Radio-labelling as a tool to investigate the absorption and bio-distribution of selected antimalarial drugs

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ABSTRACT

Previous studies have shown that the formulation of an active pharmaceutical ingredient (API) entrapped in the Pheroid® (Pheroid for simplification) delivery system enhances absorption of the API, suppresses its metabolism, and may contribute to an increase in the quantity of the API present at the site of action. Higher drug levels at the active site should particularly increase the effectiveness of a drug with a narrow therapeutic index and reduce the incidence of the resistance that may otherwise arise if the sub-therapeutic levels of the API are in contact with the site of interest.

Two approaches were followed in this study. First, the radioactive tracer molecule ^{99m}Technetium methylene diphosphonate (^{99m}Tc MDP) was used. Intravenously injected ^{99m}Tc MDP is an extremely effective bone-seeking radiopharmaceutical used in the diagnosis of bone disorders such as bone metastases in patients. However, if entrapped inside a Pheroid vesicle, it will locate to that site, usually an organ, where the Pheroid vesicles may tend to accumulate. Experiments conducted with ^{99m}Tc MDP alone or with Pheroid will therefore establish how efficiently Pheroid vesicles localize and will also indicate the preferred site of localization inside a body. The process would involve the oral administration of ^{99m}Tc MDP either alone or with Pheroid, involving an animal model. It would also involve tracking localization to particular organs, blood or other sites. The second approach requires the use of chloroquine (CQ) labeled with carbon-14 (^{14}C-CQ,) to compare absorption of the drug both with and without the Pheroid system.

The intention was to compare oral absorption and bio-distribution of ^{14}C-CQ administered either alone or entrapped in the Pheroid system. It was also possible to establish whether the Pheroid affects the biological half-lives of the CQ and residence times of CQ in the different organs of the body.

Absorption of free ^{99m}Tc MDP (orally adminsistered) through the intestinal tract is negligible but it was anticipated that increased absorption will be observed when ^{99m}Tc MDP was

entrapped in the Pheroid system. In the ^{99m}Tc MDP study, different routes of administration of ^{99m}Tc MDP, as well as ^{99m}Tc MDP entrapped and not entrapped in the Pheroid system, were investigated. The Sprague Dawley rat was used as animal model. Rats were divided into three groups of four rats each for the first part of the study. In the first group, only ^{99m}Tc MDP was injected intravenously in order to establish natural distribution of the ^{99m}Tc MDP. For the second group, ^{99m}Tc MDP was administered orally in order to establish whether there was any absorption through the intestinal tract. In the third group, the ^{99m}Tc MDP was entrapped in Pheroid vesicles and this formulation was administered orally in order to establish whether the Pheroid system enhanced oral absorption. The animals were sacrificed four hours after administration and organs were harvested and were counted for radioactivity to determine the percentage of injected/administrated dose in each organ.

After oral administration, the Pheroid system was found to have facilitated absorption of ^{99m}Tc MDP through the intestinal tract into the blood. ^{99m}Tc MDP concentrations in the femur, although lower, were still comparable with that observed after intravenous administration of ^{99m}Tc MDP in the absence of Pheroid. Thus, overall, excellent absorption of the Pheroid entrapped ^{99m}Tc MDP through the intestinal tract was seen in contrast to little or zero absorption of the compound in the reference formulations. The half-life of the radio-labelled compound in the blood was prolonged after oral administration owing to the Pheroid.

To investigate the bio-distribution of radioactive chloroquine (^{14}C-CQ) Sprague Dawley rats were divided into two groups of four rats each. In the first group, ^{14}C-CQ in deionised (DI) water was administered orally, and in the second group ^{14}C-CQ entrapped in Pheroid vesicles was administered, also orally. The animals were sacrificed one, two and four hours after administration and subjected to comprehensive macroscopic inspection. All the organs were harvested and radioactivity was determined with liquid scintillation after applicable sample preparation. The Pheroid system produced much higher organ and blood

concentrations of ^{14}C-CQ and enhanced residence times within the organs and blood in comparison with that of ^{14}C-CQ administered alone.

Commercial applications of these results are possible, as a number of radiopharmaceutical products can presently be administered only intravenously. The added potential of these new Pheroid formulations could be of significance in the treatment of malaria, as chloroquine is inexpensive and widely available. Another point of interest is that the use of these formulations may enable micromolar drug concentrations to be achieved using drug dosage regimes that usually produce only nanomolar levels. However, safety aspects would have to be carefully monitored.

Key words: Pheroid, ^{99m}Technetium Methylene-diphosphate (^{99m}Tc MDP), ^{14}C-Chloroquine, malaria, radiotracers, drug delivery.

UITTREKSEL

Vorige studies het getoon dat die formulering van 'n aktiewe farmaseutiese bestanddeel (API) vasgevang in die Pheriod®(Pheriod) draer-sisteem die absorpsie van die API verhoog, API se metabolisme verlaag en moontlik kan bydra tot 'n verhoging in die kwantiteit van die API teenwoordig by die area van belang. Verhoogde vlakke van die middel by die aktiewe area behoort die effektiwiteit van 'n middel, veral die met 'n smal terapeutiese indeks, te verhoog en die voorkoms van weerstandigheid wat moontlik kan voorkom indien sub-terapeutiese vlakke van die API teenwoordig is by die area van belang, te verlaag.

Twee benaderings is gevolg in hierdie studie. Eerstens is die radioaktiewe merker molekule ^{99m}Tegnesium metileen difosfonaat (^{99m}TcMDP) gebruik. Intraveneus gespuite ^{99m}Tc MDP is 'n uiters effektiewe been-soekende radiofarmaseuties produk wat gebruik word om been probleme soos metastatese te diagnoseer by pasiënte. Indien dit egter vasgevang is binne 'n lipied-gebaseerde Pheriod vesikel sal dit natuurlik lokaliseer na die betrokke area, normaalweg in 'n orgaan, waar die Pheriod vesikel neig om te akkumileer. Dit sal dus met behulp van studies wat uigevoer is met ^{99m}Tc MDP alleen of saam met die Pheriod, vasgestel kan word hoe effektief Pheriod lokaliseer by die gewenste area van lokalisasie binne die liggaam. Die studie het dan ook die orale toediening van ^{99m}Tc MDP alleen of met Pheriod binne 'n diermodel behels om sodoende die lokalisasie na spesifieke organe, bloed of ander areas vas te stel. Die tweede benadering behels die gebruik van chlorokien (CQ) gemerk met koolstof-14 (^{14}C-CQ,) om 'n vergelyking te tref tussen die middel se absorpsie, met en sonder die Pheriod sisteem.

