.1.

TABLE 3.1: Quantities of raw materials used in the oil phase of Pheroid™ vesicles.

	QUANTITY (w/v)	
Raw material	4 %	8 %
Vitamin F ethyl ester	2.8 %	5.6 %
Cremaphor®EL	1.0 %	2.0 %
DL-α-Tocopherol	0.2 %	0.4 %

Microsponges additionally contain Incromega 7010 and Incromega 3322 (0.25 % in a 4.5 % formulation and 0.5 % in a 9 % formulation).

The previously mentioned study concluded that erythromycin inhibited growth of *P. falciparum* at an IC₅₀ value of 58.2 ± 7 μM and azithromycin at 8.4 ± 1.2 μM (Nakornchai & Konthiang, 2006). An IC₅₀/EC₅₀ value depicts the concentration of the drug needed for 50 % of the maximum inhibitory effect in a test system (Stepniewska *et al.*, 2007). This study measured ³H hypoxanthine incorporation. Formulation was based on concentration ranges of 0 - 80 μM for erythromycin and 0 - 8 μM for azithromycin. Poor water solubility is a major difficulty in the process of drug formulation (Chingunpituk, 2007). Erythromycin estolate and azithromycin dihydrate are both particularly insoluble in water. The high molecular masses, relatively high EC₅₀ values and both drugs' poor solubility proved a challenge in the formulation process.

3.4 CHARACTERIZATION OF PHEROID™ FORMULATIONS

3.4.1 Apparatus, experimental conditions and method for particle size analysis

Sample preparation is critical, for many of the problems faced during measurement of samples are due to incorrect preparation. Samples sticking together, dissolving, floating on the surface or failure to obtain a representative sample, are all measures that could lead to unsatisfactory and incorrect results (Malvern Instruments Ltd., 1997). A Malvern mastersizer (Malvern Instruments, United Kingdom) was the instrument used for the analysis of particle size, by the method of laser diffraction. It is compulsory that the laser beam stabilises beforehand and therefore the apparatus is switched on half an hour before initialising measurements. Air bubbles are detected by the instrument and should therefore be eliminated from the sample being analysed, for they can cause variations in data (Uys, 2006). Adequate sample was added to 800 ml of distilled water to obtain a laser obscuration between 10 - 20 % at a pump speed of 2500 rpm. Data was saved automatically and results were presented in histograms. Examples of Malvern mastersizer analysis reports are illustrated in Addendum C.

3.4.2 Confocal laser scanning microscopy (CLSM)

The morphological features of Pheroid™ vesicles and Pheroid™ microsponges were assessed by both optical microscopic and confocal laser scanning microscopy (CLSM). The optical microscopy images were taken with an inverted microscope (Nikon Eclipse TE-3000). The CLSM images were taken with a Nikon D-Eclipse C1 confocal laser scanning microscope with a DXM 1200 digital camera with real time imaging and a medium (10 pm) pinhole. A 60× 1.40 ApoPlanar oil immersion objective was used. The microscope was equipped with a green krypton laser (wavelengths: excitation 488 nm, emission 515 nm) and a red helium neon laser (wavelengths: excitation 505 nm, emission 564 nm). Pheroids™ were labelled with the fluorophore Nile red (1 mg/ml). The latter has an emission wavelength of between 640 and 650 nm. Pheroids™ were placed on a microscope slide, covered with a glass coverslip and sealed with adhesive to prevent fluid loss (Du Plessis *et al.*, 2010).

3.5 EVALUATION OF THE HRP II ELISA METHOD

The motivation for the use of this method includes its advantages, such as relative simplicity of implementation, reduced requirements for laboratory equipment, ease of training of personnel and rapid performance. It also permits the testing of slow-acting drugs, like antibiotics, without changes in protocol (Noedl *et al.*, 2003). The Malaria Ag CELISA™ kit was applied in this study, employing a simple sandwich enzyme-linked immunosorbent assay (ELISA). The following steps underlie the basic principle of an ELISA (Voet & Voet, 1995):

- An HRP II antibody is immobilised on an inert solid.
- The sample being assayed for HRP II is applied to the antibody-coated surface allowing the antibody to bind to HRP II and unbound protein to be washed away.
- The resultant protein-antibody complex is reacted with a second protein-specific antibody, covalently linked to an easily assayed enzyme.
- After the washing step where any unbound antibody-linked enzyme is removed, the enzyme in the immobilised antibody-protein-antibody-enzyme complex is assayed to ultimately determine the amount of HRP II present.

3.5.1 Materials

HRP II ELISA kit (Malaria Ag CELISA, Cellabs (Pty.) Ltd., Brookvale, New South Wales, Australia).

3.5.2 Method

Prior to conducting an assay, samples were frozen after incubation times of 48 hours, 96 hours and 144 hours and then thawed at room temperature in order to ensure complete hemolysis of erythrocytes in each sample. After complete hemolysis was obtained, 100 μ l of each sample was transferred to the precoated (monoclonal antibodies against *P. falciparum* HRP II) ELISA plate. Following a one hour incubation period at room temperature the plate was washed four times manually, using the wash buffer provided with the test kit. The working strength conjugate was prepared, by adding the conjugate concentrate to the conjugate diluent, and 100 μ l of this conjugate was added to each sample. Samples were incubated for one hour at room temperature in a humid chamber. Subsequent to incubation, the working strength substrate was prepared by adding substrate chromagen to substrate buffer. The plate was once again washed four times and 100 μ l of the working strength substrate was added to each sample. Samples were incubated, in the dark, for 14 minutes. In the final step, 50 μ l of a stopping solution was added to each sample. The method is summarised in Figure 3.2.

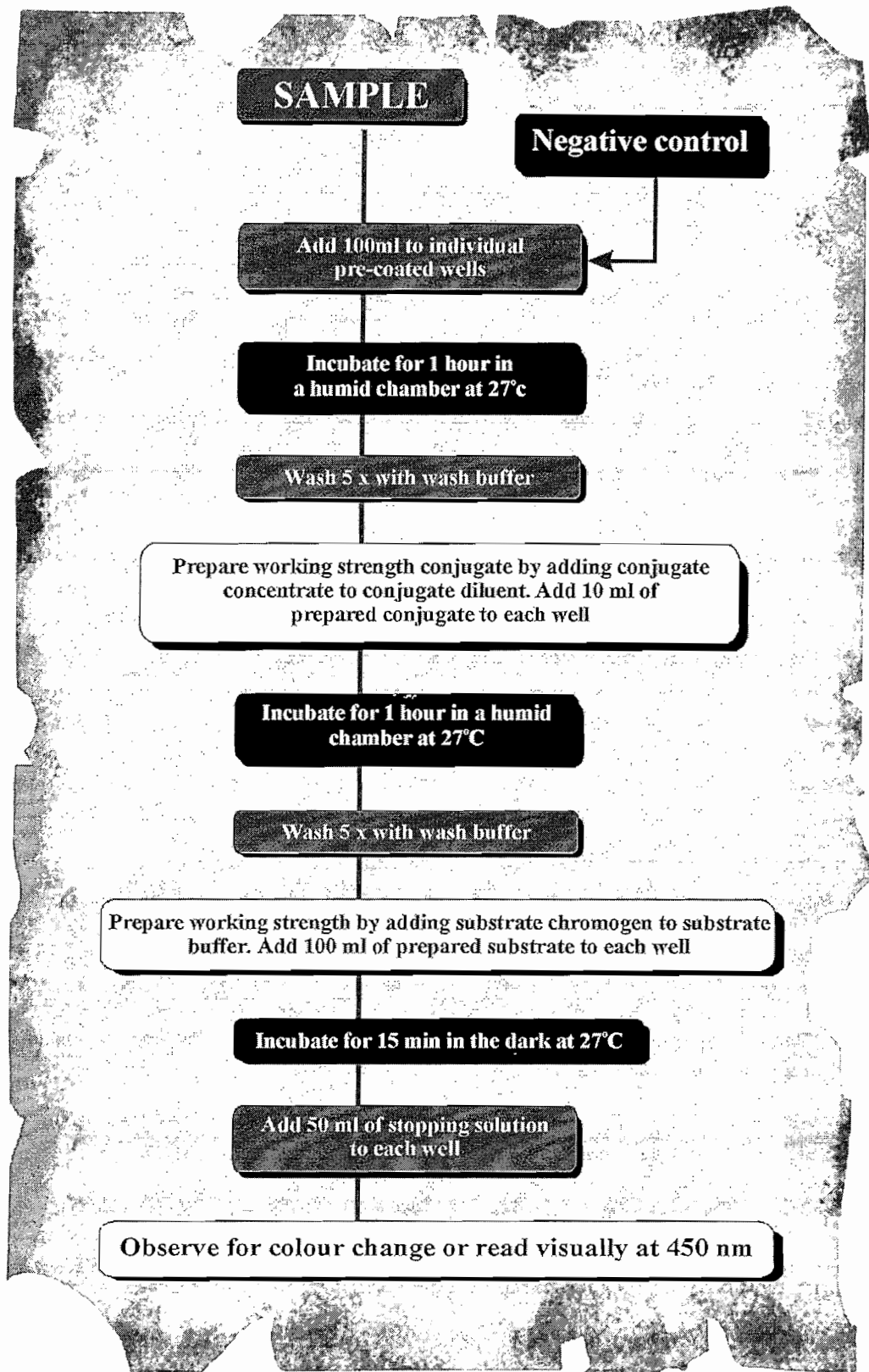


FIGURE 3.2: Summary of the ELISA method (Malaria Ag CELISA, Cellabs (Pty.) Ltd., Brookvale, New South Wales, Australia).

Quantification of this assay was based on the binding kinetics of a homogeneous recombinant histidine rich protein (rHRP II). Levels of HRP II present in culture supernatant was then calibrated by implementing a standard graph created to allow measurement of rHRP II. Levels of HRP II present in unknown malaria experimental samples was measured in reference to the calibrated material which served as the positive control. In order to evaluate the method a recombinant HRP II standard curve was generated through the serial dilution of the recombinant antigen (Recombinant Calibrator BM181, supplied with the kit). The recombinant was diluted in 100 µl of 0.1 M PBS containing 0.01 % Tween® 20 (PBS/T). A standard series (0; 0.86; 1.72; 3.44; 6.80; 13.75 and 27.50 ng/ml) was prepared in a 96-well plate. The standards were transferred to the precoated 96-well plate and the standard ELISA as described was performed. To determine the sensitivity of the method, especially at low parasitemia levels, a serial dilution of infected erythrocytes was made. The dilution ranged from 4 % to 0.25 % parasitemia at 1.5 % hematocrit. Parasitemia (%) were adjusted with culture medium and non-infected erythrocytes, as described in section 3.7.1.4.

3.6 IN VITRO MAINTENANCE OF PARASITES

The four prevalent *Plasmodium* species; *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, have all been cultivated and maintained to some extent, though the only species for which all life cycle stages have been established in culture is *P. falciparum*. The numerous strains of *Plasmodium* differ from one another; some are easily cultured *in vitro*, while others are obstinate. Isolates also undergo change in culture, for instance the gametocyte formation which is lost with long term cultivation of *P. falciparum*. Cryopreservation can preserve these characteristics (Schuster, 2002).

3.6.1 Materials

RPMI-1640, D-(+)-glucose, HEPES, hypoxanthine, sodium bicarbonate, gentamycin, sodium chloride, sodium phosphate and potassium phosphate were obtained from Sigma Aldrich® (South Africa). Albumax II was purchased from Scientific Group (South Africa) and the liquid nitrogen and special gas mixture, consisting of: 5 % oxygen (O₂), 5 % carbon dioxide (CO₂) and 90 % nitrogen gas (N₂), from Afrox (Germiston, South Africa). Methanol was obtained from MERCK (South Africa) and first grade, frosted, 1.2 mm, ground edge microscopic slides from Lasec (SA (Pty) Ltd.) or Separations (South Africa).

3.6.2 Method

Due to the unavoidable risk of infection of cultured cells, all preparations and experimental procedures were performed in a laminar flow cabinet under sterile conditions. The majority of apparatus purchased was sterile, and glass ware and pipettes were autoclaved using the liquid cycle of the autoclave. Standard operating procedures were compiled from a modified method of Trager and Jensen (1976) and De Ridder (2007). Ethics approval was obtained from the Ethics Committee of the North -West University (NWU-0008-08- S5) (Refer to addendum A).

3.6.2.1 Thawing of cultures

Isolates were cryopreserved in liquid nitrogen for long term storage. When thawing strains the cryotube, containing the isolate to be thawed, were removed from the liquid nitrogen and placed in a water bath at 37°C. The contents were transferred to a 15 ml falcon tube. A volume of 0.2 ml filtered 12 % sodium chloride was added gradually and the contents were mixed well. Thereafter, 1.8 ml of filtered 1.6 % sodium chloride was added very slowly while mixing the contents. The mixture was then centrifuged at 2000 rpm for 8 minutes. Using the vacuum system, the supernatant was removed and approximately 0.5 ml erythrocytes and 10 ml culture medium were added. The contents were transferred to a 75 cm³ culture flask and gassed with the special gas mixture for approximately 2 minutes. Culture flasks were stored in an incubator at 37°C in a CO₂ enriched atmosphere.

3.6.2.2 Preparation of culture and wash media

Dry products, as outlined in Table 3.2, were weighed and dissolved in 500 ml distilled water.

TABLE 3.2: Raw materials and the quantity of each in the preparation of culture and wash media.

RAW MATERIAL	MASS
RPMI 1640	5.2 g
D-(+)-glucose	2 g
hepes	3 g
hypoxanthine	0.022g

3.7 IN VITRO GROWTH INHIBITION STUDY OUTLINE

3.7.1 Experimental design

Individual drugs in combination with Pheroid™ vesicles were tested, in duplicate, over an extended observation period of 144 hours in 48-well plates. Additional control groups included Pheroid™ vesicles, parasitised erythrocytes, 180 µl parasitised erythrocytes with 20 µl of the diluent phosphate buffered saline (PBS) and 180 µl parasitised erythrocytes with 20 µl Pheroid™ vesicles. Experiments were conducted on a *P. falciparum* chloroquine-resistant strain (RSA 11).

3.7.1.1 Day one of the experimental method

Pheroid™ vesicles were manufactured and the formulations were agitated with the aid of a shaking apparatus (GFL Gesellschaft für Labortechnik mbH, Germany) for 24 hours. See section 3.2.

3.7.1.2 Day two of the experimental method

Particle size analysis and confocal laser scanning microscopy was conducted to evaluate stability and integrity of Pheroid™ vesicles (section 3.4.1 & 3.4.2).

3.7.1.3 Day three of the experimental method

The concentration range investigated for erythromycin estolate were 0; 2.5; 5; 10; 20; 40; 80 µM and 0; 0.25; 0.5; 1; 2; 4; 8 µM for azithromycin dihydrate. For experimental purposes test compounds were entrapped into Pheroid™ vesicles by adding the test compounds to a dilution (1:250) of manufactured Pheroid™ vesicles. The combined Pheroid™ and drug solution were shaken for 12 hours to ensure that the drug was completely entrapped by the Pheroid™ vesicles. Stock solutions were prepared and continuously diluted with phosphate buffered saline (PBS) and NW.

3.7.1.4 Day four of the experimental method

Parasitemia was determined by means of microscopy. Parasitemia is the term used to describe the percentage of parasite infected red blood cells. It is generally determined by dividing the

number of infected erythrocytes by the number of erythrocytes in a specific area under the microscope (equation 3.1).

$$\% \text{ Parasitemia} = \frac{\text{Number of parasite infected erythrocytes}}{\text{Number of erythrocytes}} \times 100 \quad \text{Eq 3.1}$$

An initial parasitemia of 0.1 % and hematocrit of 1.5 % was implemented in experimental procedures. Preliminary parasitemia was later adjusted to levels as high as 2 %. Thin blood smears were made (Fig 3.3), fixed with methanol and stained with giemsa stain. As mentioned in Chapter 1, microscopy is still one of the most reliable and easiest methods to apply in the determination of parasitemia. The problem of staining and detecting morphologically minute and similar parasites, however, requires the the need for well-trained microscopists. The labour intensity when screening multiple samples also adds to the disadvantages of this method (Singh, 1997). The method followed in the preparation of thin blood smears is illustrated in Figure 3.3. Microscopy was applied in the initial determination of parasitemia in advance of experimental procedures in order to adjust parasitemia to experimental requirements. Blood smears were fixated with methanol and stained with giemsa stain for approximately 5 minutes. Giemsa stain was prepared in a 1:1 ratio with phosphate buffer (0.65 g sodium phosphate and 0.41 g potassium phosphate to one litre of distilled water).

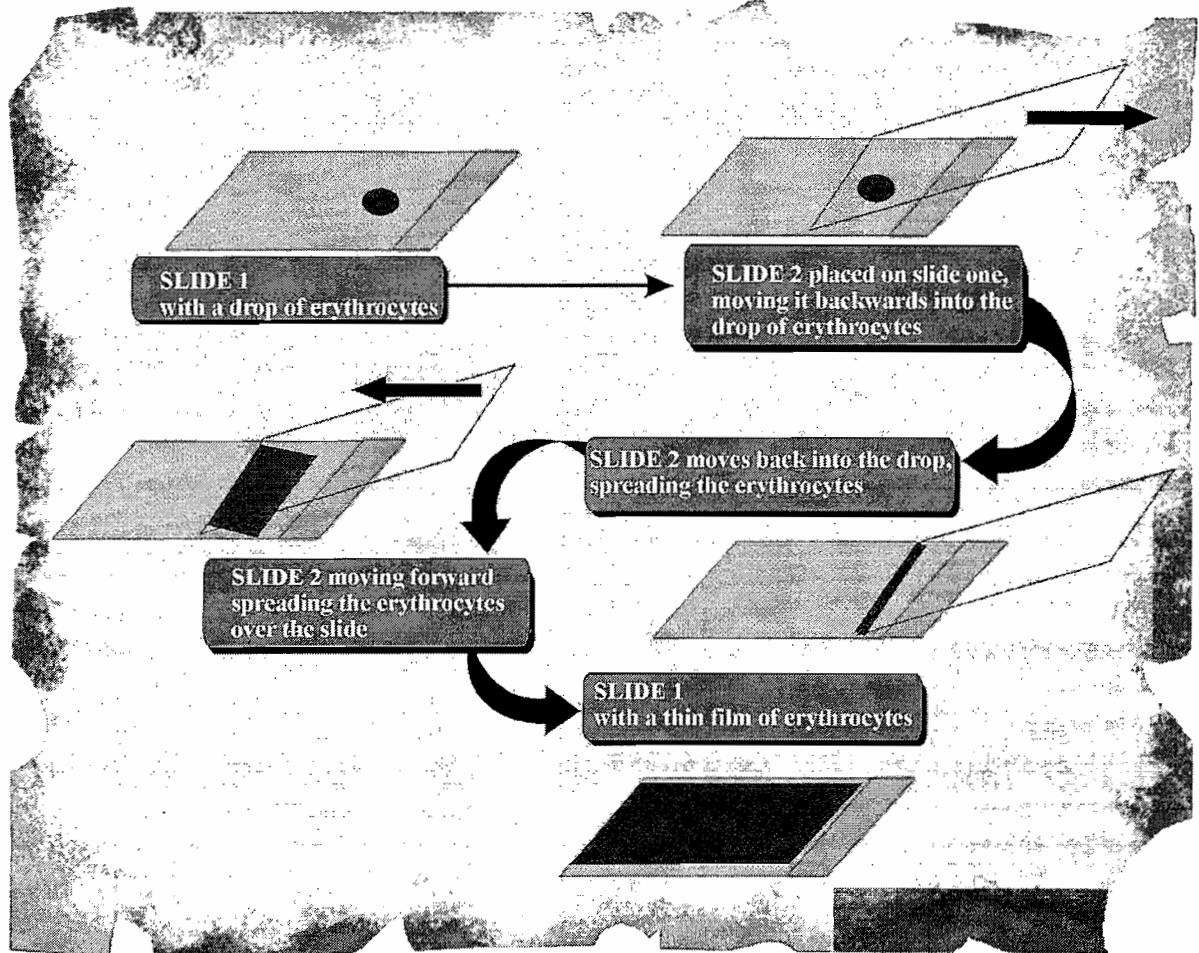


FIGURE 3.3: The procedure to follow when making a thin blood smear (Slabbert, 2008).
Used with permission from the author.