Die doel was om die orale absorpsie en die bio-distribusie van ^{14}C-CQ toegedien alleen of vasgevang binne die Pheriod sisteem te vergelyk. Dit was ook moontlik om die Pheriod se effek op die biologiese halflewe van die CQ en die tydsverloop van die CQ binne die verskillende organe van die liggaam vas te stel.

Absorpsie van die ^{99m}Tc MDP (oraal toegedien) deur die spysverteringskanale is onbeduidend en dit word verwag dat enige absorpsie slegs met die lipied-gebaseerde Pheriod sisteem opgemerk sal word. In hierdie studie is verskillende toedieningsroetes van ^{99m}Tc MDP, sowel as ^{99m}Tc MDP vasgevang in die Pheriod sisteem, ondersoek. Sprague Dawley rotte is as dieremodel gebruik: rotte is verdeel in drie groepe van vier rotte elk. In die eerste groep is ^{99m}Tc MDP slegs binneaars ingespuit om die natuurlike verspreiding van die ^{99m}Tc MDP vas te stel. In die tweede groep is ^{99m}Tc MDP oraal toegedien om vas te stel of daar enige absorpsie was deur die spysverteringkanaal. By die laaste groep is ^{99m}Tc MDP, vasgevang binne die Pheriod versikels, toegedien om vas te stel of die Pheriod die absorpsie na orale toediening verhoog. Die diere is binne vier ure na toediening uitgesit en die organe uitgehaal en 'n radioaktiwiteitstelling is gedoen om te bepaal wat die persentasie van die gespuite/toegediende dosis in elke orgaan was.

Na orale toediening is waargeneem dat die Pheriod sisteem die absorpsie van ^{99m}Tc MDP, deur die spysverteringstelsel, na die bloed in bewerkstellig het. Alhoewel ^{99m}Tc MDP konsentrasies binne die femur laer was, is dit steeds vergelykbaar met dit wat waargeneem is na binneaarse toediening van ^{99m}Tc MDP sonder die Pheroid. In die geheel gesien was daar uitstekende absorpsie van die Pheriod vasgevang binne die ^{99m}Tc MDP deur die spysverteringstelsel, in teenstelling met min of geen absorpsie van die middel in die verwysingsformulasies. Die halflewe van die radio-gemerkte middel in die bloed is verleng na orale toediening as gevolg van die Pheriod.

In die tweede studie is ^{14}C-CQ gebruik, wat internasionaal bekom is. Sprague Dawley rotte is ingedeel in twee groepe van vier rotte elk. Die eerste groep is deur die orale roete toegedien met ^{14}C-CQ in gedeioniseerde water en die tweede groep is ook oraal met ^{14}C-CQ vasgevang binne die Pheriod vesikels toegedien. Die diere is uitgesit een, twee en vier ure na toediening en onderwerp aan volledige mikroskopiese inspeksie. Alle organe is uitgehaal en radioaktiwiteit is vasgestel deur vloeistiof sintilasie (LS) na toepaslike monster voorbereiding. Die Pheroid sisteem het baie hoër bloed en orgaan konsentrasies van ^{14}C-

CQ gelewer, en die teenwoordigheid daarvan binne die organe en bloed is aansienlik verleng in vergelyking met die van ^{14}C-CQ wat alleen toegedien is.

Hierdie resultate dui op 'n moontlike kommersiële toepassing in terme van die gebruik van Pheroid om toedieningsroetes van middels te verander, aangesien 'n aantal radiofarmaseutiese produkte tans slegs binneaars toegedien kan word. Die bykomende potensiaal van hierdie nuwe formulasies kan betekenisvol wees in die behandeling van malaria, aangesien chlorokien goedkoop en algemeen beskikbaar is. 'n Ander aspek is dat die gebruik van hierdie formulasies moontlik aangewend kan word om mikromolêre middel konsentrasies te bereik, wanneer 'n aflweringsisteem soos Pheroid gebruik word, in teenstelling met nanomolêre vlakke wat verkry word onder normale omstandighede. Die veiligheids aspekte sal wel versigtig gemonitor moet word.

Sleutelwoorde: Pheroid, ^{99m}Technetium metileen difosfonaat (^{99m}Tc MDP), ^{14}C-chlorokien, malaria, radiasie, vervoersisteem.

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LIST OF ABBREVIATIONS

API	Active Pharmaceutical Ingredient
^{14}C-CQ	Chloroquine labeled with carbon 14
CQ	Cloroquine
DI	Deionised
DV	Digestive Vacuole
H_2O_2	Hydrogen Peroxide
ITLC-SG	Instant Thin Layer Chromatography Silica Gel
IV	Intravenous
keV	kiloelectron volt
LS	Liquid Scintillation
LSC	Liquid Scintillation Counting
MWT	Mann-Whitney test
Necsa	South African Nuclear Energy Corporation
N_2O	Nitrous Oxide
NTeMBI	Nuclear Technologies in Medicine and the Biosciences
NWU	North West University
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
PfMDR1	*Plasmodium falciparum* multi drug resistance 1
RL	Radiolabels
ROS	Reactive Oxygen Species
SAMRC	South African Medical Research Council
SQPI	Spectra Quench Parameter of the Isotope
STPHI	Swiss Tropical and Public Health Institute
^{99m}Tc MDP	^{99m}Tc Methylene Diphosponate

TI   Therapeutic Index

UCT   University of Cape Town

UFS   University of the Free State

USA   United States of America

WHO   World Health Organization

CHAPTER 1

PROBLEM STATEMENT AND OBJECTIVES OF THE STUDY

The two main objectives of this study were:

1. Evaluate the ability of the Pheroid® (Pheroid for simplification) drug delivery system to enhance drug absorption from the gastrointestinal tract,

2. Uncover more precisely the bio-distribution of the Pheroid system.

3. The plan was to use radioactive isotopes entrapped in the Pheroid system and to follow the distribution of these isotopes entrapped in the vesicles throughout the body.

Every 15 seconds, a child dies around the world and the reason for these deaths, malaria. Malaria remains a serious health problem, even though there has been a steady decline in mortalities in recent years (WHO, 2013). Malaria directly touches more than 40% of the world's population living in 90 countries, and the worldwide prevalence of the disease is estimated at 135-287 million clinical cases each year. Mortality due to malaria is in the range of 473 000 – 789 000 deaths per year, with the vast majority of deaths occurring among young children in Africa (Wells *et al.,* 2009; WHO, 2013; Stratton *et al.,* 2008). Eighty percent of all malaria cases are concentrated in sub-Saharan Africa (Nadjm & Behrens, 2012; WHO, 2013).

Plasmodium protozoa is the culprit responsible for malaria infections and the parasites are transmitted by an infective female Anopheles mosquito vector (WHO, 2010). The five Plasmodium species known to cause malaria in humans are *P. falciparum* (responsible for the majority of malaria infections and displays extensive resistance to normal chloroquine treatment (WHO, 2010)), *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi (*WHO, 2013).

The number of malaria cases increased globally until the turn of the millennium, owing to the rapidly escalating prevalence of drug-resistant strains of *Plasmodium falciparum* (Basco & Ringwald, 1999), the high cost of conventional antimalarial medicines (Foley & Tilley, 1998) and the limited number of widely available chemo-prophylactic and chemotherapeutic agents (Daily, 2006). However, since 2000, there has been a reduction in both incidence and

mortalities owing to extended coverage and financing of malaria control programs worldwide (WHO, 2013). Drug resistance still results in treatment failures and increased mortality, particularly among the elderly, pregnant women and children under the age of five years (Feachem *et al.,* 2010). The emergence of multi-drug-resistant malaria caused earlier antimalarial drugs to become ineffective, which poses a considerable threat to the control of the disease (White, 2004; WHO, 2010b). The drug improvement pipeline for antimalarial drugs is under increased strain due to the increase in drug resistance (Eastman & Fidock, 2009; Wells et al., 2009; Kappe et al., 2010).

The resistance of *P. falciparum to* chloroquine is well-known. In an *in vitro* study conducted with the chloroquine resistant RB-1 strain of *P. falciparum*, the reversal of resistance was observed when chloroquine was entrapped in Pheroid vesicles (Langley, 2011). This crucial finding suggests that possibly the entire vesicle containing CQ was actively transported into the erythrocytes. Furthermore, the entrapment of other antimalarial agents such as mefloquine, artemether and artesunate showed enhanced efficacy following their entrapment in Pheroid (IF 90020 1 Year Technical Report). *In vitro* studies that will be conducted using antimalarial drugs and their radio-labelled derivatives entrapped in Pheroid will provide additional evidence that the Pheroid delivery system facilitates the active transport of these drugs into target cells.

Pheroid technology, also called Phertech, is a drug delivery technology that has the ability to enhance the absorption of drugs, as has already been demonstrated in numerous *in vitro* and *in vivo* studies (Grobler, 2009; Steyn, 2011; Langley, 2011; Grobler *et al.,* 2014). Malaria is currently one of the greatest health threats in developing countries (Kappe *et al.,* 2010), and one answer to the drug development problem lies in the reformulation of existing drugs into more effective dosage forms by using innovative drug delivery technologies (Feachem *et al.,* 2010). It is consequently crucial not only to focus efforts on the research and development of new anti-malarial compounds (Baird, 2005), but also to develop 'smart'

formulations for known antimalarial drugs that may bypass resistance (Eastman & Fidock, 2009; Wells *et al.,* 2009; Kappe *et al.,* 2010).

Pheroid technology incorporates different formulations that depend on the composition and method of manufacture (Uys, 2006; Grobler, 2009; Steyn, 2011). The three types most often used are Pheroid vesicles, Pheroid micro sponges and Pro-Pheroid. The Pheroid system is a drug delivery system that has been shown to enhance the bioavailability of the antimalarial drugs in rodents (Grobler, 2009; Steyn *et al*., 2011, Aminakem *et al.,* 2012) and in vervets (Gibard, 2012; Grobler *et al.,* 2014). The studies of Steyn *et al.,* 2011 also showed improved efficacy after entrapment of artemisone in Pheroid. Overall, it is a system that effectively entraps and delivers the drug (Saunders, 1999), thereby enhancing the plasma levels of a number of anti-infective drugs (Grobler, 2009, Aminakem *et al.,* 2012, Grobler *et al.,* 2014).

The use of a Pheroid formulation for antimalarial drugs may offer important advantages. The enhanced absorption and efficacy means that the initial loading doses can be lower, which offers benefits for those drugs with a relatively narrow therapeutic index. As drug resistance may be due to the induction of drug efflux transporters in the malaria parasite (Trape *et al.,* 1998; Basco & Ringwald, 1999), the Pheroid formulation may, in principle, sidestep this problem, given that the drug is entrapped in the Pheroid.

For the same reason, the Pheroid has been shown to protect some drugs from metabolism and inactivation in the plasma, which otherwise may result in a sub-optimal drug concentrations at the site of action (Grobler, 2009, Grobler et al., 2014). Overall, use of the Pheroid system presents the possibilities of using lower drug dosage regimens over a shorter treatment period.

In order to ensure the therapeutic efficacy of antimalarial drugs entrapped in Pheroid nanovesicles, appropriate concentrations of the drugs inside the Pheroid need to be established. Further, the *in vivo* absorption, distribution, metabolism and clearance of these nano-carriers should be examined carefully. This study aimed to incorporate radioactive

isotopes into Pheroid vesicles and then to follow the distribution of the radioisotope in the Pheroid *in vivo*. This would then be compared with the *in vivo* distribution of a radiolabelled anti-malarial drug. In this way it was possible to establish the extent to which the Pheroid enhances drug absorption and influences the drug distribution in the body, if indeed it does so. The ^{99m}Tc MDP was entrapped in the Pheroid vesicle.