Figures 3.4 and 3.5 illustrate the appearance of a thin blood smear under the light microscope with the objective lens set at 100 X magnification. When adjusting the focus setting, parasites tend to obtain a “halo” or “glowing” effect, setting them apart from wasted giemsa stain. Figure 3.4 demonstrates the infested erythrocytes while in Figure 3.5 one can easily observe the “halo” effect when adjusting the focus setting of the microscope. Wasted giemsa stain is highlighted in the red block. The “halo” effect cannot be observed for the wasted stain.

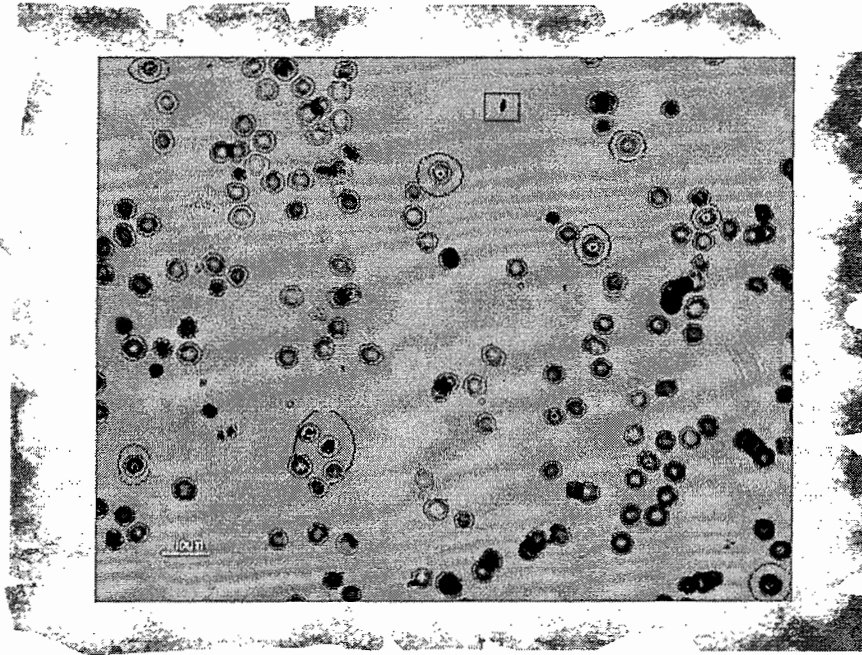


FIGURE 3.4: Light microscopic views of infected erythrocytes.

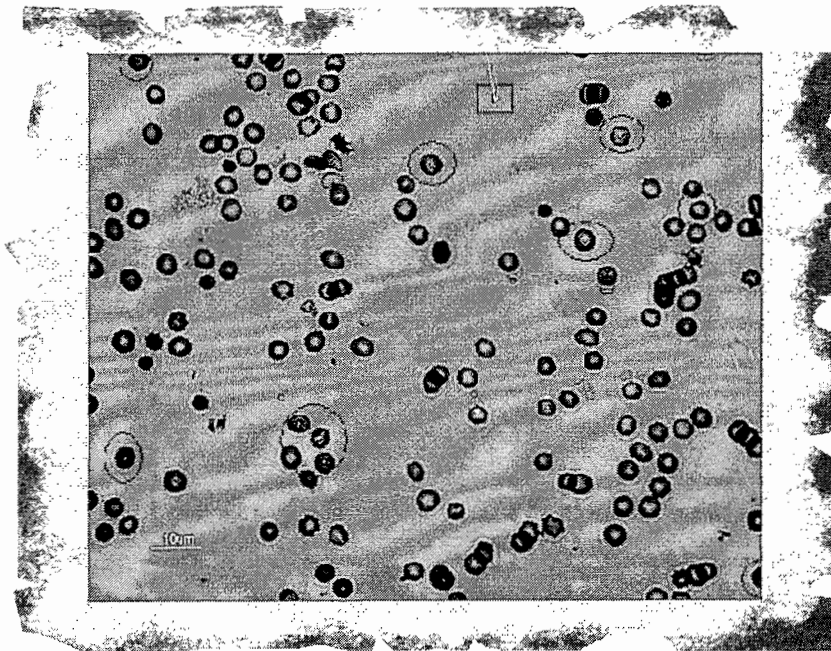


FIGURE 3.5: Light microscopic views of infected erythrocytes. When altering the focus setting parasites obtain a “halo” effect, assisting in the recognition of parasites.

The contents of the culture flask was transferred to a falcon tube and centrifuged at 2000rpm for 8 minutes. Hematocrit of the contents of the culture flask was determined as demonstrated in Equation 3.2:

$$\text{Hematocrit} = \frac{\text{Parasitised erythrocytes}}{\text{Total volume}} \times 100$$

Eq.3.2

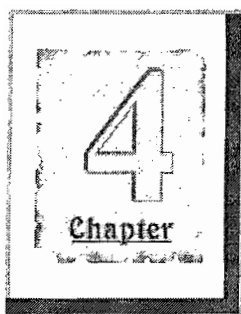
Parasitemia and hematocrit was adjusted to 0.1 % and 1.5 % respectively. A total volume of 200 μ l was transferred to three 48-well plates. The 200 μ l consisted of 180 μ l adjusted parasitised red blood cells and 20 μ l test compound in dilution. Well-plates were incubated and gassed with the special gas mixture for 5 minutes every 24 hours. Samples were frozen at -20°C, 48 hours, 96 hours and 144 hours apart.

3.8 STATISTICAL ANALYSIS

The plate reader (ELX 800™ Absorbance Microplate Reader with GEN5™ data analysis software) was applied in the assessment of the efficacy of the test compounds and reference formulations. After completion of the experiment on day ten, three ELISA assays were conducted on day 11, 12 and 13 (section 3.3.4.4). GraphPad®Prism 4.0 was used to present data as column graphs alongside each other for comparative purposes. Statistical comparisons were obtained through Statistica™ (StatSoft, Inc. (2008). STATISTICA (data analysis software system), version 8.0. www.statsoft.com) and GraphPad®Prism 4.0 and evaluated based on P-values. Test compounds (single agents as control and combinations of Pheroid™ vesicles and test compounds as experimental, referred to as Pheroid™ vesicles) were compared with Statistica™ and the effect of formulation, time, and concentration was investigated by means of a 3-way analysis of variance (ANOVA). *In vitro* tests results are expressed as Parasitemia (% of drug-free control) plotted against the macrolide antibiotic drug concentration (or log concentration) to produce a dose-response (concentration-effect) curve. EC₅₀ values were determined by means of non-linear regression. During non-linear dose-response curve fitting, the programme used (GraphPad®Prism 4.0) uses a standard method to compute the standard error and confidence interval for each parameter fit. The best fit of the different data sets (Control vs. Pheroid™ vesicles) was evaluated for statistical significant

difference using the extra sum-of-squares F test. The best fit value of the EC_{50} was chosen for comparison. The EC_{50} values was deemed significantly different when $P < 0.05$. A dose-response curve is typically sigmoid and can be illustrated through a number of parameters:

- minimum growth/uptake/production,
- maximum growth/uptake/production,
- the slope, and the
- EC_{50} concentration.



RESULTS AND DISCUSSION

4.1 INTRODUCTION

The malaria burden continues to be unacceptably high (Olumese, 2005). Research, in the field of malaria, is confined to pharmaceutical and non-pharmaceutical approaches with the investigation of artemisinin antimalarials and genetics receiving the highest attention. Research by itself will not reduce the mortality rate from malaria, though it may act as the fundamental foundation upon which public policies are based (Lewison & Srivastava, 2008). Even though there have been notable advances in malaria prevention, diagnosis, and treatment the surfacing of drug resistance and changes in the epidemiology of the infection continues to present new challenges (Stauffer & Kammat, 2003). Antibiotics that possess antimalarial activity may offer an interesting option for the treatment of multidrug-resistant *falciparum* malaria (Noedl *et al.*, 2001). In Chapter 4, results obtained through methods followed in Chapter 3 will be discussed and compared.

- Results from formulation processes are presented in terms of examples of confocal microscopy images and Malvern mastersizer results.
- The evaluation of the HRP II assay as drug efficacy assay is discussed.
- The results of the efficacy of erythromycin on *P. falciparum* will be assessed and compared to the efficacy of this test compound in combination with Pheroid™ vesicles.
- The results of the efficacy of azithromycin on *P. falciparum* will be assessed and compared to the efficacy of this test compound in combination with Pheroid™ vesicles.
- The effect of extended exposure and concentration, of both erythromycin and azithromycin, will be compared.
- Erythromycin and azithromycin will be compared in terms of EC₅₀ values.

4.2 PHEROID™ FORMULATION

The specific aim of this section was to obtain an acceptable formulation of Pheroids™ and test compounds to be applied in an antimalarial drug efficacy assay. Pheroid™ vesicles typically range between 0.5 - 1.5 µm in size and microsponges between 1.5 - 5 µm. Pheroids™ are polyphylic, enabling these carrier systems to entrap both lipid and water soluble molecules to a great extent (85 - 90 %) (Grobler, 2004). Erythromycin and azithromycin are both particularly insoluble in water and consequently the aim was to entrap these drugs in the oil phase of the Pheroid™ vesicles (4 %). Table 4.1 illustrates some of the physical-chemical properties of both test compounds.

TABLE 4.1: Physical-chemical properties of erythromycin and azithromycin (Drugbank, 2010).

	ERYTHROMYCIN	AZITHROMYCIN
Melting point	191°C	113-115°C
Predicted water solubility	4.59e-01 mg/ml	5.14e-01 mg/ml
Predicted LogP	2.37	3.03
pKa/Isoelectric Point	8.88	8.74

After the co-formulation of both erythromycin and azithromycin with Pheroid™ vesicles (4 %), particle sizes were evaluated by means of results obtained from the Malvern mastersizer. Results, as demonstrated in Table 4.2, proved the co-formulation of both test compounds with Pheroid™ vesicles unsuitable when comparing sizes obtained to that of the typical range of Pheroid™ vesicles which is 0.5 - 1.5 µm.

TABLE 4.2: Malvern mastersizer analysis of particle size (volume weighted data) of co-formulated test compound and Pheroid™ vesicles.

PARAMETERS	ERYTHROMYCIN	AZITHROMYCIN
D (v, 0.1)	1.750 µm	0.832 µm
D (v, 0.5)	72.758 µm	100.621 µm
D (v, 0.9)	161.856 µm	255.835 µm

D (v, 0.1); (v, 0.5); (v, 0.9) represents standard 'percentile' readings as obtained from the analysis. D (v, 0.1) is the size of the particle for which 10 % of the sample is smaller than this size, being 1.750 μm in the case of erythromycin and 0.832 μm in the case of azithromycin. D (v, 0.5) is also known as the mass median diameter (MMD) and is the size where 50 % of the sample is smaller and 50 % of the sample is larger than this particular size (72.758 μm for erythromycin and 100.621 μm for azithromycin). D (v, 0.9) represents the size of particle where 90 % of the sample is smaller than this size (161.856 μm in the case of erythromycin and 255.835 μm in the case of azithromycin). Based on the discussion above it is thus reasonable to assume that because D (v, 0.9) is larger than the typical size of Pheroid™ vesicles, neither formulation proved ideal. The span, representing the measurement of the width of the distribution can be calculated by means of equation 4.1 and is demonstrated for each test compound in Table 4.3. A larger span represents a wider spectrum.

$$\text{Span} = \frac{D(v, 0.9) - D(v, 0.1)}{D(v, 0.5)}$$

Eq 4.1

TABLE 4.3: Measurement of the width of the distribution (span) of Pheroid™ vesicles and test compounds.

PHEROID™ VESICLES	ERYTHROMYCIN	AZITHROMYCIN
7.132 μm	2.201 μm	2.534 μm

The large particle sizes indicate that the test compounds did not dissolve in the Pheroid™ vesicles during co-formulation. A saturated solution is a solution where equilibrium between undissolved and dissolved solute is established in a dissolution process. Aulton, (2002) defines the solubility of a substance as the amount of substance passing into a solution to establish equilibrium at constant temperature and pressure to ultimately produce a saturated solution. A supersaturated solution is unstable and the excess solute tends to precipitate readily (Aulton, 2002), as was the case with both test compounds. Based on results from particle analysis and the supersaturated nature of both formulations, it was decided to continue formulation on azithromycin, since formulation was going to be especially complicated in the case of this compound. Azithromycin was co-formulated with various strengths of Pheroid™ vesicles and Pheroid™ microsponges. The test compound was added

to the oil phase in each case and left to stir overnight on a hot plate. Heating the mixture to 41°C seemed to improve solubility. The NW was added after 12 hours. This compound proved especially hydrophobic, for when the NW was added azithromycin readily precipitated. Figure 4.1 (a) and (b) demonstrates light microscopic and CLSM images of the formation of crystals during co-formulation of azithromycin and Pheroid™ microsponges. Ultimately, calculations and volume adjustments were re-evaluated and it was decided to continue this study by entrapping test compounds into diluted Pheroid™ vesicles (4 %). This formulation proved promising due to the absence of crystal formation (Figure 4.1, c and d).

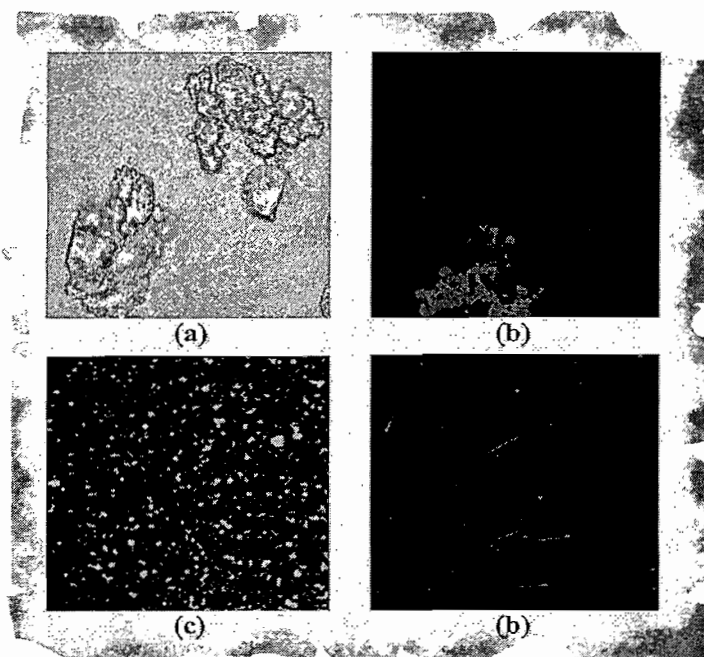


FIGURE 4.1: (a) Light microscopic image displaying crystal formation of azithromycin during entrapment into Pheroid™ vesicles. (b) CLSM image displaying crystal formation of azithromycin during entrapment into Pheroid™ microsponges. (c) CLSM image of Pheroid™ vesicles. (d) CLSM image illustrating an example of diluted test compound co-formulated with Pheroid™ vesicles.

In the formulation process, optimisation and planning are vital to obtain satisfactory data. Goals need to be clear, evaluation procedures need to be selected, initial compositions ought to be defined, products are then prepared and evaluated appropriately and the prospective formulation is modified and implemented. Though the scientist can control the variables to obtain acceptable product results, the absence of a mathematically or statistically meticulous approach to optimisation could lead to a temporarily satisfactory product, but not necessarily

the optimal formulation (Block, 1996). Because of the difficulty of formulation when co-formulating the macrolide antibiotics together with the Pheroid™ formulation, Pheroid™ vesicles were manufactured and drugs were entrapped into the Pheroid™ vesicles (4 %) in a 1:1 ratio on day three of the experimental procedure by adding the test compounds to a dilution of Pheroid™ vesicles (1:250). This approach allows the scientist better control due to the freedom of volume adjustments that are not possible during co-formulation. The formulation of entrapped test compounds in Pheroid™ vesicles allowed for a temporarily satisfactory product, but was in many ways still not ideal.

4.3 THE HRP II METHOD

The specific aim of this section was to evaluate the applicability of the HRP II ELISA in the drug efficacy assays. The sensitivity of the assay, the influence of Pheroid™ vesicles and the extended observation period was determined.

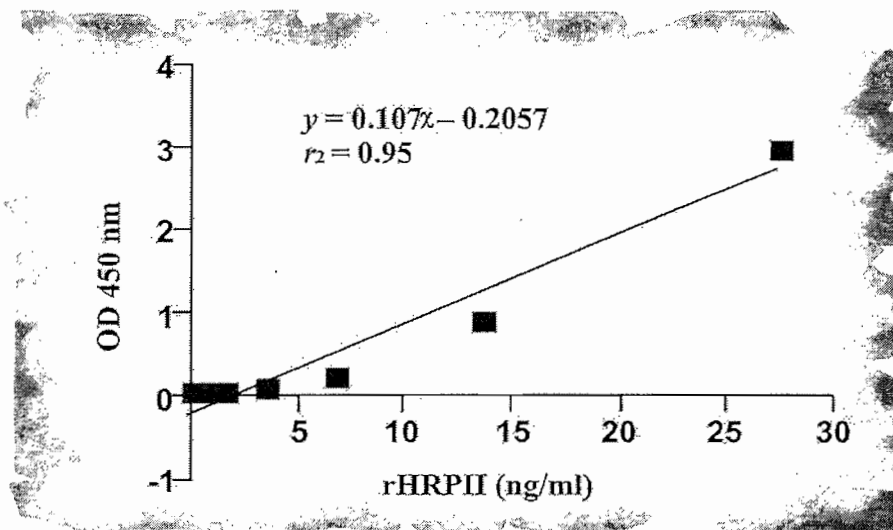


FIGURE 4.2: Shows a typical standard curve obtained with recombinant HRP II supplied by the manufacturer of the kit.

Figure 4.2 shows a typical standard curve obtained with recombinant HRP II supplied by the manufacturer of the kit. Linear regression analysis was performed to quantify the amount of HRP II protein in the samples. A sensitivity assay was performed to evaluate the applicability of this assay in this study. A serial dilution of infected erythrocytes was made and the standard ELISA performed as described in Section 3.5.2. The absorbance (OD 450 nm) values of the parasitemia dilutions are shown in Figure 4.3 A and the corresponding HRP II (ng/ml) values in Figure 4.3 B. These results indicate that the HRP II ELISA seems to be less

sensitive at low parasitemia levels. Similar results were obtained by Kifude *et al.*, (2008) who demonstrated that the lower level of quantification i.e. the point at which the analysis is feasible, was 3.19 ng/ml, corresponding to an OD of 0.05. This was confirmed in this assay where the linear part of the response can only be seen from 1 % parasitemia. Therefore, although the assay is recommended to be performed at parasitemia levels of 0.5 %, all further experiments were performed with 1 - 2 % parasitemia.