Technetium is a gamma emitter, and technetium compounds have been used for many years as imaging agents (Bouquot *et al*., 2012; Schwochau, 1994; Rey, 2010; Rudd *et al.,* 1977; Wang *et al.,* 1979). Different isotopes, depending on the nature of the ligand, tend to concentrate in particular organs. ^{99m}Tc MDP accumulates in bone (Subramanian *et al*., 1973; Blake *et al.,* 2011; Kung *et al.,* 1978). The distribution of the isotopes will be reflected by the radiation emitted in the different tissues (Kaye & Hayward, 2002). The radiation can also be imaged on a gamma camera, where the bio-distribution can be followed in real-time. It is hypothesized that the entrapment of these radioactive compounds in Pheroid vesicles will re-direct the distribution of the isotopes to other organs or tissue types. The isotope used has a short half-life, and its decay takes place rapidly, so damage to cells or subjects is minimized (Weber *et al.,* 1989).

This study also intended to determine the *in vivo* distribution of Pheroid containing the radioactive isotope with and without entrapped antimalarial drugs in a rodent model by using conventional radiochemical counting. A pilot study was conducted to establish whether the Pheroid system enhanced the absorption of the radioisotope from the gut and the site where the radioisotope would accumulate. Studies were undertaken to establish whether the Pheroid nanovesicles and microsponges would incorporate ^{99m}Tc compounds and, if so, to establish the *in vivo* distribution of the Pheroid containing the radioisotope with and without entrapped anti-malarial drugs, and the anti-malarial drug on its own, through results of the *ex vivo* analyses of the organs, making use of a C-14 labelled CQ.

The long-term objective of this research was to investigate the potential use of Pheroid entrapped antimalarial compounds for both the prophylaxis and the treatment of malaria.

The development the Pheroid into a cost-effective anti-infective medicines geared to African conditions would contribute to the health of the nation and that of its neighbours.

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CHAPTER 2

MANUSCRIPT 1

FUTURE PROSPECTS FOR THE PHEROID® REGARDING MALARIA

AND CHLOROQUINE

Future prospects for the Pheroid® regarding malaria and chloroquine

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Abstract

The treatment of Malaria in sub-Saharan Africa is hampered by chloroquine resistant parasites, unsatisfactory patient compliance and the ever-increasing breeding grounds for the parasite's vector. This review will provide background to malaria, chloroquine and the Pheroid®. The future prospects for malaria and the possibilities of resolving the associated problems will be explored.

1. Malaria

1.1 Introduction

Malaria is a life-threatening disease caused by infection by *Plasmodium* protozoa. The parasites are transmitted by an infective female *Anopheles* mosquito vector [1,2]. The characteristic symptoms associated with malaria are fevers and headaches, which, in their harsher form, can result in coma or death. Malaria is normally restricted to tropical and subtropical regions, most of which are in developing and poor countries [1,2]. This increases the burden of the disease.

The five Plasmodium species known to cause malaria in humans are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* [3,4]. *P. Knowlesi,* the zoonotic species, is to a great extent present only in Southeast Asia. In principle it is responsible only for malaria in macaques, although harsh human infections have been reported. *P. falciparum* is responsible for the majority of malaria infections and also exhibits widespread resistance to standard chloroquine treatment [5].

Microscopic blood analysis and antigen-based rapid diagnostic tests are used to diagnose malaria in endemic areas [6]. Polymerase chain reaction, or PCR, in which the parasite's DNA is detected, is a modern and very effective technique [7,8], but its high cost and complexity discourages wide use of the technique.

Malaria is a public health problem in more than 90 countries, inhabited by 40% of the world's population. The worldwide prevalence of the disease is estimated at approximately 135-287 million clinical cases annually [2]. The mortality rate for malaria is in the range of 473-789 thousand deaths per year. The vast majority of deaths in Africa occur among young children and pregnant women, while more than 80% of all malaria cases occur in sub-Saharan Africa [9]. The large numbers of maternal deaths are due to both malaria during pregnancy and

poor immunological protection from a vast number of childhood infections. New-born deaths resulting from low birth weight are also high [10].

The emergence of multi-drug-resistant malaria meant that earlier antimalarial drugs became ineffective, posing an enormous threat to the control of malaria [5,11]. The global increase in the number of malaria cases is exacerbated by the high cost of some of the currently used combination therapy and the low number of widely-available chemo-prophylactic and chemotherapeutic agents (see also Section 2 below).

It is consequently crucial to focus efforts on research and development when it comes to new anti-malarial compounds [12], as well as developing 'smart' formulations for known antimalarial drugs able to bypass resistance [4,13,14].

1.2 Epidemiology

Malaria currently kills more people worldwide than any other disease [15]. In the 2012 World Malaria Report, it was estimated that the disease had killed 627 000 people, while more than 207 million were infected [15]. Another study, using a slightly different model to predict the number of deaths from malaria, estimated the number at 1.24 million [16].