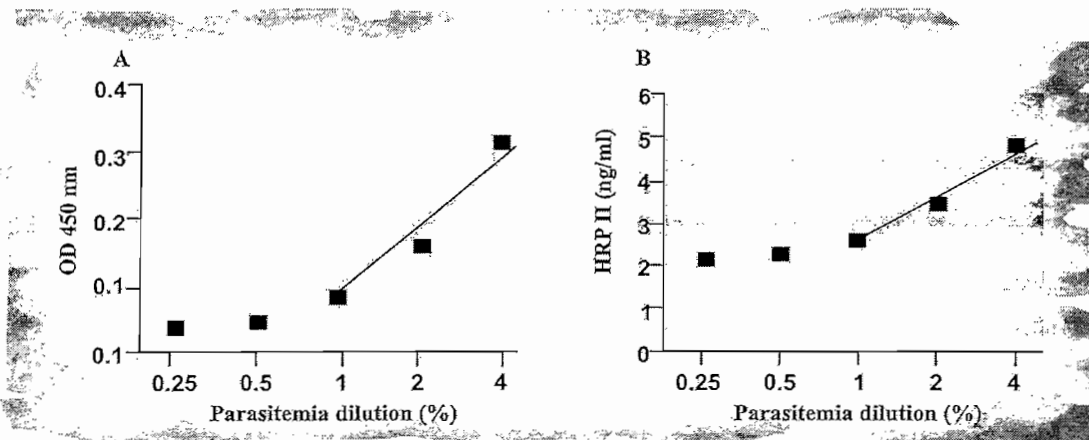


FIGURE 4.3: Sensitivity of the HRP II ELISA. A) Absorbance (OD 450 nm) values of the parasitemia dilution (%). B) Corresponding HRP II (ng/ml) levels of the parasitemia (%) dilution. The fitted regression line indicated the linear range of the graphs.

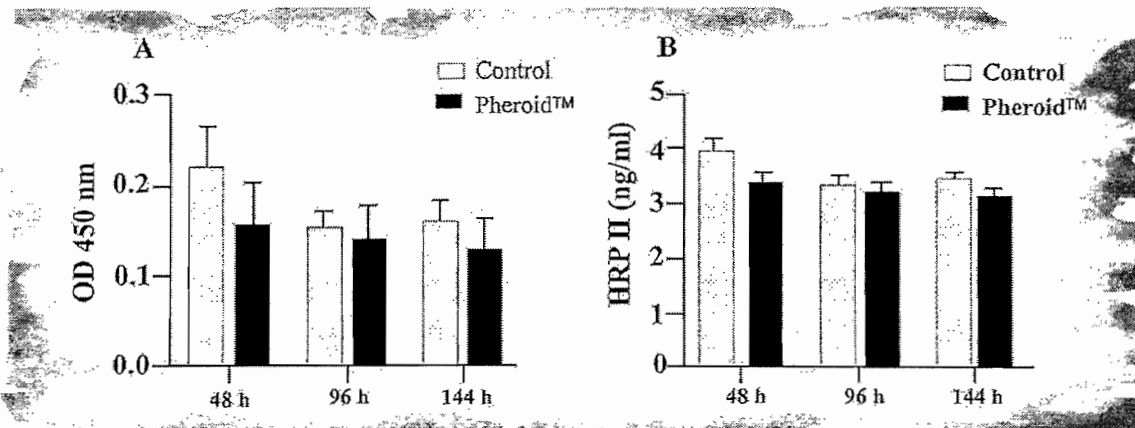


FIGURE 4.4: Effect of Pheroid™ vesicles and time on absorbance (A) and HRP II (B) levels.

To determine the applicability of the assay with Pheroid™ vesicles, infected erythrocytes (2 % parasitemia, 1.5 % hematocrit) were incubated with PBS (control) and diluted Pheroid™ (1:2500). The plates were incubated for 48 hours, 96 hours and 144 hours, frozen and analysed using the HRP II ELISA. Figure 4.4 A shows the absorbance (OD 450 nm) values obtained and Figure 4.4 B the corresponding HRPII (ng/ml) levels. These results obtained corresponded well with the results obtained with the parasitemia dilution. Similar absorbance and HRP II values were seen for the 2 % parasitemia dilution and the values obtained with this experiment. Pheroid™ vesicles resulted in lower absorbance and HRP II levels although this was not significant. Slightly lower absorbance and HRP II levels were also seen with the extended time periods, but this was also not significant. The life cycle of *P. falciparum* is completed within 48 hours. Thereafter infected erythrocytes burst to release more merozoites, each capable of invading a red blood cell. This phenomenon leads to increased parasite densities (Slabbert, 2008). Each well contained additional control samples in duplicate. The control group, consisting of parasitised erythrocytes, did however, not exude the expected increase in parasite density. Instead, the parasite density tended to stay the same or decrease to a small degree. The strain on which experiments were conducted has been maintained in culture for a couple of years, possibly contributing to parasitemia not increasing as expected. During cultivation of this strain it was evident that parasitemia levels maintained a percentage of between 1 - 3 %. Prolonged cultivation may also lead to changes in the parasite biology.

4.4 ERYTHROMYCIN

Erythromycin was dissolved in 2 ml of 95 % ethanol (EtOH) where after 98 ml PBS was added to obtain a 1600 μM solution. Two stock solutions were then prepared:

- a control stock solution (800 μM) consisting of test compound in PBS, and
- a Pheroid™ stock solution (800 μM), consisting of test compound entrapped in Pheroid™ vesicles.

CLSM images displayed complete dissolution of test compound due to the absence of crystal formation. The control stock solution was continuously diluted with PBS, and the Pheroid™ stock solution with Pheroid™ vesicles (1:250). A concentration range of 0 - 80 μM was tested throughout. ELISA-based assays were conducted, 48 hours apart. Results from the HRP II ELISA at optical density (OD) values at a wavelength of 450 nm are demonstrated in Table 4.4. Figure 4.5 illustrates data depicted in column graphs as mean absorbance values at

a wavelength of 450 nm on the y-axis, plotted against the various concentrations of the test compound (μM) on the x-axis.

TABLE 4.4: Absorbance values, mean and standard deviation (SD) measured in optical density at a wavelength of 450 nm following 48 hours of incubation.

48 h CONTROL : 450 nm (OD)								
μM							MEAN	SD
0	0.267	0.29	0.093	0.114	0.135	0.157	0.176	0.083
2.5	0.191	0.360	0.092	0.108	0.155	0.153	0.177	0.097
5	0.370	0.367	0.096	0.094	0.136	0.160	0.204	0.130
10	0.180	0.650	0.118	0.095	0.152	0.152	0.224	0.211
20	0.381	0.166	0.095	0.097	0.146	0.157	0.174	0.106
40	0.197	0.129	0.089	0.108	0.140	0.144	0.134	0.037
80	0.261	0.108	0.096	0.102	0.153	0.157	0.146	0.062
48 h PHEROID™ : 450 nm (OD)								
0	0.474	0.096	0.006	0.035	0.036	0.032	0.113	0.179
2.5	0.684	0.213	0.012	0.057	0.034	0.028	0.171	0.262
5	0.324	0.000	0.038	0.029	0.024	0.028	0.074	0.123
10	0.105	0.000	0.057	0.042	0.183	0.033	0.070	0.065
20	0.283	0.422	0.035	0.031	0.029	0.026	0.138	0.172
40	0.051	0.514	0.032	0.031	0.032	0.029	0.115	0.196
80	0.039	0.000	0.035	0.038	0.028	0.031	0.029	0.015

Figure 4.5 indicate that absorbance values of the control at 0 μM were 0.176 ± 0.083 . With increasing concentration, absorbance values for the control remained steady at 2.5, 5, 10 and 20 μM . At 40 and 80 μM absorbance values decreased slightly to 0.134 ± 0.037 and 0.146 ± 0.062 , respectively. Absorbance values of the Pheroid™ vesicles were consistently lower than the control group, although not concentration dependant. Absorbance values decreased to 0.074 ± 0.123 and 0.070 ± 0.065 at 5 and 10 μM (erythromycin in combination with Pheroid™ vesicles) and increased to 0.138 ± 0.172 and 0.115 ± 0.196 , respectively for 20 and 40 μM . At 80 μM the absorbance value was significantly lower than the drug-free control (0.029 ± 0.015).

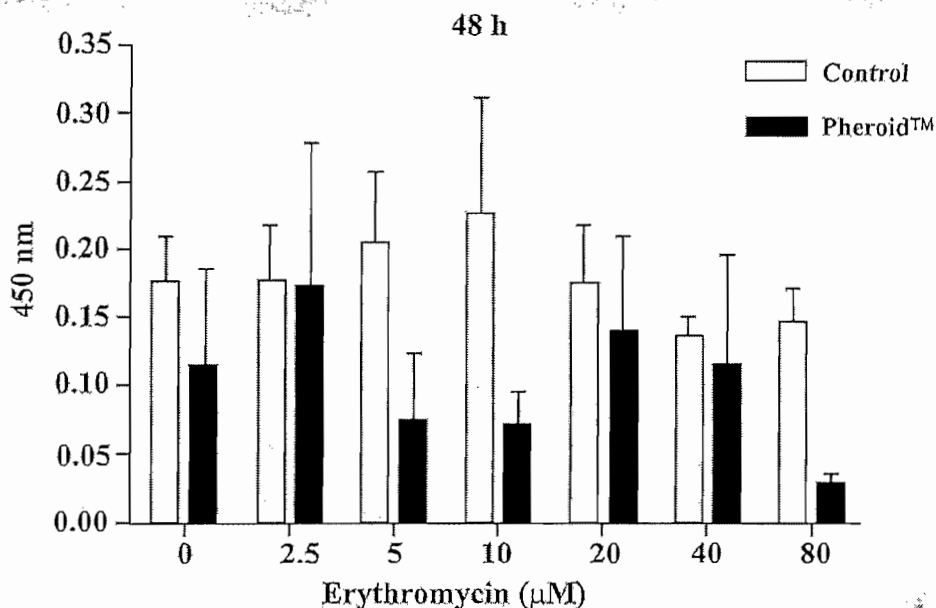


FIGURE 4.5: Mean \pm SD 450nm absorbance values of the control group and Pheroid™ group analysed at 48 hours (n = 6).

The results from the HRP II ELISA (absorbance) were expressed as the percentage parasite growth (relative to drug-free controls) plotted against erythromycin (log concentration) to give a dose-response curve. A typical representative dose-response curve is demonstrated in Figure 4.6. More resistant strains of parasites give a response that lies at the extreme value of the concentration range (high drug concentrations). Concentrations are usually used as two-fold serial dilutions which indicate that there are less measurements of drug effect at the high end of the range. The relatively large intervals between the highest drug concentration and the low drug concentrations indicate that the middle range of the inhibitory effect is often not seen. As an example, for one concentration there may be no drug effect and for the next dilution the maximum effect is measured. This was clearly observed in the dose-response curves for erythromycin (Addendum D) and in the column graph (Figure 4.5). It is therefore important to log transform (x-axis) and normalise the data relative to the drug-free controls (y-axis). The EC_{50} can then be calculated more accurately.

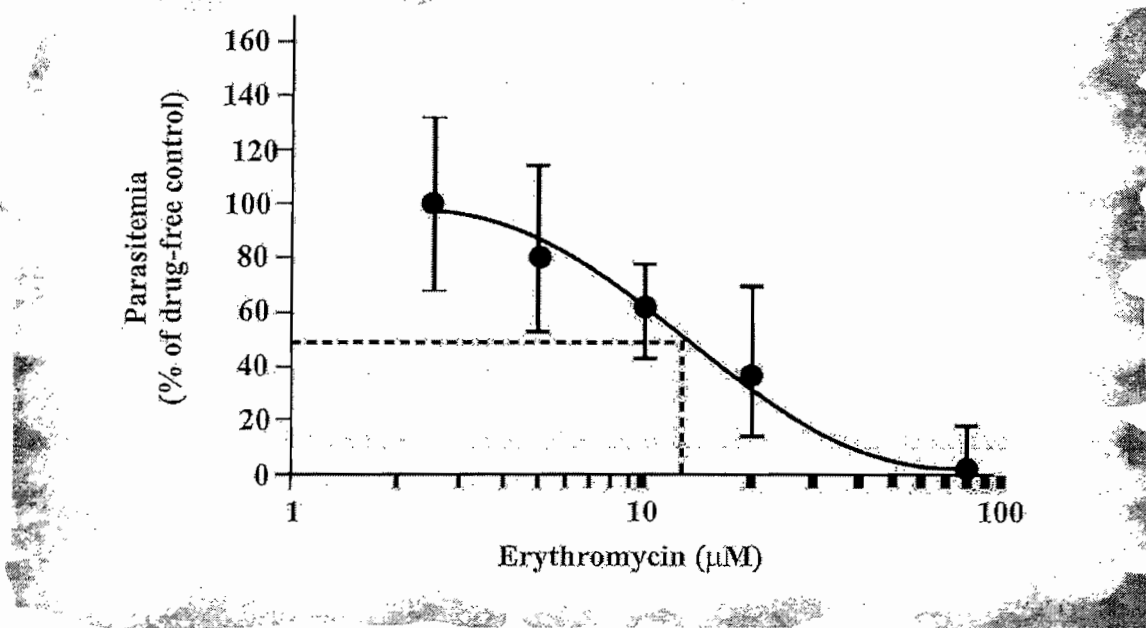


FIGURE 4.6: Representative dose response curve of erythromycin, illustrating the EC_{50} with the dotted line.

Figure 4.7 demonstrates a comparison of the EC_{50} values of the control and Pheroid™ vesicles with erythromycin, evaluated for 48 hours. The EC_{50} decreased from $13.85 \pm 0.34 \mu\text{M}$ for the control to $3.14 \pm 1.17 \mu\text{M}$ for the Pheroid™ vesicles.

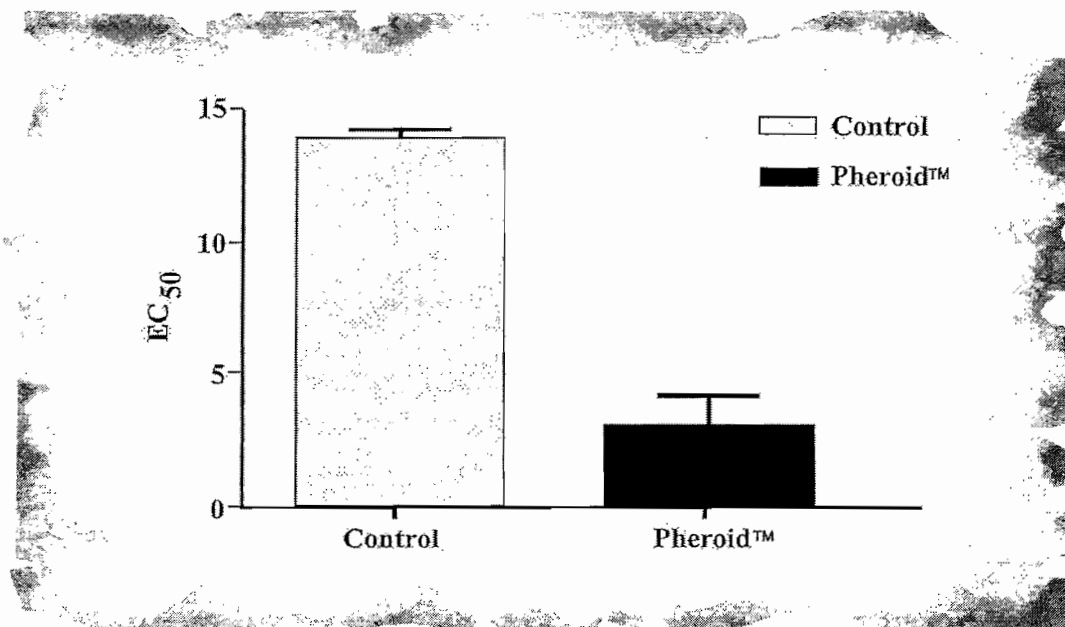


FIGURE 4.7: Comparison of EC_{50} values between the control and Pheroid™ after an incubation period of 48 hours.

A second well-plate was prepared during the experimental procedure, on the same day and under the exact circumstances as mentioned above. This plate was, however, incubated for a time period of 96 hours. Prolonged incubation generally has some major advantages, including better imitation of physiological conditions, they usually lead to a clearer increase in parasitemia and biomass, they allow for the testing of slow acting drugs, such as antibiotics, without changes in protocol, and the success rate will be considerably higher (Noedl, 2002). The aim was to determine the effect of prolonged exposure, since antibiotics are slow antiparasitodal agents. Results from the HRP II ELISA measured at 96 hours (OD values at 450 nm) are demonstrated in Table 4.5. Figure 4.8 illustrates data depicted in column graphs as mean \pm SD absorbance values, plotted against the various erythromycin concentrations.

TABLE 4.5: Absorbance values, mean and standard deviation (SD) measured in optical density at a wavelength of 450 nm following 96 hours of incubation.

96 h CONTROL : 450 nm (OD)								
μ M							MEAN	SD
0	0.119	0.122	0.107	0.121	0.198	0.173	0.140	0.037
2.5	0.121	0.167	0.101	0.311	0.239	0.188	0.188	0.078
5	0.157	0.157	0.123	0.108	0.179	0.171	0.149	0.028
10	0.165	0.125	0.111	0.095	0.172	0.174	0.140	0.034
20	0.166	0.137	0.104	0.102	0.199	0.178	0.148	0.040
40	0.158	0.130	0.114	0.112	0.226	0.164	0.151	0.043
80	0.123	0.131	0.105	0.102	0.224	0.197	0.147	0.051
96 h PHEROID : 450 nm (OD)								
0	0.009	0.016	0.073	0.048	0.281	0.022	0.075	0.104
2.5	0.081	0.020	0.055	0.058	0.032	0.005	0.042	0.028
5	0.042	0.038	0.098	0.051	0.008	0.009	0.041	0.033
10	0.035	0.007	0.050	0.050	0.013	0.011	0.028	0.020
20	0.038	0.012	0.045	0.051	0.011	0.036	0.032	0.017
40	0.007	0.008	0.053	0.063	0.033	0.009	0.029	0.025
80	0.022	0.047	0.055	0.056	0.015	0.077	0.045	0.023

Examining Figure 4.8, a decrease in 450 nm values can be observed for the control at 5 μM (0.149 ± 0.028). Following this incidence, levels seem to remain constant (~ 0.147). The Pheroid™ group displayed a more remarkable reduction in 450 nm values than the control group.