Children are the most susceptible to the disease, with 65% of cases reported for those under the age of [15,9,16]. Pregnant women are another vulnerable group, with 200 000 infant deaths from maternal malaria occurring annually [17] in Sub-Saharan Africa.
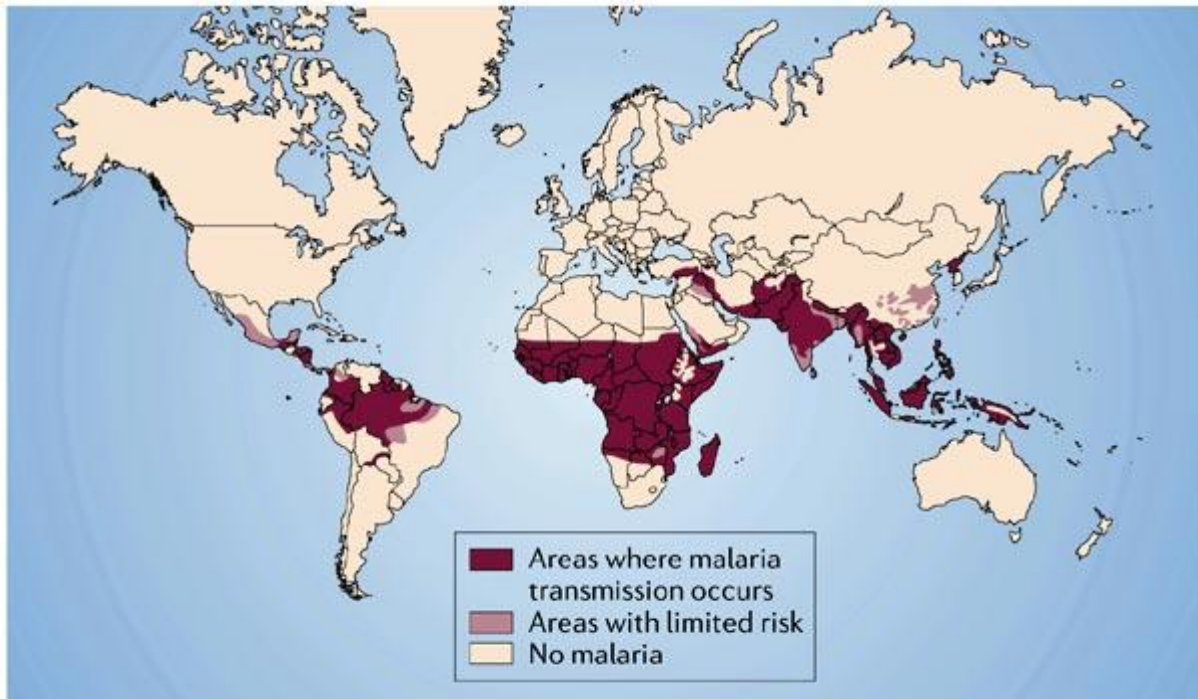
Figure 1.1: Global distribution of malaria (Adapted from Bell *et al.,* 2006:(4)7-20 with permission from Nature Publishing Group).

The African countries Burkina Faso, Nigeria, the Democratic Republic of the Congo, Mozambique, Côte d'Ivoire and Mali account for the highest number of deaths from malaria, [9] and these are some of the poorest countries in the world. Figure 1.1 illustrates these facts.

With the exception of South-East Asia, where malaria is also problematic, the rest of the world is less vulnerable, with about 10,000 malaria cases per year in Western Europe, and 1300 to 1500 in the United States [18]. From 1993 to 2003, the death toll for malaria in Europe was only 900 [19].

It has been reported that 2.3 % of the disease burden worldwide is caused by malaria, which is responsible for between 5% and 10% of the disease burden in 34 African countries [20,21]. More people die from malaria today than 40 years ago [22].

One of the paramount risk factors associated with malaria is poverty. More than 60% of malaria infections take place in poor, developing countries [22]. Factors responsible for the disease burden are: maintenance of clinics, the high cost of treatment, patients who are unable to work, vector controlling insecticides, and the high death toll [1]. Malaria is an extremely difficult disease to treat, as poverty is a key factor, while malaria is a key factor

contributing to poverty [22]. The reintroduction of chloroquine within a Pheroid formulation could be an inexpensive solution to some of these problems.

With all these challenges, there are nevertheless attempts to control malaria. The widespread dispersal and use of insecticide-treated mosquito nets and indoor residual spraying is one strategy for preventing the disease [22]. Draining the mosquito breeding grounds is another approach that has had good results in many areas [22]. In contradiction to these worthy efforts and results, mosquitos developing a resistance to insecticide halted the progress made in many circumstances. The development of an effective vaccine for the chemoprophylaxis and treatment of malaria is another important item on the agenda for the fight against malaria. The complex physiology of malaria parasites, together with the multifaceted nature of the parasite-host interaction, makes the development of an effective vaccine a very complex task [23]. In spite of these challenges, encouraging vaccines are currently undergoing clinical trials. For example, in a phase 3 study of 15,460 children with the candidate malaria vaccine RTS,S/AS01 in seven African countries, the RTS,S/AS01 vaccine provided on average 45% protection against both clinical and severe malaria in African children [23,24].

1.3 Pathophysiology

Four of the *Plasmodium* species are primarily responsible for infections in humans [25]. They are *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is responsible for the highest percentage of deaths and severe infections [25]. Although P. vivax is less deadly, it is responsible for 25-40% of the malaria burden, particularly in Central and South America, and in South and South-East Asia [26]. The Duffy blood group antigen is an essential receptor for *P. vivax* invasion and its absence from Africa is the reason why *P. vivax* is not prevalent in Africa [23]. Because *P. vivax* and *P. ovale* can persist in a dormant state in the form of hypnozoites in the liver for periods ranging from weeks to years, it is difficult to eradicate these parasites. Results of a recent study [27] show the Pheroid's ability to enhance tissue localization of an anti-malarial drug in the liver, the Pheroid, in combination with an anti-malarial active against *P. vivax* and *P. ovale*, could play an important role in the treatment of these species of the malaria parasite.

As pointed out, there is a fifth human malaria parasite, *Plasmodium knowlesi,* which was thought to be restricted to macaques. However, it can also be transmitted to humans by the

mosquito *Anopheles abaoenais* [3,10,28]. The other plasmodium species able to infect non-human mammals are *Plasmodium berghii* and *Plasmodium yeolii* [29].

The female Anopheles mosquito is the vector responsible for transmission, because only female mosquitoes feed on blood, while males feed on plant nectar, and thus do not transmit the disease. The female mosquito prefers to feed at night. Their feeding time typically begins at dusk and continues until a blood meal has been consumed [30]. Transmission of the disease by blood transfusions has been reported, even though this is extremely rare [31].

Malaria infection is divided into two phases, the exoerythrocytic phase and the erythroctic phase. Figure 1.2 provides a graphic illustration of these two stages. The first involves the liver (exoerythrocytic phase) and the other involves red blood cells or erythrocytes (erythrocytic phase). The Pheroid has been shown to increase drug levels in both the blood and the liver, so in theory it could help to eradicate the parasite in both the above-mentioned phases [27]. When an infected *Anopheles* mosquito, the definitive host, takes a blood meal, it injects a motile infected form, called sporozoites, into the bloodstream of the secondary host from the salivary glands [32]. These sporozoites use the blood stream to migrate to the liver and infiltrate the hepatocyte cells, where it reproduces asexually and develops into tissue schizonts. This process is known as the exoerythrocytic cycle.

After a potentially dormant period in the liver, these organisms differentiate to yield thousands of merozoites, which, following the rupture of their host cells, escape into the blood and infect red blood cells to begin the erythrocytic stage of the life cycle [33]. The parasite escapes undetected from the liver by wrapping itself in the cell membrane of the infected host liver cell [34]. During the erythrocytic cycle, the merozoites infect the erythrocytes, using haemoglobin as their nutrient.
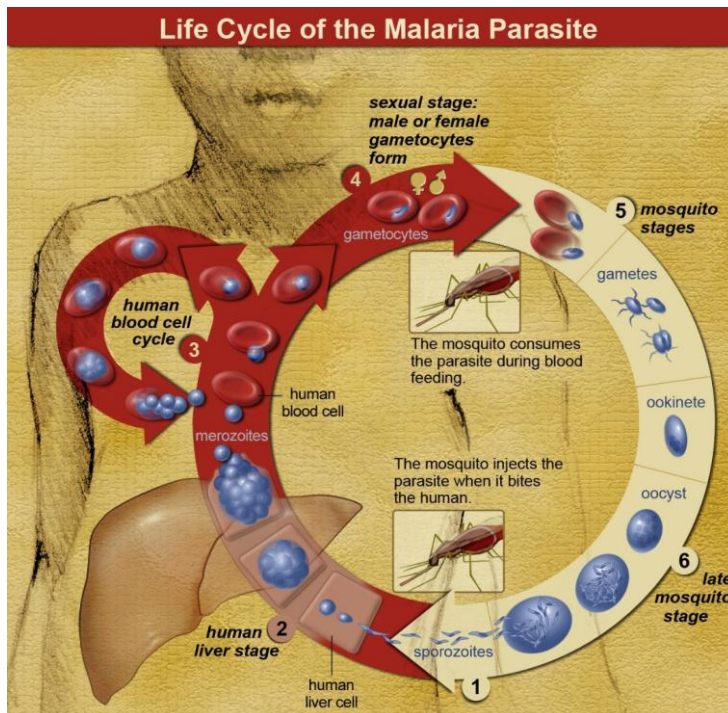
Figure 1.2: Life Cycle of the Malaria Parasite (Reprinted from http://www.niaid.nih.gov/topics/Malaria/Pages/lifecycle.aspx with permission from National Institute of Allergy and Infectious Diseases).

Within the red blood cells or erythrocytes the parasites multiply further by means of asexual development from juvenile ring forms to trophozoites, and finally mature schizonts. The erythrocytes containing schizonts rupture and release merozoites, thereby starting the whole cycle again [20,33,35]. *P. falciparum, P. vivax* and *P. ovale* take 48 hours to complete the erythrocytic cycle. Microscopic evaluation of the early stages of *P. knowlesi* and *P. malariae* shows that they are very similar. *P. malariae* has a three-day lifecycle, but dangerous blood levels are never reached. *P. knowlesi,* on the other hand, has a mere 24-hour lifecycle, but it is extremely dangerous due to rapid disease progress [36].

The erythrocytic stages of the parasite are responsible for the disease pathology and are most vulnerable to antimalarial drugs [37]. The sexual development of the merozoites into male and female gametocytes takes place in the bloodstream. When the mosquito takes a blood meal, the gametocytes in turn develop into sporozoites in the mosquito's stomach. The sporozoites are transferred to the salivary gland and the mosquito re-infects another subject [20,35].

2. Antimalarial drugs

Antimalarial drugs, also known as antimalarials, are used in the following situations:

- Individuals for whom malaria infection is suspected or confirmed

- As prophylaxis for individuals who have no natural immunity and who are visiting a malaria-endemic region

- Intermittent preventive therapy for individuals living in endemic regions.

Malaria is presently treated with a combination of treatment/therapy. The advantages of combination therapy include: reduced treatment failures; condensed resistance development; improved expediency and fewer side-effects. As mentioned above, malaria is diagnosed either by parasitological authentication by microscopy or by rapid diagnostic tests. The sooner the onset of treatment commences after a confirmation, the less the chance of complications in the disease [15]. If neither of these two diagnostic tools are available, but there is a strong clinical suspicion that the disease is present, treatment is generally started [15].

Antimalarials currently in use can be divided into six classes: quinolines and arylaminoalcohols, antifols, artemisinin derivatives, hydroxynaphthaquinones, antibacterial agents and sulfonamides. In addition, the drugs can be grouped in two more ways. First, is their intended use i.e. treatment of the disease or prophylaxis for the disease. Secondly, they can be classified by targeting part of the lifecycle of the parasite [35]. The lifecycle of the parasite is in three distinct sections: the exoerythrocytic cycle, the erythrocytic cycle or asexual development and the gametocytes cycle, or sexual development [28,35]. Table 1.1 gives further information about the three targets.

Table 2.1: The three targeted stages of malaria with the name of the stage, parasite that can be targeted in the stage as well as drugs that is effective against the stage of the parasite.

Stage	Name	Parasite	Drugs
Exoerythrocytic cycle	Tissue schizonticides	*P. Ovale* and *p. Vivax* infections to eradicate all liver merozoites[37]	Primaquine and proguanil[28]
Erythrocytic cycle	Blood schizonticides	All	Mefloquine, chloroquine, quinine, pyrimethamine and artemisinins
Gametocytes cycle	The gametocides	All	Primaquine[28]

2.1 Quinolines and arylaminoalcohols

Quinolines are heterocyclic aromatic organic compounds. Quinine was the original chemotherapy against malaria, while quinolines have been the pillar for anti-malarial chemotherapy for the last 300 years. Quinolines attack the parasite during the intra-erythrocytic stages of the life cycle, except for primaquine, which has the capacity to attack hepatic stages as well [23]. For this reason it is used for the treatment of *P. vivax* [38,39]. Research on quinolines has led to many related compounds being synthesized and tested for anti-malarial activity [35].