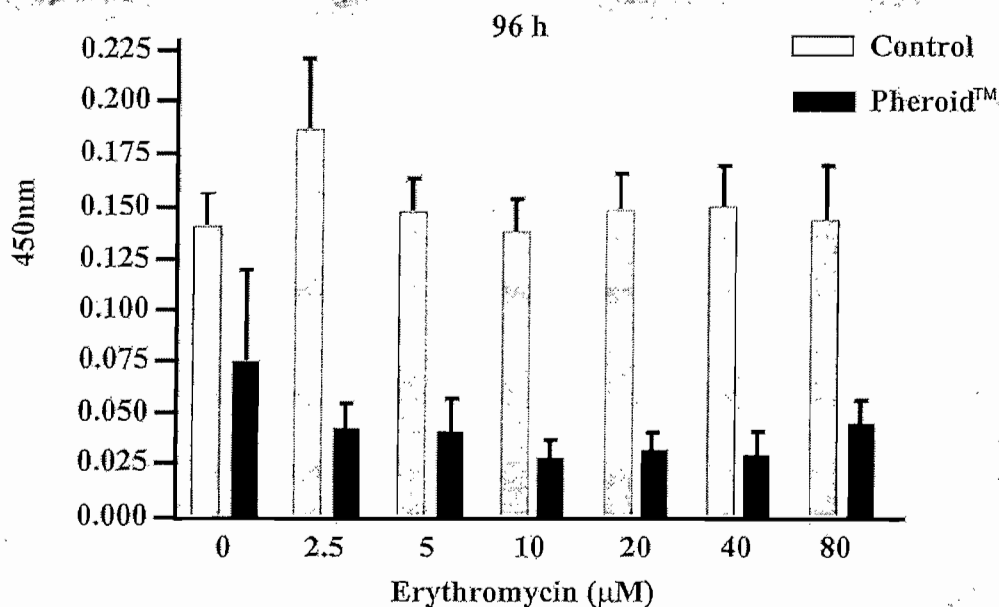


FIGURE 4.8: Mean \pm SD 450 nm absorbance values of the control and Pheroid™ group analysed at 96 hours (n = 6).

Compared to 48 hour incubation absorbance values (for both the control and Pheroid™) were similar to values obtained with 80 μM erythromycin. This indicates that the extended observation period of 96 hour did not have a marked effect on parasite levels. Similar results were seen with the dose-response curves (refer to addendum D). Figure 4.9 shows a comparison of the EC_{50} values. The EC_{50} value decreased from $12.61 \pm 0.50 \mu\text{M}$ for the control to $4.43 \pm 0.82 \mu\text{M}$ for Pheroid™ vesicles.

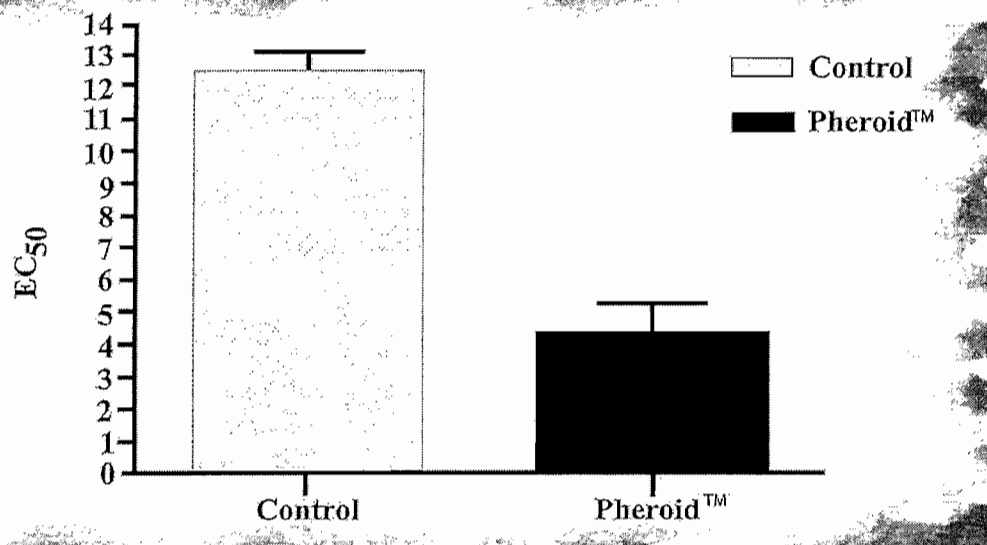


FIGURE 4.9: Comparison of EC₅₀ values between the control group and Pheroid™ group after an incubation period of 96 hours.

Results from the HRP II ELISA (OD values at 450 nm) measured at 144 hours are shown in Table 4.6 and illustrated in Figure 4.10. The results indicate that for the control, absorbance values remained constant at approximately similar values as 96 hours with slight increases at 2.5 and 5 μM . Similar results are seen with Pheroid™ vesicles with absorbance of 0.064 ± 0.043 and 0.057 ± 0.03 at 0 and 80 μM , respectively.

TABLE 4.6: Absorbance values, mean and standard deviation (SD) measured in optical density at a wavelength of 450 nm following 96 hours of incubation

144 h CONTROL : 450 nm (OD)								
μM							MEAN	SD
0	0.111	0.112	0.106	0.114	0.273	0.195	0.152	0.068
2.5	0.139	0.149	0.108	0.103	0.270	0.256	0.171	0.074
5	0.109	0.110	0.100	0.109	0.189	0.429	0.174	0.129
10	0.106	0.117	0.167	0.120	0.205	0.184	0.150	0.041
20	0.130	0.102	0.115	0.117	0.195	0.219	0.146	0.048
40	0.098	0.101	0.120	0.116	0.166	0.199	0.133	0.040
80	0.103	0.106	0.121	0.112	0.172	0.187	0.133	0.036

144 h PHEROID™: 450 nm (OD)								
0	0.070	0.030	0.069	0.105	0.000	0.112	0.064	0.043
2.5	0.036	0.037	0.076	0.071	0.067	0.053	0.057	0.017
5	0.042	0.054	0.056	0.049	0.041	0.060	0.050	0.008
10	0.032	0.114	0.085	0.056	0.057	0.083	0.071	0.029
20	0.029	0.022	0.095	0.077	0.085	0.104	0.069	0.035
40	0.027	0.018	0.116	0.066	0.057	0.064	0.058	0.035
80	0.029	0.023	0.061	0.073	0.052	0.105	0.057	0.030

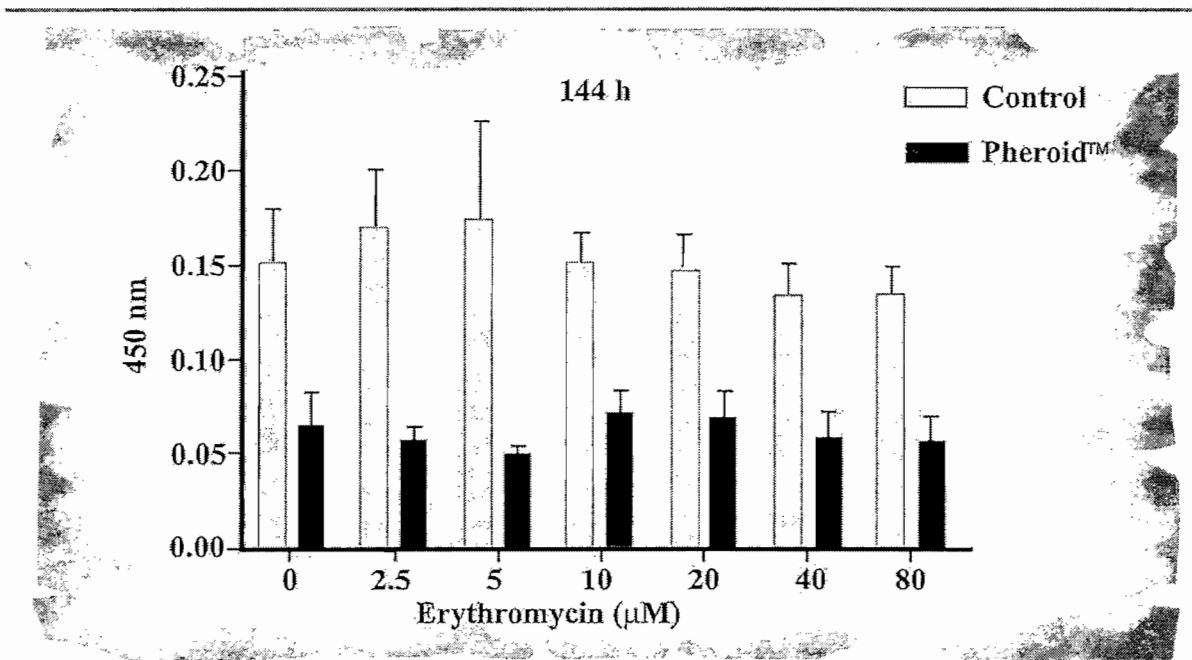


FIGURE 4.10: Mean ± SD 450 nm absorbance values of the control and Pheroid™ group analysed at 144 hours (n = 6).

Figure 4.11 shows the comparison of EC₅₀ values between control and Pheroid™ with erythromycin after 144 hours. The EC₅₀ value decreased from 12.69 ± 0.25 μM for the control to 4.09 ± 3.3 μM for Pheroid™ vesicles.

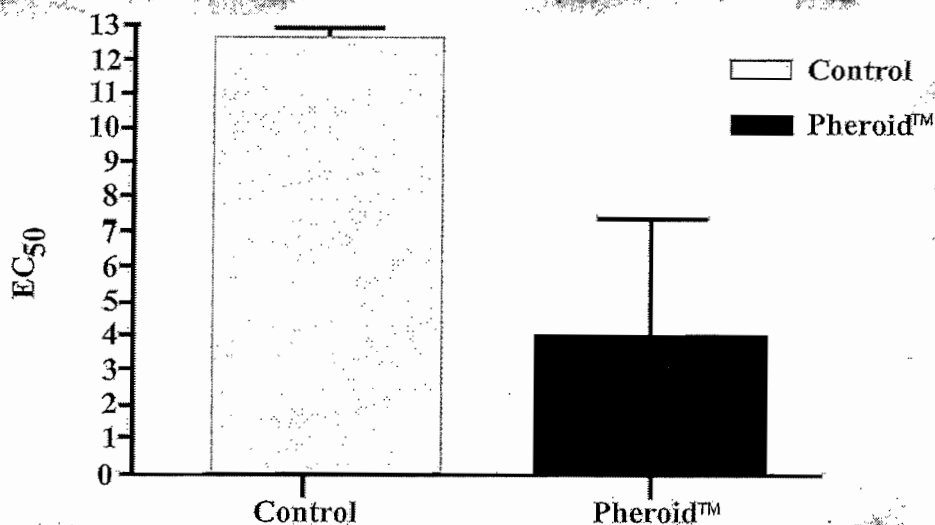


FIGURE 4.11: Comparison of EC50 values between the control group and Pheroid™ group after an incubation period of 144 hours.

4.5 STATISTICAL COMPARISON REGARDING ERYTHROMYCIN

Three variables between the control and Pheroid™ group were statistically compared (time vs. concentration vs. formulation) with the help of Statistica™ (addendum D). A P-value is a probability, with a value ranging from zero to one. If the P-value is small enough a conclusion can be drawn that the difference between sample means is unlikely to be due to chance. The threshold value (α) is usually set to $P = 0.05$ (an arbitrary value that has been widely adopted) (Motulsky, 2003). In the experiments with erythromycin, formulation and time proved statistically significant with P-values of respectively $P = 0.000000$ concerning the type of formulation and $P = 0.008268$ regarding the extended exposure period. Concentration, however, displayed no significant effect ($P = 0.628424$). This confirms the results obtained with the dose-response curves.

4.6 AZITHROMYCIN

Azithromycin was dissolved in 1ml dimethyl sulfoxide (DMSO) and diluted with PBS to obtain a 160 μM solution. Two stock solutions were prepared:

- an 80 μM Pheroid™ stock solution, consisting of diluted test compound and Pheroid™ vesicles, and
- an 80 μM control stock solution, consisting of sole diluted test compound.

Pheroid™ vesicles were diluted with NW (1:125 ratio), taking into consideration the double dilution when adding the azithromycin stock solution. The test compound was entrapped in the diluted Pheroid™ vesicles for 12 hours. CLSM images displayed no crystal formation, thereby concluding that the test compound has dissolved properly. The concentration range of 0–80 μM was tested over an extended incubation period of 144 hours. Samples were analysed at intervals of 48 hours. Results from the HRP II ELISA at optical density (OD) values at a wavelength of 450 nm are demonstrated in Table 4.7. Figure 4.12 illustrates data depicted in column graphs as mean absorbance values at a wavelength of 450 nm on the y-axis, plotted against the various concentrations of the test compound (μM) on the x-axis.

TABLE 4.7: Absorbance values, mean and standard deviation (SD) measured in optical density at a wavelength of 450 nm following 144 hours of incubation.

48 h CONTROL : 450 nm (OD)								
μM							MEAN	SD
0	0.713	0.136	0.193	0.168	0.188	0.136	0.256	0.225
0.25	0.171	0.212	0.216	0.173	0.180	0.200	0.192	0.020
0.5	0.224	0.198	0.161	0.208	0.152	0.170	0.186	0.029
1	0.169	0.197	0.161	0.175	0.167	0.290	0.193	0.049
2	0.152	0.204	0.144	0.153	0.245	0.187	0.181	0.039
4	0.183	0.193	0.143	0.169	0.229	0.174	0.182	0.029
8	0.187	0.170	0.150	0.170	0.153	0.196	0.171	0.018
48 h PHEROID™ : 450 nm (OD)								
0	0.069	0.055	0.118	0.045	0.115	0.049	0.075	0.033
0.25	0.072	0.648	0.040	0.046	0.050	0.064	0.153	0.243
0.5	0.066	0.047	0.036	0.058	0.048	0.042	0.049	0.011
1	0.089	0.000	0.035	0.029	0.050	0.043	0.041	0.029
2	0.072	0.000	0.033	0.026	0.055	0.057	0.040	0.026
4	0.000	0.021	0.031	0.031	4.064	0.050	0.700	1.648
8	0.060	0.032	0.033	0.042	0.047	0.072	0.048	0.016

The absorbance value of the control at a concentration of 0 μM is 0.256 ± 0.225 . This value represents the normal growth of parasites, as no drug concentration is added. Concentrations of 0.25 and 0.5 μM lowered 450 nm values to 0.192 ± 0.020 and 0.186 ± 0.029 , respectively. As indicated in Figure 4.12, 450 nm values remained steady between concentrations of 1 - 4 μM . At 8 μM , 450 nm values decreased to 0.171 ± 0.018 . The effect of the Pheroid™ vesicles following 48 hours of incubation did not seem to be concentration dependant, albeit lower than the control. The 450 nm values remained relatively constant. Figure 4.12 indicates a drastic increase in 450 nm values (0.700) at a concentration of 4 μM which was most likely the result of experimental error.

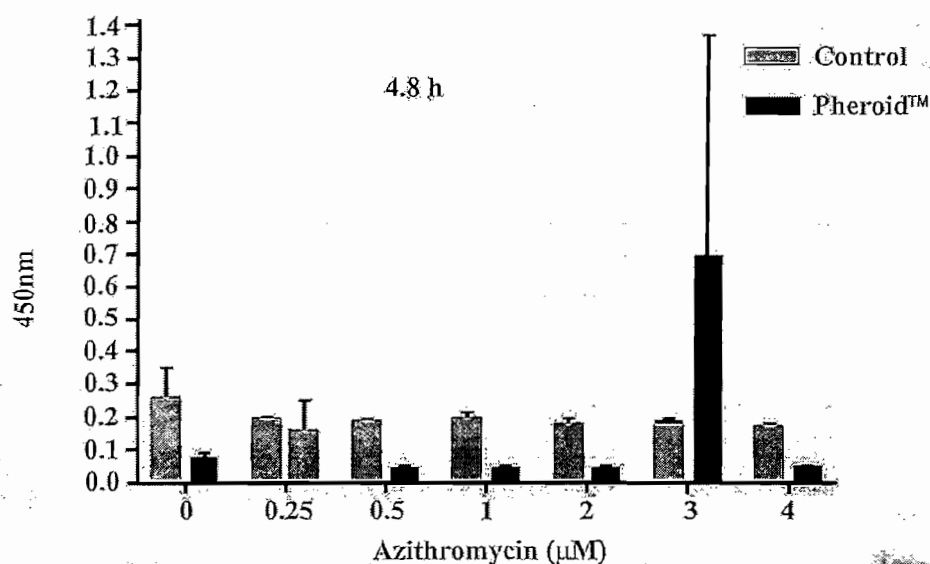


FIGURE 4.12: Mean \pm SD 450nm absorbance values of the control and Pheroid™ vesicles analysed at 48 hours (n = 6).

The EC_{50} values, as demonstrated in Figure 4.13, decreased from $0.39 \pm 0.25 \mu\text{M}$ for the control to $0.27 \pm 0.52 \mu\text{M}$ for the Pheroid™.

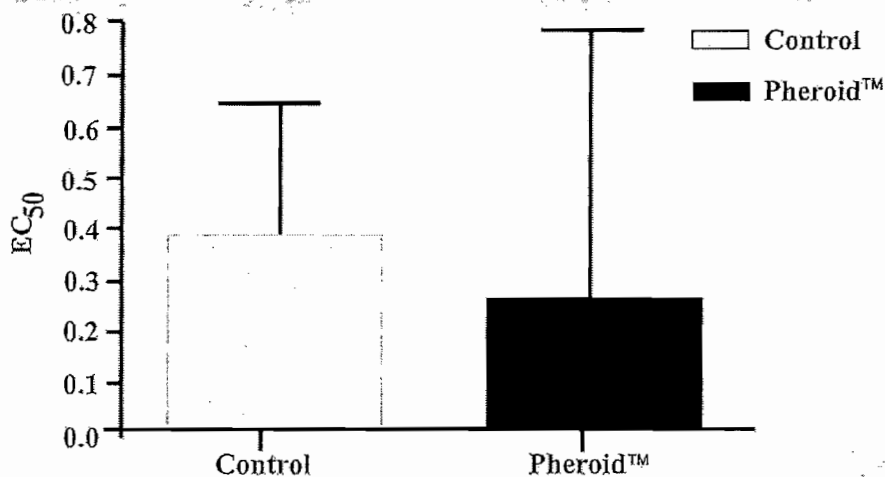


FIGURE 4.13: Comparison of EC₅₀ values between the control group and Pheroid™ group after an incubation period of 48 hours.

Results from the HRP II ELISA measured at 96 hours (OD values at 450 nm) are demonstrated in Table 4.8. Figure 4.14 illustrates data depicted in column graphs as mean ± SD absorbance values, plotted against the various azithromycin concentrations.

TABLE 4.8: Absorbance values, mean and standard deviation (SD) measured in optical density at a wavelength of 450 nm following 96 hours of incubation.

96 h CONTROL : 450 nm								
μM							MEAN	SD
0	0.113	0.112	0.173	0.169	0.210	0.213	0.165	0.045
0.25	0.128	0.117	0.164	0.167	0.212	0.280	0.178	0.060
0.5	0.134	0.111	0.166	0.167	0.198	0.215	0.165	0.039
1	0.166	0.140	0.180	0.335	0.195	0.212	0.205	0.068
2	0.126	0.152	0.175	0.139	0.182	0.211	0.164	0.031
4	0.240	0.171	0.168	0.185	0.214	0.403	0.230	0.089
8	0.169	0.167	0.150	0.179	0.188	0.218	0.179	0.023

96 h PHEROID™ : 450 nm								
0	0.038	0.063	0.184	0.029	0.062	0.034	0.068	0.058
0.25	0.047	0.052	0.029	0.027	0.067	0.059	0.047	0.016
0.5	0.092	0.049	0.004	0.011	0.060	0.064	0.047	0.034
1	0.048	0.051	0.018	0.009	0.038	0.059	0.037	0.020
2	0.035	0.043	0.013	0.000	0.065	0.043	0.033	0.023
4	0.059	0.048	0.031	0.031	0.061	0.053	0.047	0.013
8	0.055	0.037	0.066	0.029	0.054	0.048	0.048	0.013

Results obtained, following 96 hours of incubation, do not seem to vary from results following 48 hours of incubation. The 450 nm values of the control fluctuate, while Figure 4.14 illustrates a slight decrease in 450 nm values for the Pheroid™ vesicles between concentrations of 1 and 2 μM (0.037 ± 0.020 and 0.033 ± 0.023).