2.2 Quinine

Quinine is an old drug that has been derived from the bark of the cinchona tree and has been used to treat malaria since the 1630s (Figure 2.1) [25,37,40]. Initial drug developments produced only a few compounds that were of use to humans [37]. In the 1920s, two 8-aminioquinolines were synthesized. The toxic pamaquine was used as a radical cure, which led to the development of primaquine and was less toxic. The most important discovery was made in the 1940s when the synthetic 4-aminoquinoline chloroquine was developed.
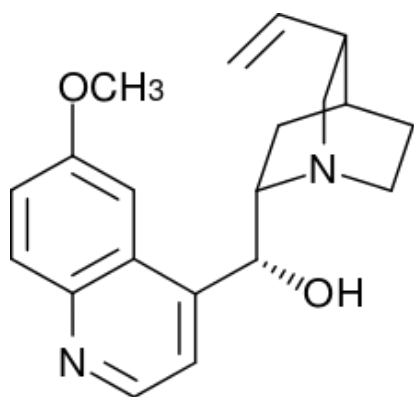
Figure 2.1: Structure of quinine

Common side effects include tinnitus, hearing impairment, dizziness and vertigo. Quinine may also cause hypoglycaemia owing to its stimulation of insulin production, which can be extremely risky during pregnancy [41]. Exceptionally rare adverse effects, such as renal failure, intravascular coagulation and cardio toxicity, may also prevail. *P. falciparum* infections can be treated with quinine, when contracted in regions where there is still reduced sensitivity to quinine, in combination with an antibiotic-like tetracycline or doxycycline or clindamycin [42]. Intravenous or intramuscular quinine is used for the treatment of hyperparasitaemic infections and for severe malaria.

2.3 Chloroquine

Chloroquine (Figure 2.2) is a 4-aminoquinolone compound with a complex and still relatively uncertain mechanism of action. Initial studies proposed that chloroquine interacts with DNA, proteases, metabolic enzymes or phospholipases, but these proposed mechanisms would entail higher concentrations of chloroquine than those that can be reached *in vivo* [43].
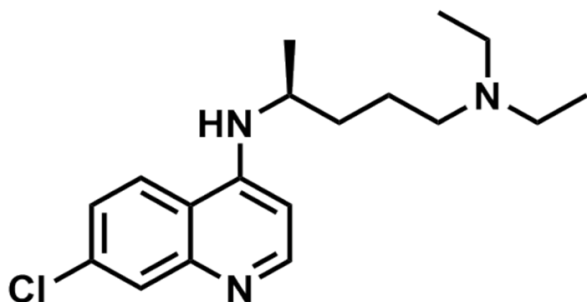
Figure 2.2: Structure of chloroquine

Chloroquine accumulates within the vacuoles of the parasite in high concentrations and owing to its alkaline nature, increases the internal pH of the vacuoles [32,44]. A few mechanisms of action have emerged. One suggestion is that chloroquine inhibits the conversion of toxic haem to haemozoin by inhibiting its biocrystallization. The haem concentration swells to toxic levels, which in turn interrupts cell membrane function [32,35,37,45]. Another possible mechanism of chloroquine is that it hinders the feeding process of the parasites. The parasite feeds on haemoglobin, and chloroquine causes swelling of the food vacuole and undigested haemoglobin accumulation [37,46]. Other probable mechanisms include interfering with the biosynthesis of parasitic nucleic acids and the formation of a chloroquine-haem or chloroquine-DNA complex [37,47].

All forms of the schizonts (except chloroquine-resistant *P. falciparum* and *P. vivax* strains) and the gametocytes of *P. vivax, P. malariae* and *P. ovale,* as well as the immature gametocytes of *P. falciparum,* are targeted by chloroquine. When *P. vivax* infections are treated with chloroquine, an added anti-pyretic and anti-inflammatory effect is observed and it could therefore remain useful, even with established resistance.

The malaria parasite degrades haemoglobin in the mid-ring and early trophozoite stages within the digestive vacuole (DV) [48,49,50,51]. During this process, Ferriprotoporphyrin IX (heme-Fe^{3+}) is released, which is cytotoxic [52] and responsible for reactive oxygen species (ROS) production. In turn, it prompts peroxidation of lipid membranes and may induce cell lysis [50,51,53]. However, the malaria parasite has an effective heme-Fe^{3+} detoxification system. One crucial system is that found in the DV. Within the DV, heme-Fe^{3+} is detoxified to malaria pigment hemozoin and β-haematin by a biomineralization process [23,50,54].

In the above process, CQ exerts one of its mechanisms of action. This is accomplished by interference with the sequestration of heme-Fe^{3+}. CQ binds to heme-Fe^{3+} to avoid the creation of hemozoin [55,56]. Inhibition of this conversion to hemozoin results in a build-up of heme in the DV. This brings about the formation of the CQ-heme-Fe^{3+} complex, which transfers across a steeper concentration gradient into the cytosol [57]. The order is that the CQ-heme-Fe^{3+} diffuses out of the fairly acidic DV (pH ~5.2) [48] into the cytosol, where, at a higher pH (pH ~ 7.4), it disassociates into the deprotonated heme and chloroquine. CQ may then go back into the DV to convey even more heme into the cytosol. For this reason, CQ results in the redistribution of monomeric heme within the parasite, intensifying cytotoxicity [57].

Until recently, chloroquine was the most extensively used anti-malarial [58,59]. It was the novel model from which the majority of treatments were derived. It is also the most inexpensive and broadly tested, as well as the safest of all the currently available drugs. However, for some time now drug-resistant parasitic strains have swiftly been diminishing its effectiveness. Nevertheless, chloroquine is still the first-line of defence against malaria in most sub-Saharan African countries. The resistance has been linked to numerous polymorphisms in the PfCRT gene on chromosome 7 [60]. A new suggestion is that it should be used in combination with other antimalarial drugs to extend its effective use.