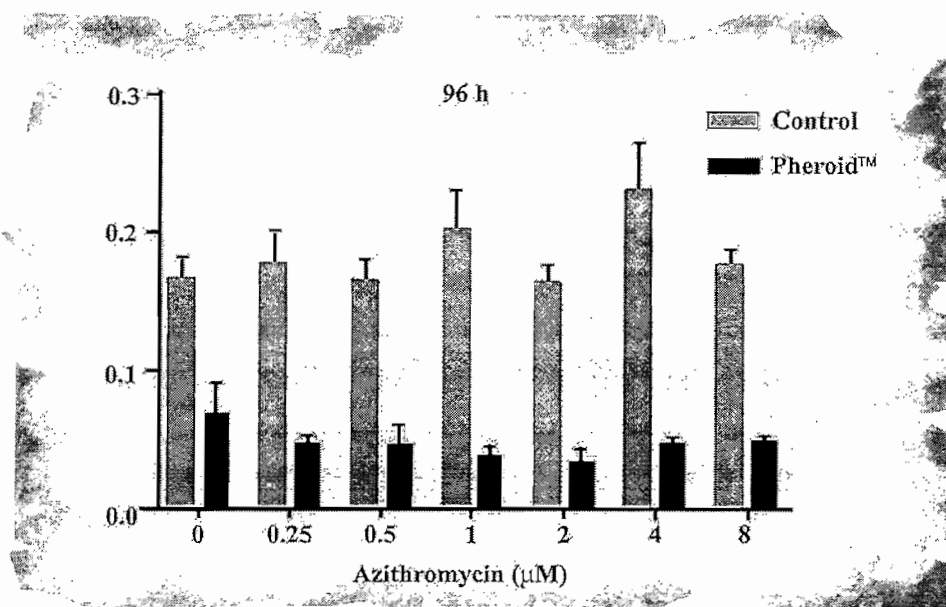


FIGURE 4.14: Mean \pm SD 450nm absorbance values of the control and Pheroid™ group analysed at 96 hours (n = 6).

In the comparison of EC_{50} values (Figure 4.15) between the control and Pheroid™ vesicles, following 96 hours of incubation, values decreased from $3.88 \pm 0.50 \mu\text{M}$ for the control to $0.40 \pm 0.60 \mu\text{M}$ for the Pheroid™ vesicles.

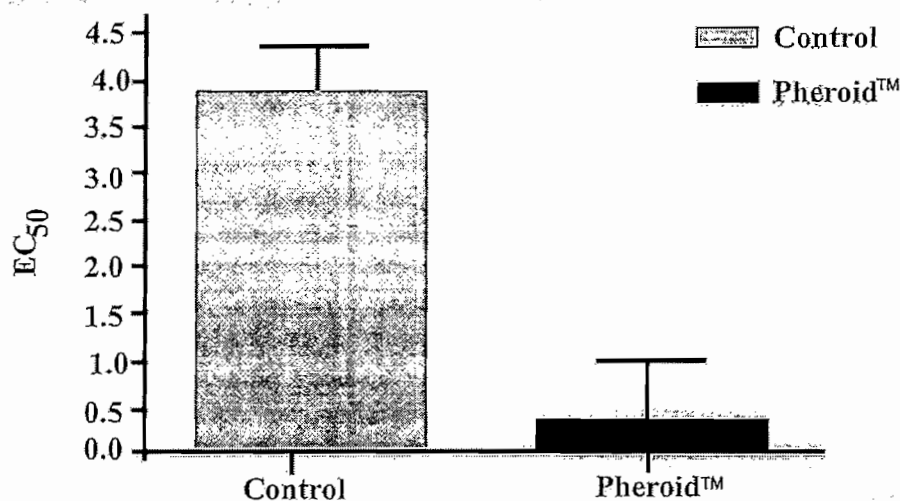


FIGURE 4.15: Comparison of EC₅₀ values between the control group and Pheroid™ group after an incubation period of 96 hours.

Results from Table 4.9 prove that the 450 nm values of the control fluctuate between concentrations of 0 - 1 μM, but Figure 4.16 indicates a decrease between 2 μM (0.193) and 8 μM (0.167). The Pheroid™ vesicles display a decrease in 450 nm values from concentrations 0 - 1 μM and seem to be concentration dependant. At concentrations 2 μM and 4 μM, 450 nm values increased to 0.053 ± 0.03 and 0.55 ± 0.037 , respectively. Values were slightly lower at 8 μM (0.047 ± 0.023).

TABLE 4.9: Absorbance values, mean and standard deviation (SD) measured in optical density at a wavelength of 450 nm following 144 hours of incubation.

144 h CONTROL : 450 nm								
μM							MEAN	SD
0	0.111	0.114	0.190	0.191	0.188	0.231	0.171	0.048
0.25	0.156	0.160	0.186	0.190	0.230	0.227	0.191	0.032
0.5	0.165	0.142	0.169	0.196	0.198	0.183	0.175	0.021
1	0.140	0.156	0.179	0.199	0.216	0.214	0.184	0.031
2	0.191	0.129	0.182	0.190	0.267	0.202	0.193	0.044
4	0.148	0.133	0.157	0.196	0.218	0.203	0.176	0.034
8	0.116	0.116	0.210	0.191	0.198	0.172	0.167	0.042

144 h PHEROID™ : 450 nm								
0	0.016	0.038	0.151	0.133	0.015	0.022	0.063	0.062
0.25	0.048	0.025	0.074	0.080	0.017	0.050	0.049	0.025
0.5	0.037	0.076	0.043	0.086	0.016	0.015	0.045	0.030
1	0.036	0.082	0.068	0.061	0.000	0.006	0.042	0.034
2	0.025	0.101	0.055	0.061	0.060	0.019	0.053	0.030
4	0.030	0.042	0.067	0.048	0.122	0.020	0.055	0.037
8	0.028	0.031	0.071	0.080	0.045	0.030	0.047	0.023

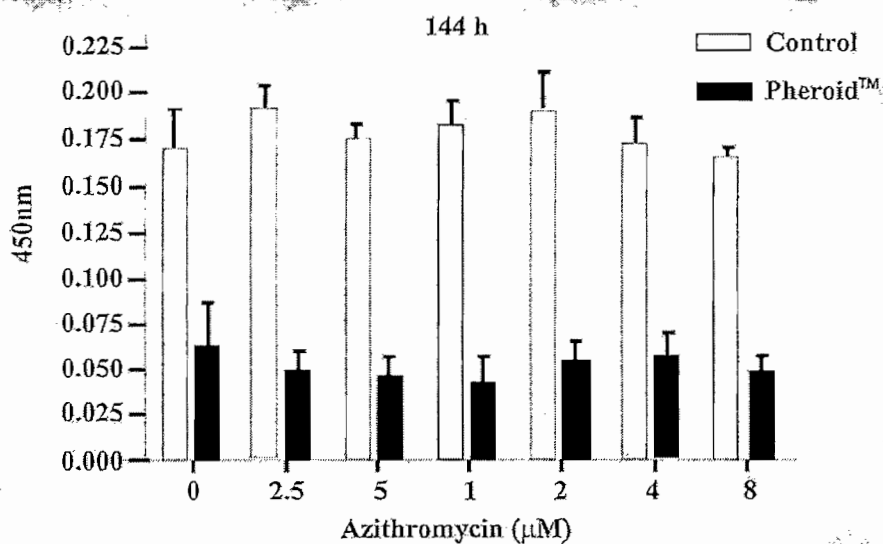


FIGURE 4.16: Mean ± SD 450 nm absorbance values of the control and Pheroid™ group analysed at 144 hours (n = 6).

Figure 4.17 demonstrates that the EC_{50} values decreased from 2.69 ± 0.43 for the control and 0.18 ± 0.21 for the Pheroid™ vesicles.

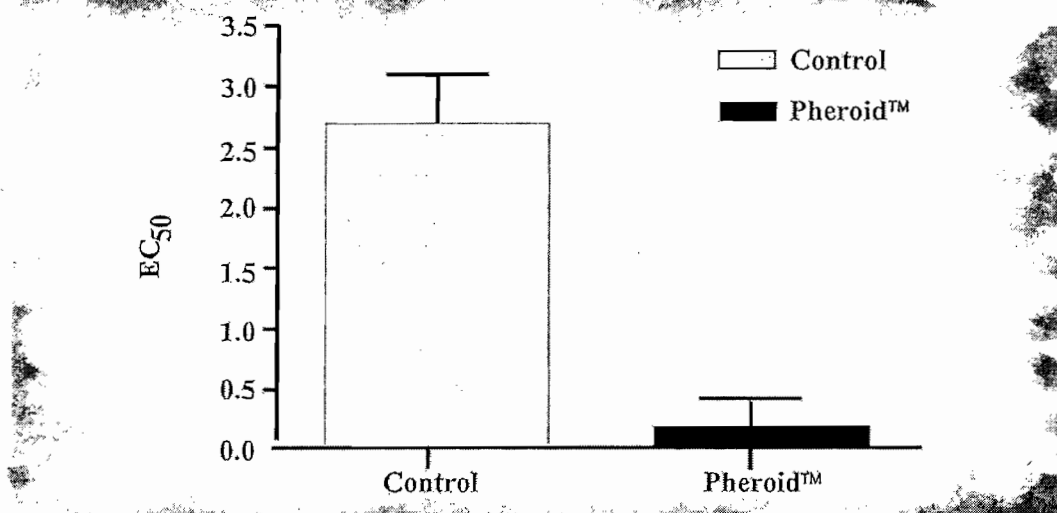


FIGURE 4.17: Comparison of EC₅₀ values between the control group and Pheroid™ group following an incubation period of 144 hours.

4.7 STATISTICAL COMPARISON REGARDING AZITHROMYCIN

In the comparison between the two groups (calculated with Statistica™) the type of formulation (control group vs. Pheroid™ group) proved to be statistically significant ($P = 0.002572$). Concentration deemed statistically insignificant ($P = 0.427731$), while the extended observation period did not deliver the expected results either ($P = 0.221941$).

4.8 COMPARISON BETWEEN TEST COMPOUNDS AND PROLONGED EXPOSURE

Table 4.10 illustrates the EC₅₀ values of the control and Pheroid™ of both test compounds presented in terms of mean ± SEM calculated with GraphPad®Prism 4.0. The P-value is also demonstrated.

TABLE 4.10: EC₅₀ values of the control and Pheroid™ of erythromycin and azithromycin formulations.

ERYTHROMYCIN	MEAN	SEM	P
Control 48 h	13.85	0.34	
Pheroid 48 h	3.14	1.17	0.7616
Control 96 h	12.61	0.50	
Pheroid 96 h	4.43	0.82	0.4028
Control 144 h	12.69	0.25	
Pheroid 144 h	4.09	3.30	0.4079
Azithromycin			
Control 48 h	0.39	0.25	
Pheroid 48 h	0.27	0.52	0.9992
Control 96 h	3.88	0.50	
Pheroid 96 h	0.40	0.60	0.3307
Control 144 h	2.69	0.43	
Pheroid 144 h	0.18	0.21	0.7291

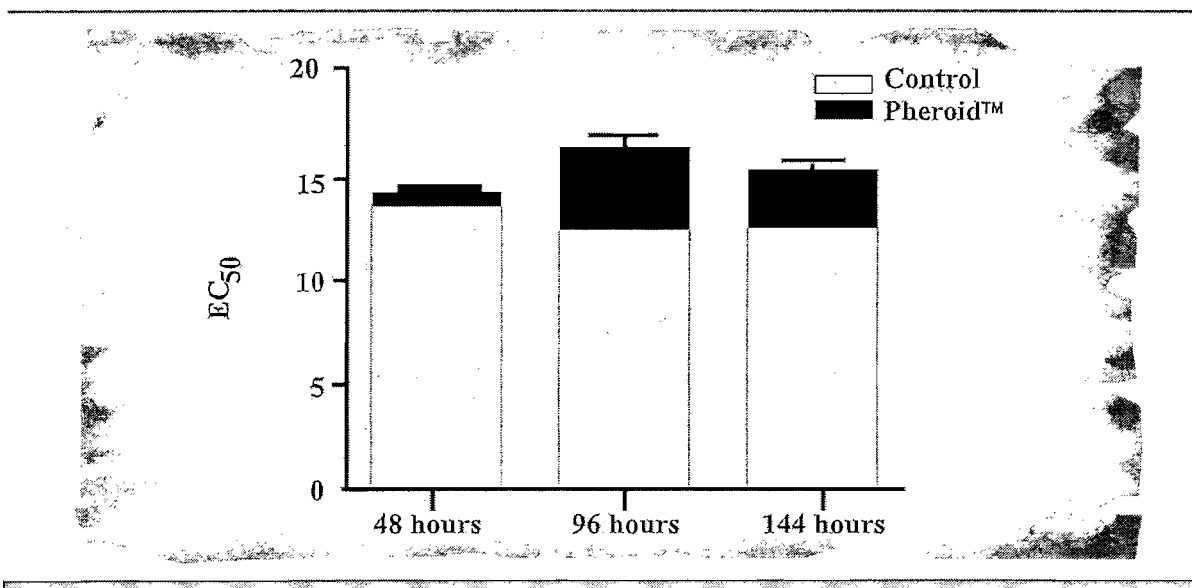


FIGURE 4.18: Comparison of the EC₅₀ values of the control of erythromycin and azithromycin at 48 hour intervals.

Figure 4.18 indicate that erythromycin demonstrated better antimalarial activity following 48 hours of incubation. At 144 hours the effect remained the same concluding that exposure need not exceed 96 hours. These results compare well to a study conducted by Yeo & Rieckman, (1994) which demonstrated a marked reduction in minimum inhibitory concentration, of azithromycin, between the first (48 hours) and second (96 hours) asexual erythrocytic cycles. Results demonstrate that erythromycin is a slow antiplasmodial agent and should be combined with a rapid acting antimalarial compound. Azithromycin, however, displayed fluctuating results and in this study prolonged exposure did not seem statistically significant.

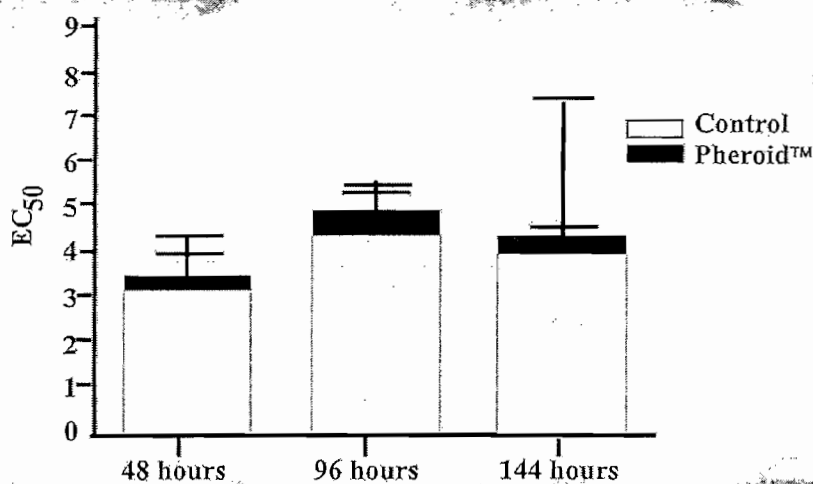


FIGURE 4.19: Comparison of the EC₅₀ values of the Pheroid™ vesicles of erythromycin and azithromycin at 48 hour intervals.

Neither of the test compounds exuded better antiplasmodial effects after prolonged exposure to infected erythrocytes in combination with Pheroid™ vesicles (Figure 4.19). These fluctuating results might be due to various factors such as experimental error and altered parasitic life cycle. Conditions, to which samples were exposed, could also influence experimental results. Samples were taken from the incubator every 48 hours and the remaining samples were gassed at room temperature and placed in the incubator for extended observation purposes. The estimation of relative potency in biological assay is very important in pharmaceutical and toxicological quality control. Government authorisation is required for inspection and control in cases where a pharmaceutical company is about to place a new drug on the market. One part of inspection is measuring a drugs' potency, relative to a known preparation (standard) as to estimate the relative potency of the new (or test) preparation to

the standard preparation. If the test preparation is as potent as the standard preparation, the relative potency is equal to 1 (Chen, 2006). In this study, the effective concentration of the Pheroid™ vesicles was compared to the effective concentration of the control.

$$\text{Relative potency} = \frac{\text{EC}_{50} \text{ Pheroid vesicles}}{\text{EC}_{50} \text{ Control}}$$

Eq 4.2

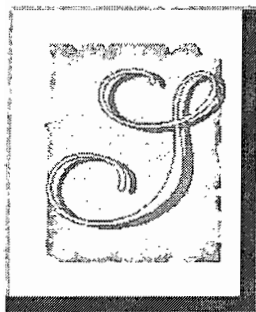
TABLE 4.11: The relative potency of erythromycin and azithromycin at 48 hours, 96 hours and 144 hours.

ERYTHROMYCIN	
48 hours	0.227
96 hours	0.351
144 hours	0.322
AZITHROMYCIN	
48 hours	0.692
96 hours	0.103
144 hours	0.067

4.9 CONCLUSION

The formulation of Pheroid™ vesicles with the macrolide antibiotics proved difficult. The large molecular masses of both test compounds and their poor solubility was some of the major obstacles that had to be overcome. In the end erythromycin and azithromycin was entrapped in Pheroid™ vesicles. It could, however, not be proved that the test compounds was completely entrapped in Pheroid™ vesicles and assumptions was made on the absence of crystal formation. Formulation was, nevertheless, not perfected and there are various aspects that could be improved upon in future studies. The HRP II ELISA method proved to be sensitive only at relatively high parasitemia levels (1 - 2 %). Pheroid™ vesicles and time did not appear to influence either absorbance of HRP II levels. Drug efficacy assays were conducted with both erythromycin and azithromycin over a period of 48 hours, 96 hours and 144 hours. The results indicated that Pheroid™ vesicles decreased the EC₅₀ values of both

test compounds, but not statistically significantly. The relative potency of erythromycin in combination with Pheroid™ vesicles proved to be higher after 96 and 144 hours. Azithromycin in combination with Pheroid™ vesicles had a high relative potency at 48 hours, but very low at 96 and 144 hours (Refer to Table 4.11).



SUMMARY AND FUTURE PROSPECTS

The development of chloroquine and the residual insecticide dichloro-diphenyl-trichloroethane (DDT) more than fifty years ago, enthused an international malaria eradication effort. This global campaign was successful in many endemic countries, especially outside Africa. Unfortunately, the surfacing and spread of chloroquine-resistant *Plasmodium* parasites and DDT-resistant *Anopheles* mosquitoes lead to a worldwide revival of the disease, including areas where malaria had been largely eradicated (Greenwood *et al.*, 2008). Risk groups, including young children and particularly pregnant woman, cause difficulties in the effective medicinal treatment of malaria. The pharmacokinetics, dosing regimen, efficacy and safety profile of currently available antimalarials in adults and children are well known, however this is not the case in pregnancy where limited data complicates prevention and treatment of the infection. *P. falciparum* infection is responsible for maternal, perinatal and neonatal morbidity. Sulphadoxine-pyrimethamine is currently the drug of choice in the intermittent preventive treatment regime in areas of medium or high malaria transmission, recommended by the WHO. The threat of mounting resistance to this combination is urgently pressing for the development of alternative drug regimens (Vallely *et al.*, 2007).

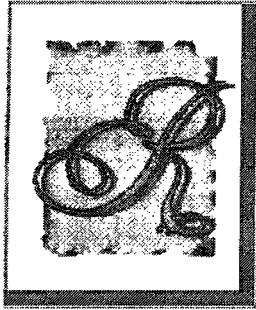
The Pheroid™ drug delivery system has demonstrated various advantages in previous studies (Grobler, 2004). The increased efficacy of active compounds when combined with Pheroids™ prompted the investigation into the possible enhancement of the antimalarial efficacy of the macrolide antibiotics, erythromycin and azithromycin. The formulation of the test compounds together with Pheroid™ vesicles proved challenging due to the large molecules of both macrolides and their poor solubility. Since formulation was not the main focus point of this study, the mere aim was to formulate a suitable working formula for conducting *in vitro* assays by means of the HRP II ELISA method. The entrapment of the test compounds into Pheroid™ vesicles delivered a temporarily satisfying product, though this formulation was in many ways still not ideal. Pheroids™ hold great potential for further investigation into formulation with erythromycin and azithromycin.

The HRP II ELISA method was evaluated and interpreted by means of the effective concentrations (EC_{50}) of test compounds. The EC_{50} for the Pheroid™ vesicles was consistently lower than the control, though decreases did not prove statistically significant. The effect of the Pheroid™ vesicles on the HRP II ELISA is not clear and also encourages further investigation. The results of the HRP II ELISA was depicted in terms of absorbance values measured in optical density at a wavelength of 450 nm. This study investigated the effects of a control and Pheroid™ formulation of test compounds, concentration and incubation time on a chloroquine-resistant *P. falciparum* strain (RSA 11). Formulation proved statistically significant for both of the macrolide antibiotics, indicating that the Pheroid™ increased the antimalarial efficacy of erythromycin and azithromycin. This positive result encourages further investigation. Neither of the formulations seemed to be concentration dependant and higher concentrations did not seem to alter the 450 nm values. Prolonged exposure was not statistically significant but various factors could have added to this outcome. The parasite strain has been maintained in culture for a number of years, and it is probable that the parasitic lifecycle was altered. Nevertheless, antibiotics remain slow acting antimalarials and a more extensive study is advisable.

Future prospects and recommendations that became evident through this study is:

- Extensive investigation into the optimal formulation of erythromycin and azithromycin. Microsponges, co-formulation and entrapment could be examined.
- Investigation into the effect the Pheroids™ exude on the HRP II ELISA assay.
- Examining a more extensive concentration range of erythromycin and azithromycin and prolonging exposure.
- Combination therapy. Most studies aim to combine antibiotics with rapid acting antimalarials. Pheroid™ technology further enhances efficacy, enabling lower dosages and consequently less serious side-effects.

Erythromycin and azithromycin have both demonstrated antimalarial properties. Their safety profile and relatively few side-effects encourage more extensive research into their applicability in malaria.



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ADDENDUM A :
ETHICS APPROVAL



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

Private Bag X6001, Potchefstroom
South Africa 2520

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Web: <http://www.nwu.ac.za>

**UNIT FOR DRUG RESEARCH AND
DEVELOPMENT**

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

Research Ethics Committee
North-West University
Potchefstroom Campus
Box116

15 April 2008

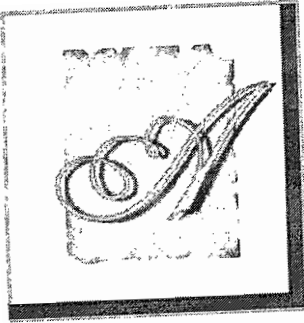
Dear Ms Botha

FINAL RESPONSE: Ethics application: NWU-0008-08-S5

The abovementioned application has reference.

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

**PROF. J. DU PLESSIS
DIRECTOR**



ADDENDUM B : CERTIFICATES OF ANALYSIS

LUPIN
CHEMICALS (THAILAND) LTD.

Factory Address : 309, Soi 6 Bangsoo Industrial Estate Sukhumvit Rd., Samutprakarn
Tel. : 324-0478-80, 324-0245, 7099657-3
Fax : 324-0892
Head Office : 8/17-18 4th Fl. Cathay House Sathorn Rd., Sathorn,
Bangkok, Bangkok 10500, Thailand
Tel : (662) 633-8101-3, (662) 236-6933
Fax : (662) 237-2654, 237-0871
E-mail : lupin@lupinchem.co.th

CERTIFICATE OF ANALYSIS DB/2535

Product : Erythromycin Estolate Ph.Eur.5th Ed 2005,sup 5.4. Q.C.report No. : 2870507
B.No. : EET/B,FP-043/07 Quantity : 500 kg.
Mfg.date : May 2007 Date of release : 12/05/07
Exp.date : April 2012 GMP certificate : by FDA-Thailand, No.1-2-07-17-06-00059

Sr.No.	Tests	Observations	Specifications
1	Characteristics	A white,crystalline powder.	White or almost white,crystalline powder.
2	Solubility	Soluble in alcohol and acetone	Practically insoluble in water, freely soluble in alcohol(96%), soluble in acetone.It is practically insoluble in dilute HCl.
3	Identification	Conform to std.IR	As per Ph.Eur.2005
4	Related substances (by HPLC)		
	: Impurity A	0.03%	3.0% (Max)
	: Impurity B	0.04%	3.0% (Max)
	: Impurity C	0.63%	3.0% (Max)
	: Impurity D	nil	3.0% (Max)
	: Impurity E	0.22%	3.0% (Max)
	: Impurity F	0.05%	3.0% (Max)
	: Impurity G	0.02%	3.0% (Max)
	: Any individual other impurity	Less than 0.2%	0.2% (Max)
	: Total Impurities	1.88%	6.0% (Max)
5	Free erythromycin	0.32%	6.0% (Max)
6	Dodecyl Sulphate	23.86%	23.0 - 25.6 % (anhydrous basis)
7	Water (by K.F)	2.94%	4.0 % (Max)
8	Sulphated ash	0.02%	0.5 % (Max)
9	: Assay (anhydrous basis)	652 I.U./mg.	610.0 I.U/mg. (Min)
	: Erythromycin estolate (anhydrous basis)	90.78%	86.0 - 102 % (anhydrous basis)
	: Erythromycin B (anh.basis)	0.72%	5.0 % (Max)(anhydrous basis)
	: Erythromycin C (anh.basis)	0.73%	5.0 % (Max)(anhydrous basis)
10	Other tests		
	: Residual solvents		
	: Butyl acetate	95.58 ppm	2000 ppm (Max)
	: Methylene chloride	0.85 ppm	500 ppm (Max)
	: Particle size	11.55%	10 - 15% > 125 microns
		88.45%	85 - 90% < 125 microns

Remarks : The product complies as per Ph.Eur.5th Edition-2005 / supplement 5.4.

We, Linaria Chemicals (Thailand) Ltd.,as a manufacturer of Erythromycin Estolate, hereby certify, that this batch has been produced by us, in full compliance with the GMP requirements of the local Regulatory authority (FDA-Thailand).

GMP Certificate no.1-2-07-17-03-00059, valid till December 24,2008, Issued by FDA-Thailand as per WHO Guidelines.

Analysed by
(Ms.Arporn Boonsang)
Q.C.-Chemist

Checked by
(Ms.Jantana Nanta)
Senior Chemist



member group

MAJ. A. S.



益飞(香港)有限公司

IFFECT CHEMPHAR (HK) COMPANY LIMITED

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

DB259C.

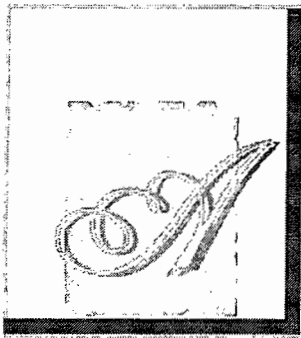
CERTIFICATE OF ANALYSIS

PRODUCT NAME:	AZITHROMYCIN DIHYDRATE	BATCH NO.:	IF-AZ-080506
QUANTITY:	2KGS	CAS NO.:	117772-70-0
MFG. DATE:	MAY.06, 2008	EXPIRY DATE:	MAY.05, 2012
ITEMS	SPECIFICATION	RESULTS	
DESCRIPTION	WHITE OR ALMOST CRYSTALLINE POWDER	CONFORMS	
SOLUBILITY	PARTICALLY INSOLUBLE IN WATER, FREELY SOLUBLE IN ETHANOL AND CHLOROFORM CHLORIDE	CONFORMS	
IDENTIFICATION	A. IR: SHOULD BE IDENTICAL TO THAT OF RS B. HPLC: SHOULD BE IDENTICAL TO THAT OF RS	CONFORMS	
CRYSTALLINE	MEETS THE REQUIREMENTS	CONFORMS	
SPECIFIC ROTATION	-45° ~ -49°	-47.4°	
PH	9.0 ~ 11.0	10.25	
WATER	4.0% ~ 5.0%	4.3%	
RESIDUE ON IGNITION	NOT MORE THAN 0.3%	CONFORMS	
HEAVY METALS	NOT MORE THAN 0.0025%	<0.0025%	
ACETONE	NOT MORE THAN 0.5%	0.21%	
ASSAY (ON DRIED BASIS)	945 µG/MG ~ 1030 µG/MG (C ₃₈ H ₇₂ N ₂ O ₁₂)	965 µG/MG	
CONCLUSION:	THE RESULTS CONFORM TO THE STANDARD OF USP26.		

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

STORAGE: PRESERVE IN WELL-CLOSED, LIGHT-RESISTANT AND TIGHT CONTAINERS.
 STORE IN COOL & DRY PLACE.

Iffect Chemphar (HK) Company Limited



ADDENDUM C :

MALVERN MASTERSIZER RESULT ANALYSIS REPORTS



MASTERSIZER



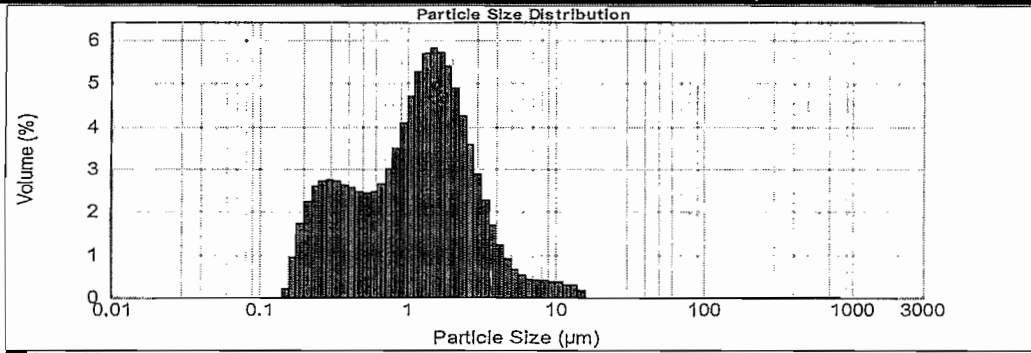
Result Analysis Report

Sample Name: Pheroid sponges skoon	SOP Name: Pheroid vesicles using Hydro 2000 MU	Measured: 03 June 2009 06:52:27 PM
Sample Source & type: microsponges	Measured by: Anel	Analysed: 03 June 2009 06:52:28 PM
Sample bulk lot ref: S09013	Result Source: Measurement	

Particle Name: Oleic Acid	Accessory Name: Hydro 2000MU (A)	Analysis model: General purpose	Sensitivity: Enhanced
Particle RI: 1.458	Absorption: 0.1	Size range: 0.020 to 2000.000 um	Obscuration: 14.22 %
Dispersant Name: Water	Dispersant RI: 1.330	Weighted Residual: 6.617 %	Result Emulation: Off

Concentration: 0.0036 %Vol	Span : 2.381	Uniformity: 0.854	Result units: Volume
Specific Surface Area: 8.41 m ² /g	Surface Weighted Mean D[3,2]: 0.714 um	Vol. Weighted Mean D[4,3]: 1.645 um	

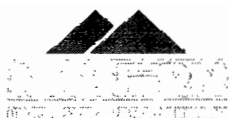
d(0.1): 0.278 um d(0.5): 1.220 um d(0.9): 3.183 um



Pheroid sponges skoon, 03 June 2009 06:52:27 PM

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.20	1.002	4.72	7.090	0.41	50.238	0.00	355.656	0.00
0.022	0.00	0.159	0.84	1.125	5.29	7.992	0.39	58.368	0.00	390.052	0.00
0.025	0.00	0.178	1.72	1.262	8.69	8.934	0.38	63.246	0.00	447.744	0.00
0.028	0.00	0.200	2.25	1.416	5.84	10.024	0.36	70.963	0.00	502.377	0.00
0.032	0.00	0.224	2.58	1.589	5.74	11.247	0.31	78.621	0.00	563.677	0.00
0.036	0.00	0.252	2.73	1.783	5.41	12.619	0.30	88.337	0.00	632.456	0.00
0.040	0.00	0.283	2.75	2.000	4.89	14.158	0.17	100.237	0.00	709.627	0.00
0.045	0.00	0.317	2.70	2.244	4.28	15.887	0.00	112.468	0.00	795.214	0.00
0.050	0.00	0.356	2.63	2.519	3.58	17.825	0.00	126.191	0.00	893.367	0.00
0.056	0.00	0.399	2.54	2.825	2.89	20.000	0.00	141.585	0.00	1002.374	0.00
0.063	0.00	0.448	2.46	3.170	2.28	22.440	0.00	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	2.42	3.557	1.70	25.179	0.00	178.250	0.00	1261.918	0.00
0.080	0.00	0.564	2.47	3.991	1.25	28.251	0.00	200.000	0.00	1416.892	0.00
0.089	0.00	0.632	2.85	4.477	0.81	31.698	0.00	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	2.99	5.024	0.67	35.596	0.00	251.785	0.00	1782.602	0.00
0.112	0.00	0.796	3.48	5.637	0.52	39.905	0.00	282.508	0.00	2000.000	0.00
0.126	0.00	0.893	3.48	6.325	0.46	44.774	0.00	316.879	0.00		
0.142	0.00	1.002	4.09	7.096		50.238	0.00	355.656	0.00		

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



MASTERSIZER



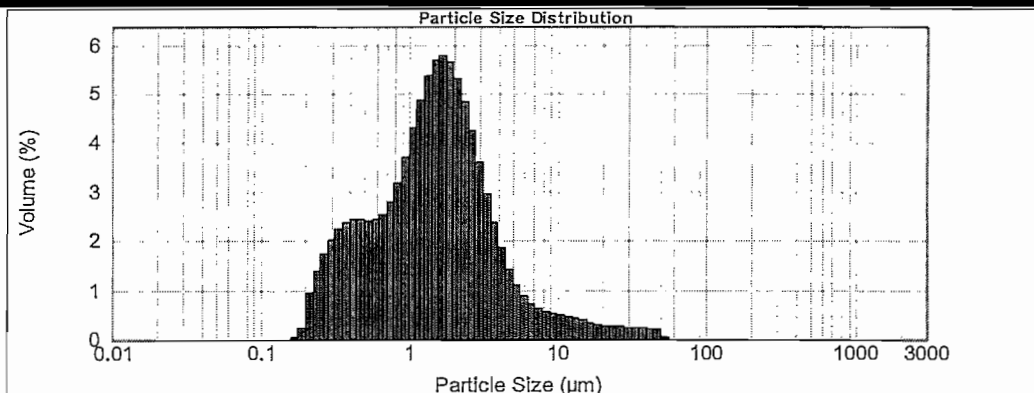
Result Analysis Report

Sample Name: Pheroid vesicles
SOP Name: Pheroid vesicles using Hydro 2000 MU
Measured: 24 April 2009 09:22:52 AM
Sample Source & type: V09010
Measured by: Rw
Analysed: 24 April 2009 09:22:53 AM
Sample bulk lot ref:
Result Source: Measurement

Particle Name: Oleic Acid
Accessory Name: Hydro 2000MU (A)
Analysis model: General purpose
Sensitivity: Enhanced
Particle RI: 1.458
Absorption: 0.1
Size range: 0.020 to 2000.000 um
Obscuration: 16.67 %
Dispersant Name: Water
Dispersant RI: 1.330
Weighted Residual: 5.510 %
Result Emulation: Off

Concentration: 0.0047 %Vol
Span : 2.807
Uniformity: 1.3
Result units: Volume
Specific Surface Area: 6.34 m²/g
Surface Weighted Mean D[3,2]: 0.947 um
Vol. Weighted Mean D[4,3]: 2.728 um

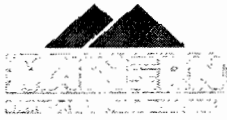
d(0.1): 0.381 um d(0.5): 1.493 um d(0.9): 4.571 um



Pheroid vesicles, 24 April 2009 09:22:52 AM

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.00	1.002	4.28	7.096	0.62	50.238	0.04	355.656	0.00
0.022	0.00	0.159	0.05	1.125	4.88	7.962	0.56	56.368	0.00	399.052	0.00
0.025	0.00	0.178	0.23	1.262	5.38	8.934	0.53	63.246	0.00	447.744	0.00
0.028	0.00	0.200	0.94	1.416	5.70	10.024	0.49	70.963	0.00	502.377	0.00
0.032	0.00	0.224	1.39	1.589	5.80	11.247	0.46	79.621	0.00	563.677	0.00
0.036	0.00	0.252	1.73	1.783	5.66	12.619	0.42	89.337	0.00	632.456	0.00
0.040	0.00	0.283	2.04	2.000	5.31	14.159	0.38	100.237	0.00	709.627	0.00
0.045	0.00	0.317	2.24	2.244	4.82	15.887	0.34	112.468	0.00	796.214	0.00
0.050	0.00	0.356	2.37	2.518	4.23	17.825	0.31	126.191	0.00	893.367	0.00
0.056	0.00	0.399	2.43	2.825	3.59	20.000	0.29	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	2.43	3.170	2.96	22.440	0.27	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	2.41	3.557	2.37	25.179	0.26	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	2.43	3.991	1.86	28.251	0.25	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	2.54	4.477	1.43	31.698	0.25	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	2.78	5.024	1.11	35.666	0.23	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	3.16	5.637	0.87	39.905	0.21	282.508	0.00	2000.000	0.00
0.126	0.00	0.893	3.68	6.325	0.72	44.774	0.22	316.975	0.00		
0.142	0.00	1.002		7.096		50.238		355.656	0.00		

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



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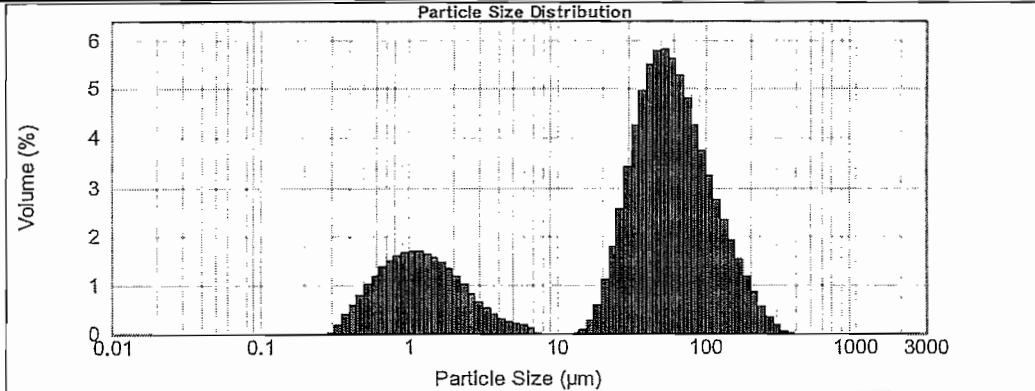
Result Analysis Report

Sample Name: Pheroid vesicles
 SOP Name: Pheroid vesicles using Hydro 2000 MU
 Measured: 15 May 2009 11:03:38 AM
 Sample Source & type: V09017a
 Measured by: Liezl-Marié
 Analysed: 15 May 2009 11:03:39 AM
 Sample bulk lot ref: 4% EFA's
 Result Source: Measurement

Particle Name: Oleic Acid
 Accessory Name: Hydro 2000MU (A)
 Analysis model: General purpose
 Sensitivity: Enhanced
 Particle RI: 1.458
 Absorption: 0.1
 Size range: 0.020 to 2000.000 um
 Obscuration: 14.77 %
 Dispersant Name: Water
 Dispersant RI: 1.330
 Weighted Residual: 1.551 %
 Result Emulation: Off

Concentration: 0.0133 %Vol
 Span: 2.654
 Uniformity: 0.823
 Result units: Volume
 Specific Surface Area: 1.59 m²/g
 Surface Weighted Mean D[3,2]: 3.778 um
 Vol. Weighted Mean D[4,3]: 54.234 um

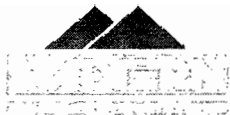
d(0.1): 0.985 um d(0.5): 44.950 um d(0.9): 120.294 um



Pheroid vesicles, 15 May 2009 11:03:38 AM

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.00	1.002	1.68	7.096	0.03	50.238	5.84	355.656	0.01
0.022	0.00	0.159	0.00	1.125	1.67	7.962	0.00	56.368	5.64	399.052	0.00
0.025	0.00	0.178	0.00	1.262	1.63	8.934	0.00	63.246	5.27	447.744	0.00
0.028	0.00	0.200	0.00	1.416	1.63	10.024	0.00	70.963	5.27	502.377	0.00
0.032	0.00	0.224	0.00	1.589	1.55	11.247	0.00	79.621	4.79	563.577	0.00
0.036	0.00	0.252	0.00	1.783	1.45	12.619	0.00	89.337	4.26	632.456	0.00
0.040	0.00	0.283	0.01	2.000	1.32	14.159	0.00	100.237	3.72	709.627	0.00
0.045	0.00	0.317	0.19	2.244	1.17	15.887	0.00	112.468	3.22	796.214	0.00
0.050	0.00	0.356	0.39	2.518	1.00	17.825	0.26	126.191	2.75	893.367	0.00
0.056	0.00	0.399	0.59	2.825	0.83	20.000	0.61	141.589	2.32	1002.374	0.00
0.063	0.00	0.448	0.80	3.170	0.67	22.440	1.11	158.866	1.91	1124.683	0.00
0.071	0.00	0.502	1.00	3.557	0.53	25.179	1.78	178.250	1.53	1261.915	0.00
0.080	0.00	0.564	1.19	3.991	0.41	28.251	2.56	200.000	1.17	1415.892	0.00
0.089	0.00	0.632	1.35	4.477	0.32	31.699	3.42	224.404	0.85	1588.856	0.00
0.100	0.00	0.710	1.49	5.024	0.26	35.566	4.25	251.785	0.57	1782.502	0.00
0.112	0.00	0.796	1.59	5.637	0.21	39.905	4.97	282.508	0.34	2000.000	0.00
0.126	0.00	0.893	1.66	6.325	0.17	44.774	5.51	316.979	0.17		
0.142	0.00	1.002	1.66	7.096	0.13	50.238	5.80	355.656	0.04		

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



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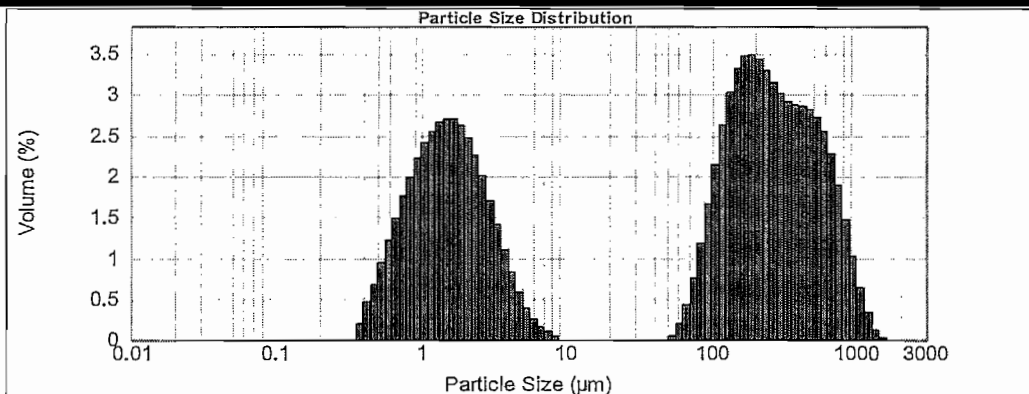
Result Analysis Report

Sample Name: Pheroid vesicles
SOP Name: Pheroid vesicles using Hydro 2000 MU
Measured: 15 May 2009 11:11:41 AM
Sample Source & type: vo9017b
Measured by: Liezl-Marié
Analysed: 15 May 2009 11:11:42 AM
Sample bulk lot ref: 8% epa
Result Source: Measurement

Particle Name: Oleic Acid
Accessory Name: Hydro 2000MU (A)
Analysis model: General purpose
Sensitivity: Enhanced
Particle RI: 1.458
Absorption: 0.1
Size range: 0.020 to 2000.000 μm
Obscuration: 24.95 %
Dispersant Name: Water
Dispersant RI: 1.330
Weighted Residual: 2.314 %
Result Emulation: Off

Concentration: 0.0176 %Vol
Span : 4.389
Uniformity: 1.4
Result units: Volume
Specific Surface Area: 1.94 m^2/g
Surface Weighted Mean D[3,2]: 3.086 μm
Vol. Weighted Mean D[4,3]: 204.395 μm

d(0.1): 0.952 μm d(0.5): 130.344 μm d(0.9): 573.039 μm



Pheroid vesicles, 15 May 2009 11:11:41 AM

Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %	Size (μm)	Volume In %
0.020	0.00	0.142	0.00	1.002	2.41	7.096	0.11	50.238	0.05	355.656	2.88
0.022	0.00	0.159	0.00	1.125	2.56	7.962	0.04	56.368	0.21	399.052	2.85
0.025	0.00	0.178	0.00	1.262	2.66	8.934	0.00	63.246	0.44	447.744	2.82
0.028	0.00	0.200	0.00	1.416	2.71	10.024	0.00	70.963	0.77	502.377	2.73
0.032	0.00	0.224	0.00	1.589	2.70	11.247	0.00	79.621	1.19	563.677	2.56
0.036	0.00	0.252	0.00	1.783	2.82	12.619	0.00	89.337	1.66	632.456	2.28
0.040	0.00	0.283	0.00	2.000	2.48	14.169	0.00	100.237	2.16	709.827	1.91
0.045	0.00	0.317	0.00	2.244	2.27	15.897	0.00	112.468	2.63	796.214	1.48
0.050	0.00	0.356	0.20	2.518	2.01	17.825	0.00	126.191	3.03	893.367	1.03
0.056	0.00	0.399	0.47	2.825	1.71	20.000	0.00	141.589	3.32	1002.374	0.65
0.063	0.00	0.448	0.69	3.170	1.41	22.440	0.00	158.866	3.47	1124.683	0.33
0.071	0.00	0.502	0.96	3.557	1.11	25.179	0.00	178.250	3.51	1261.916	0.12
0.080	0.00	0.564	1.23	3.991	0.83	28.251	0.00	200.000	3.43	1415.892	0.02
0.089	0.00	0.632	1.50	4.477	0.59	31.698	0.00	224.404	3.30	1588.656	0.00
0.100	0.00	0.710	1.76	5.024	0.40	35.566	0.00	251.785	3.15	1782.502	0.00
0.112	0.00	0.796	2.00	5.637	0.26	39.905	0.00	282.508	3.02	2000.000	0.00
0.126	0.00	0.893	2.22	6.325	0.16	44.774	0.00	316.979	2.93		
0.142	0.00	1.002		7.096		50.238		355.656			

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



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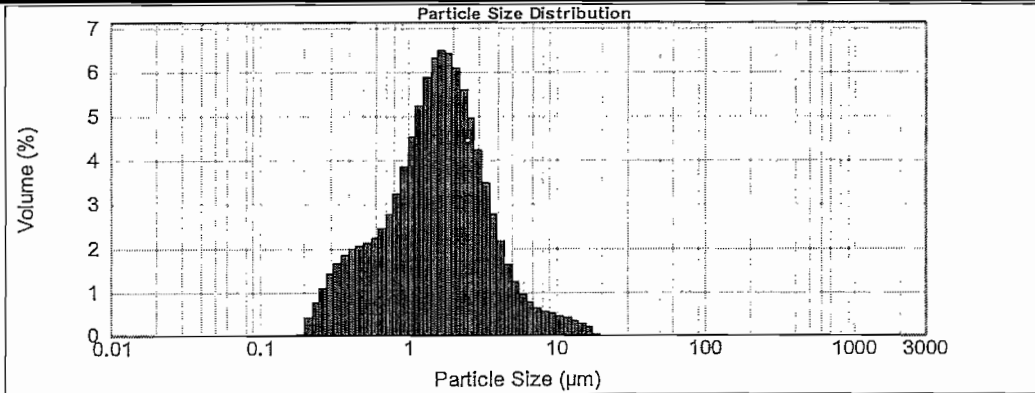
Result Analysis Report

Sample Name: Pheroid vesicles skoon
SOP Name: Pheroid vesicles using Hydro 2000 MU
Measured: 03 June 2009 06:42:21 PM
Sample Source & type: Vesicles
Measured by: Anel
Analysed: 03 June 2009 06:42:22 PM
Sample bulk lot ref: V09024
Result Source: Measurement

Particle Name: Oleic Acid
Accessory Name: Hydro 2000MU (A)
Analysis model: General purpose
Sensitivity: Enhanced
Particle RI: 1.458
Absorption: 0.1
Size range: 0.020 to 2000.000 um
Obscuration: 13.21 %
Dispersant Name: Water
Dispersant RI: 1.330
Weighted Residual: 5.258 %
Result Emulation: Off

Concentration: 0.0036 %Vol
Span : 2.224
Uniformity: 0.769
Result units: Volume
Specific Surface Area: 5.54 m²/g
Surface Weighted Mean D[3,2]: 1.083 um
Vol. Weighted Mean D[4,3]: 2.130 um

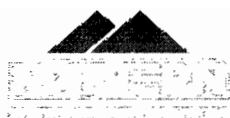
d(0.1): 0.469 um d(0.5): 1.595 um d(0.9): 4.017 um



Pheroid vesicles skoon, 03 June 2009 06:42:21 PM

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.00	1.002	4.53	7.096	0.63	50.238	355.656	0.00	
0.022	0.00	0.159	0.00	1.125	5.23	7.962	0.55	56.368	399.052	0.00	
0.025	0.00	0.178	0.02	1.262	5.86	8.934	0.50	63.246	447.744	0.00	
0.028	0.00	0.200	0.40	1.416	6.30	10.024	0.46	70.963	502.377	0.00	
0.032	0.00	0.224	0.77	1.589	6.50	11.247	0.41	79.821	563.677	0.00	
0.036	0.00	0.252	1.11	1.783	6.43	12.619	0.34	89.337	632.456	0.00	
0.040	0.00	0.283	1.41	2.000	6.11	14.159	0.28	100.237	709.627	0.00	
0.045	0.00	0.317	1.65	2.244	5.60	15.887	0.21	112.468	798.214	0.00	
0.050	0.00	0.356	1.84	2.518	4.95	17.825	0.16	126.191	893.367	0.00	
0.056	0.00	0.399	1.97	2.825	4.22	20.000	0.11	141.589	1002.374	0.00	
0.063	0.00	0.448	2.06	3.170	3.49	22.440	0.08	158.865	1124.683	0.00	
0.071	0.00	0.502	2.14	3.557	2.78	25.179	0.06	178.250	1261.915	0.00	
0.080	0.00	0.564	2.25	3.991	2.16	28.251	0.04	200.000	1415.892	0.00	
0.089	0.00	0.632	2.44	4.477	1.64	31.698	0.03	224.404	1588.656	0.00	
0.100	0.00	0.710	2.76	5.024	1.24	35.566	0.02	251.785	1782.502	0.00	
0.112	0.00	0.796	3.22	5.637	0.95	39.905	0.01	282.508	2000.000	0.00	
0.126	0.00	0.893	3.83	6.325	0.75	44.774	0.01	318.975			
0.142	0.00	1.002		7.096		50.238		355.656			

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



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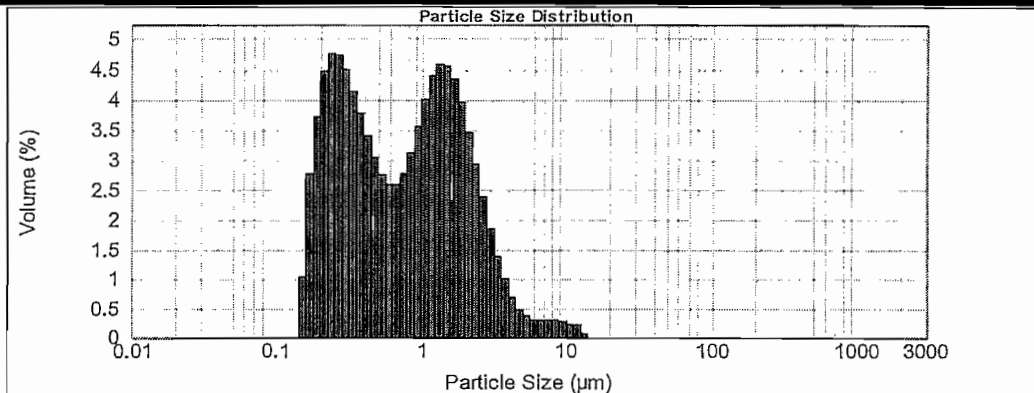
Result Analysis Report

Sample Name: Pheroid vesicles
 SOP Name: Pheroid vesicles using Hydro 2000 MU
 Measured: 21 August 2009 09:45:20 AM
 Sample Source & type: V09038
 Measured by: Chrizaan
 Analysed: 21 August 2009 09:45:21 AM
 Sample bulk lot ref:
 Result Source: Measurement

Particle Name: Oleic Acid
 Accessory Name: Hydro 2000MU (A)
 Analysis model: General purpose
 Sensitivity: Enhanced
 Particle RI: 1.458
 Absorption: 0.1
 Size range: 0.020 to 2000.000 um
 Obscuration: 15.63 %
 Dispersant Name: Water
 Dispersant RI: 1.330
 Weighted Residual: 8.327 %
 Result Emulation: Off

Concentration: 0.0037 %Vol
 Span: 3.034
 Uniformity: 1.11
 Result units: Volume
 Specific Surface Area: 12.1 m²/g
 Surface Weighted Mean D[3,2]: 0.494 um
 Vol. Weighted Mean D[4,3]: 1.206 um

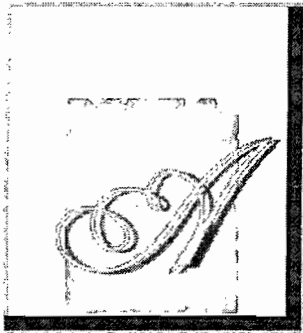
d(0.1): 0.214 um d(0.5): 0.765 um d(0.9): 2.535 um



Pheroid vesicles, 21 August 2009 09:45:20 AM

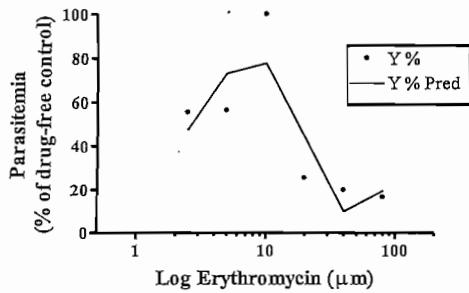
Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	1.04	1.002	4.01	7.096	0.29	50.238	0.00	355.656	0.00
0.022	0.00	0.159	2.76	1.125	4.39	7.962	0.29	56.368	0.00	399.052	0.00
0.025	0.00	0.178	3.70	1.262	4.59	8.934	0.27	63.246	0.00	447.744	0.00
0.028	0.00	0.200	4.47	1.416	4.56	10.024	0.23	70.963	0.00	502.377	0.00
0.032	0.00	0.224	4.77	1.589	4.33	11.247	0.22	79.621	0.00	563.677	0.00
0.036	0.00	0.252	4.74	1.783	3.95	12.619	0.07	89.337	0.00	632.456	0.00
0.040	0.00	0.283	4.60	2.000	3.46	14.159	0.00	100.237	0.00	709.827	0.00
0.045	0.00	0.317	4.14	2.244	2.91	15.887	0.00	112.468	0.00	796.214	0.00
0.050	0.00	0.356	3.76	2.518	2.37	17.829	0.00	126.191	0.00	893.367	0.00
0.056	0.00	0.399	3.39	2.825	1.85	20.000	0.00	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	3.04	3.170	1.39	22.440	0.00	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	2.75	3.557	1.00	25.179	0.00	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	2.58	3.991	0.70	28.251	0.00	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	2.58	4.477	0.50	31.898	0.00	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	2.77	5.024	0.37	35.566	0.00	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	3.10	5.637	0.31	39.905	0.00	282.508	0.00	2000.000	0.00
0.126	0.00	0.893	3.55	6.325	0.29	44.774	0.00	316.979	0.00		
0.142	0.00	1.002		7.096		50.238	0.00	355.656	0.00		

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

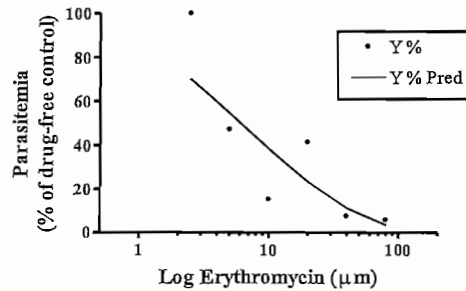


ADDENDUM D :

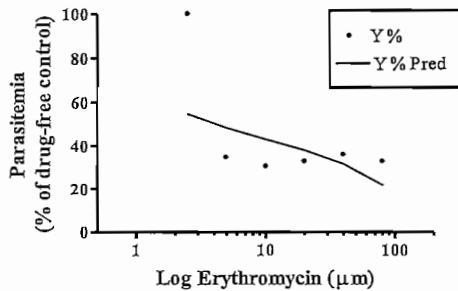
STATISTICAL ANALYSIS AND DOSE RESPONSE CURVES



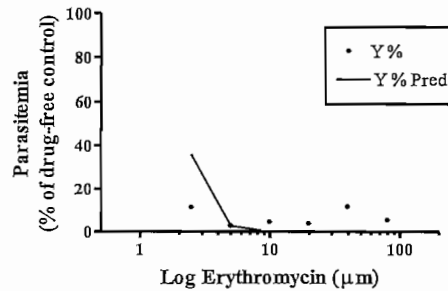
(a)



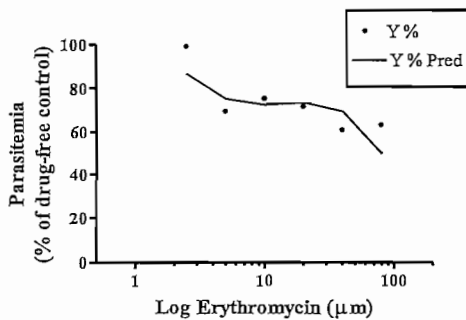
(b)



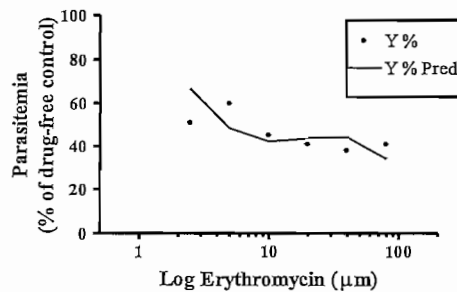
(c)



(d)



(e)



(f)

FIGURE D1: Erythromycin dose-response curves of the (a) control group and (b) Pheroid™ group following 48 hours of incubation, the (c) control group and (d) Pheroid™ group following 96 hours of incubation, the (e) control group and (f) Pheroid™ group following 144 hours of incubation.

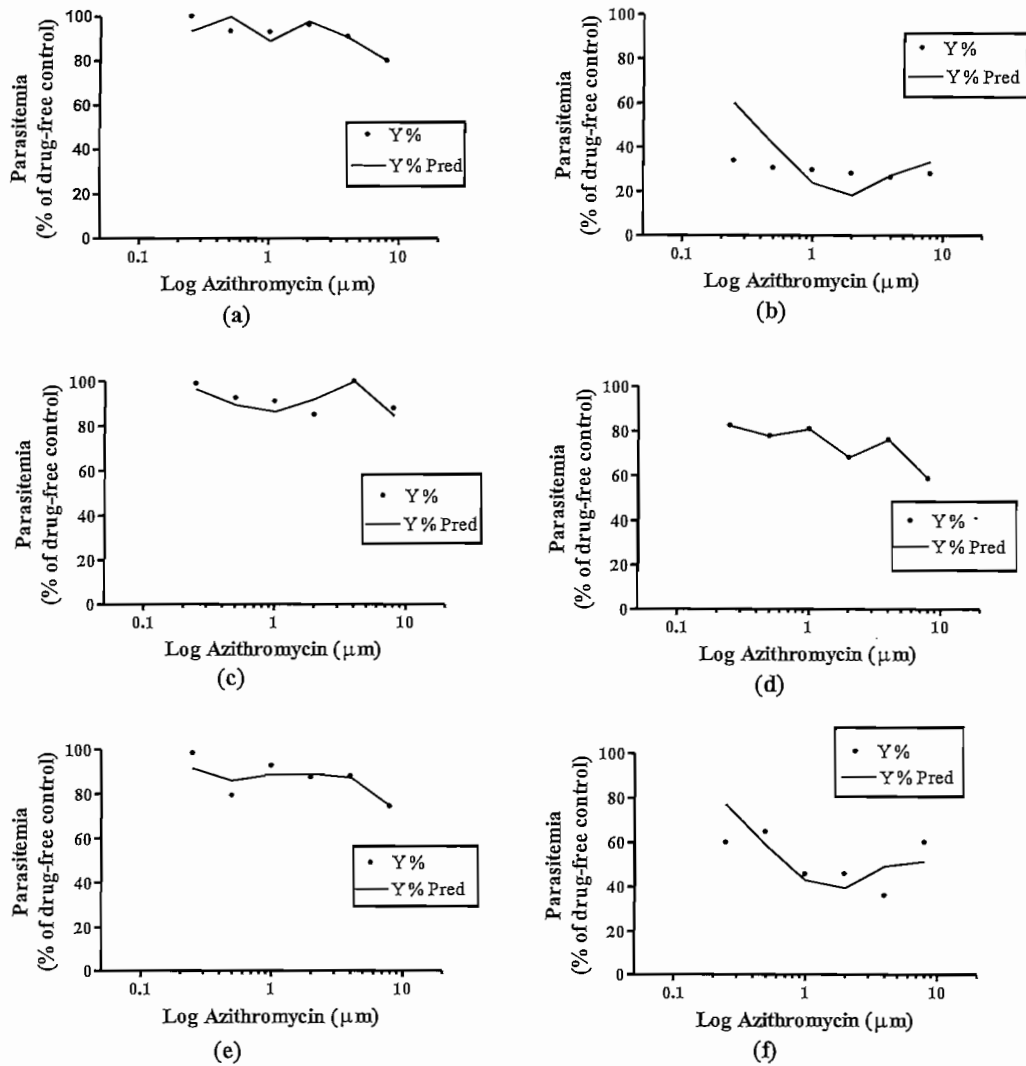
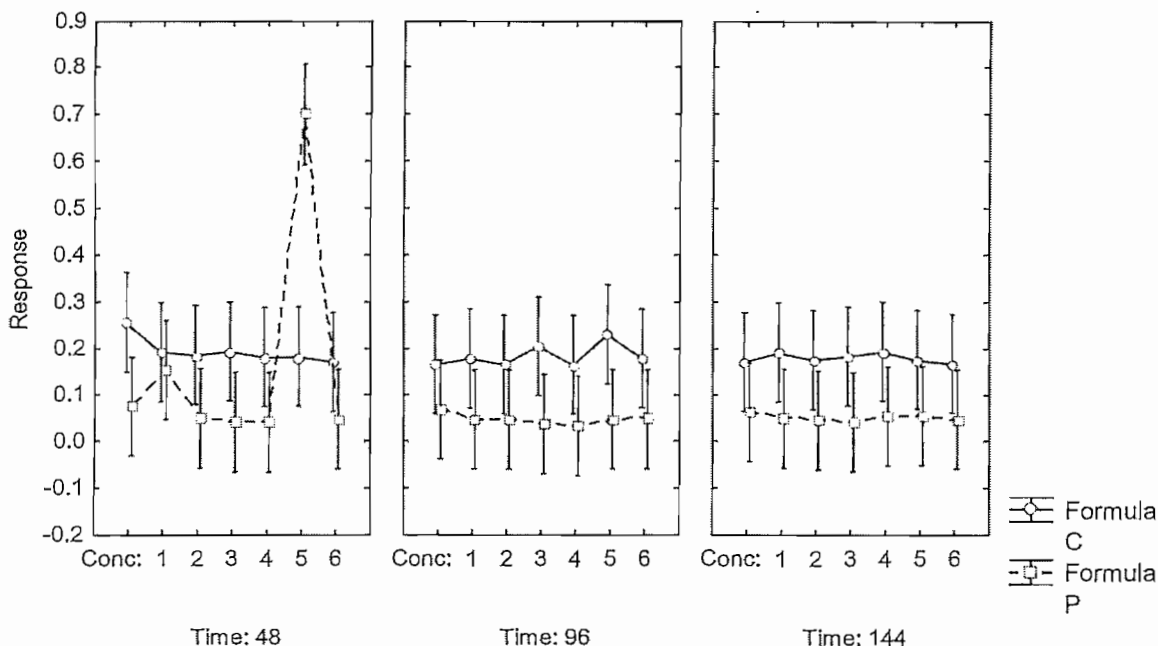


FIGURE D2: Azithromycin dose-response curves of the (a) control group and (b) Pheroid™ group following 48 hours of incubation, the (c) control group and (d) Pheroid™ group following 96 hours of incubation and the (e) control group and (f) Pheroid™ group following 144 hours of incubation.

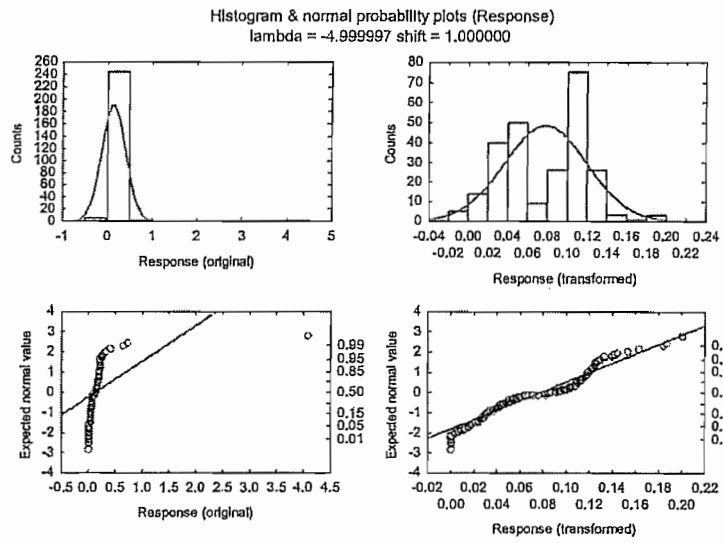
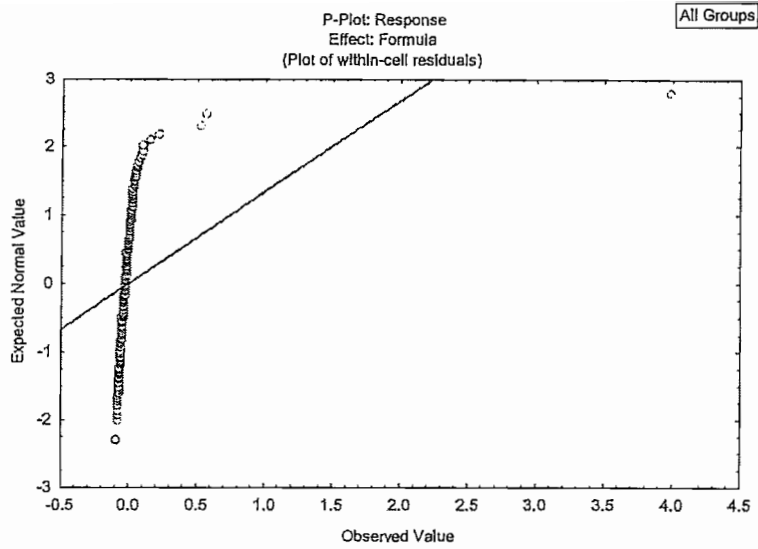
Univariate Tests of Significance, Effect Sizes, and Powers for Response (Azithromycin dihydrate.sta)								
Sigma-restricted parameterization								
Effective hypothesis decomposition								
Effect	SS	Degr. of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	4.63031	1	4.630307	67.47428	0.000000	0.243173	67.47428	1.000000
Formula	0.63893	1	0.638931	9.31071	0.002572	0.042454	9.31071	0.859349
Time	0.20809	2	0.104046	1.51619	0.221941	0.014234	3.03237	0.320512
Conc	0.41088	6	0.068480	0.99791	0.427731	0.027721	5.98745	0.390360
Formula*Time	0.13176	2	0.065878	0.96000	0.384567	0.009060	1.92000	0.215336
Formula*Conc	0.32228	6	0.053713	0.78273	0.584322	0.021874	4.69636	0.306738
Time*Conc	0.63215	12	0.052679	0.76766	0.683320	0.042023	9.21186	0.441905
Formula*Time*Conc	0.80457	12	0.067048	0.97704	0.471887	0.052879	11.72450	0.561958
Error	14.41089	210	0.068623					

Formula*Time*Conc; LS Means
 Current effect: $F(12, 210) = .97704, p = .47189$

Effective hypothesis decomposition
 Vertical bars denote +/- standard errors

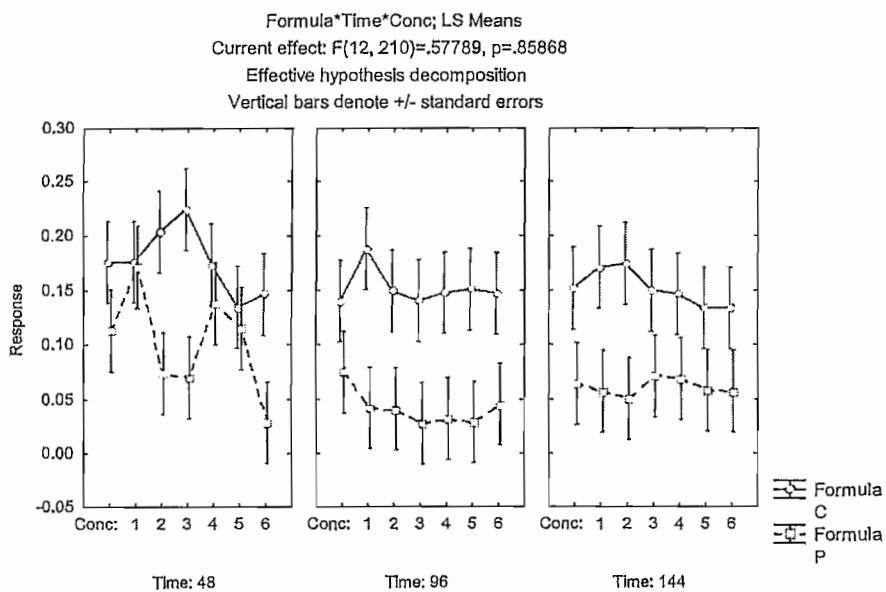


Levene's Test for Homogeneity of Variances (Azithromycin dihydrate.sta)				
Effect: Formula				
Degrees of freedom for all F's: 1, 250				
	MS Effect	MS Error	F	p
Response	0.119628	0.054079	1.866900	0.173058



	Data statistics (Azithromycin dihydrate.sta)					
	Note: Cells in red indicate that the search for optimal lambda did not yield a satisfactory result within the user specified range. It may be possible to overcome this problem by expanding the specified search range for lambda.					
	Lambda	Shift	Mean	Standard deviation	Lower Confidence Limit	Upper Confidence Limit
Transformed variable(s) Response	-5.00000	1.000000	0.077596	0.041452		

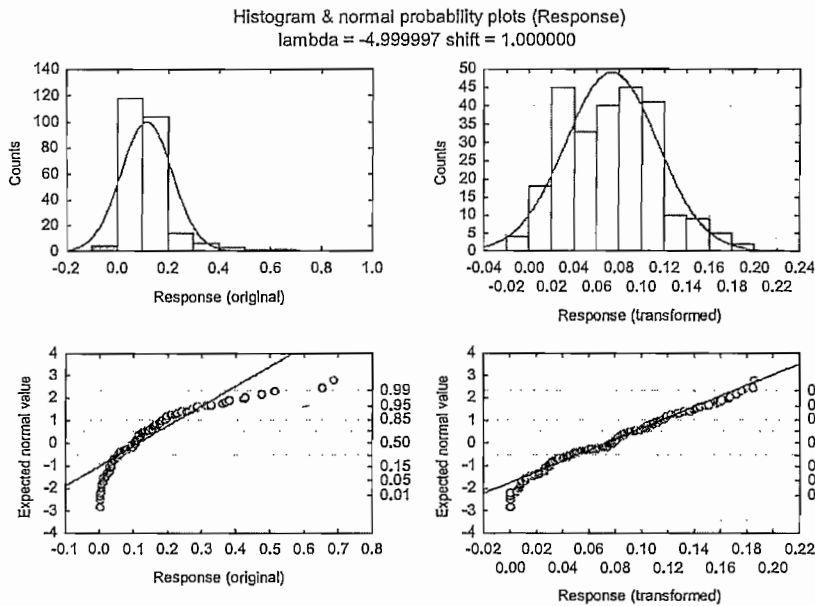
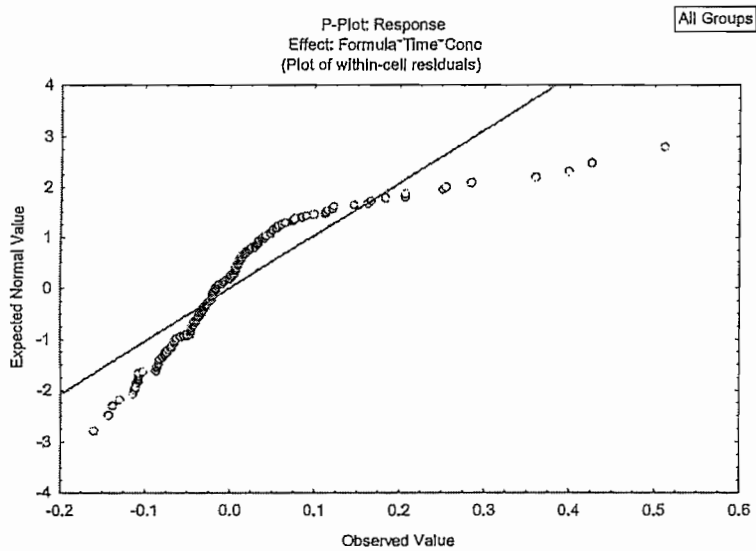
Univariate Tests of Significance, Effect Sizes, and Powers for Response (Erythromycin estolate.sta)								
Sigma-restricted parameterization								
Effective hypothesis decomposition								
Effect	SS	Degs of Freedom	MS	F	p	Partial eta squared	Non-centrality	Observed power (alpha=0.05)
Intercept	3.271117	1	3.271117	390.3524	0.000000	0.650205	390.3524	1.000000
Formula	0.532404	1	0.532404	63.5334	0.000000	0.232269	63.5334	1.000000
Time	0.082234	2	0.041117	4.9066	0.008268	0.044643	9.8132	0.801704
Conc	0.036547	6	0.006091	0.7269	0.628424	0.020345	4.3612	0.285169
Formula*Time	0.012942	2	0.006471	0.7722	0.463296	0.007301	1.5444	0.180443
Formula*Conc	0.022156	6	0.003693	0.4407	0.851043	0.012434	2.6439	0.179247
Time*Conc	0.029700	12	0.002475	0.2954	0.989651	0.016597	3.5442	0.169555
Formula*Time*Conc	0.058112	12	0.004843	0.5779	0.858685	0.031967	6.9347	0.328042
Error	1.759781	210	0.008380					



Levene's Test for Homogeneity of Variances (Erythromycin estolate.sta)				
Effect: Formula*Time*Conc				
Degrees of freedom for all F's: 41, 210				
MS Effect	MS Error	F	p	
Response	0.010539	0.003093	3.407482	0.000000

Levene's Test for Homogeneity of Variances (Erythromycin estolate.sta)				
Effect: Time				
Degrees of freedom for all F's: 2, 249				
	MS	MS	F	p
	Effect	Error		
Response:	0.062714	0.004612	13.59655	0.000002

Levene's Test for Homogeneity of Variances (Erythromycin estolate.sta)				
Effect: Formula				
Degrees of freedom for all F's: 1, 250				
	MS	MS	F	p
	Effect	Error		
Response:	0.000026	0.005304	0.004972	0.943842



*Everything comes from God alone.
Everything lives by His power and everything is
for His glory.'*

Romans 11:36

(Living Bible. Wheaton, IL: Tyndale House Publishers, 1979)