2.3.1 Pharmacokinetics of chloroquine

Owing to the longevity and extensive use of CQ, its pharmacokinetic properties are thoroughly characterized in humans. Considerable variations in peak plasma concentrations have been reported over the years, and in humans, chloroquine has a bioavailability of roughly 80%. CQ is comprehensively distributed into body tissues as it diffuses into the body's adipose tissue, as well as the placenta and breast milk, which results in a volume of distribution of 132-261 L/kg. Plasma protein binding is extensive, with 60% of CQ bound. The drug is excreted gradually from the body via the kidneys, which results in a half-life of 1-2 months [35,61].

2.3.2 Chloroquine toxicity

CQ is a relatively safe drug, especially when used within the therapeutic dose range of 10-20 mg/kg. Adverse effects detected include retinal toxicity, mood changes, blurred vision, pruritus, gastro intestinal problems, headaches, depression and anxiety [28,35,62]. Chloroquine is known to exacerbate psoriasis. Fatal cardiac arrhythmias may be caused by an overdose [35,37]. It is not safe during pregnancy because it is a category C drug and has adverse effects on the fetus [62]. It is safe for children, but the bitter taste makes oral administration complicated [37,62]. Cumulative doses of chloroquine over years can cause retinal damage [63]. Rare blood disorders, such as aplastic anaemia, have been reported, but are very unusual [35,64].

2.3.3 Drug resistance

Drug resistance can be explained as the drug losing its ability to effectively cure the associated disease. When it comes to malaria, the parasite survives, even if an increased dosage, compared to normal dosage, of the drug is administered [65,66]. Drug resistance has had an increased effect on the global malaria burden because resistant forms of the parasite has been reported to nearly all of the antimalarial drugs used currently [11,15]. The burden has increased to levels beyond its position two decades ago [67]. The basis for drug resistance can be attributed to: the inept use of drugs in both treatment and prophylaxis, [67,68] mutations in the genetic code of the parasites and the express growth of numbers in some species [67]. Two factors play a role in the genetic mutations: changes of the antimalarial target site and an increase in the effectiveness of the influx/efflux pump [11,65,67]. The drug's ability to bind the active site is decreased in two ways - altered gene expression and mutation of protein structure [65]. These changes are responsible for the mutated parasite being able to nullify the effect of the drug [69]. It takes about ten years for a parasite strain to become resistant in an endemic area [70]. The table shows the drugs to which there are resistant parasite strains, as well as the year in which this was first noticed.

Table 2.2 Quinolines and arylaminoalcohols that have shown resistance and the date when this was first observed [65].

Antimalarial drug	Date of first observed resistance
Quinine	1910
Proguanil	1949
Chloroquine	1957
Sulfadoxine-pyrimethanime	1967
Mefloquine	1982
Atovaquone	1996

Thus far, resistance has occurred in only two of the Plasmodia species, *P. falciparum* and *P. vivax* [70]. Combination drug therapy is a way of circumventing resistance, [9] owing to the combination of different mechanisms of action [11,65]. There have been cases of multidrug resistance, especially in South Asia, but they are limited [71].

2.3.4 Development of chloroquine resistance

Chloroquine resistance is effectively universal and is prevalent virtually everywhere where *P. falciparum* infections transpire. Malawi was the first country to discontinue CQ treatment in 1993, because of the ever-increasing failure of treatment of uncomplicated malaria. A steady decline in the presence of the PfCRT molecular marker of CQ resistance was reported from 1992 to 2002. A study by Kublin [72] reported its complete disappearance in 2001. These findings were supported by Laufer, [73] who added that there could yet be a future for this safe, inexpensive and long-acting drug. However, such a future would have to occur either in combination with other drugs or in new smart formulations to avoid the recurrence of resistance.

Drug-resistance results from the elimination of the CQ from the active site and has been seen in CQ-resistant parasites, where they accumulate significantly less CQ than CQ-sensitive parasites [74,75]. Two genes are linked with CQ resistance because of point mutations of the *Plasmodium falciparum* genome. They are: the *Plasmodium falciparum* CQ resistant transporter (PfCRT) gene and, to a lesser extent, the *Plasmodium falciparum* multi drug resistance 1 (PfMDR1) gene [70,76].

Mutations in the PfCRT gene, which is located on chromosome 7, and codes for *P. falciparum* CQ- resistant transport protein (PfCRT) are mainly responsible for the lowered amounts of CQ in CQ-resistant parasites [70]. The gene is positioned on the membrane of the digestive vacuole of malaria parasites, and is responsible for the active transport of CQ out of the digestive vacuole [77].

2.3.5 Spread of resistance

The spread of resistance cannot be attributed to any single reason so a few are listed below. They include factors like: economics, human behaviour, pharmacokinetics and the biology of both vectors and parasites [9,11,66,70].

- The pharmacokinetics of antimalarials are vital when making use of combination therapy. Incompatible drug combinations, in which one drug is dominant, thus leaving a vulnerable period, can amplify the prospect of selection for resistant parasites [72,74].

- There is an ecological relation between the intensity of transmission and the enlargement of resistance, although the mechanism remains uncertain [15].

- Treatment regimens can have a significant influence on resistance development. Factors like drug intake, combinations and interactions, as well as a drug's pharmacokinetic and dynamic properties, play a role [9].

- The biological influences are due to the parasite's resistance to an antimalarial and it is thus able to survive in the presence of the drug and spread even further. Under the usual conditions, the parasites that survive the drug therapy are destroyed by our natural immune systems. Therefore, any factor that reduces the parasite eradication would assist in the progress of resistance formation. This is also why immune compromised subjects, such as pregnant women and young children, are more susceptible to the disease and the parasite and to drug resistance if you follow through on this reasoning [69,70,77].

- Antimalarials developed from the same or related chemical compounds amplify the rate at which resistance is acquired. Two examples are cross-resistance between chloroquine and amiodiaquine and mefloquine conferring resistance to quinine and halofantrine [74].

- Some Plasmodia present phenotypic plasticity ability, where fast track resistance to a new drug can be acquired, even though the drug has not been used on previous occasions [15].

- Another problem lies in choosing between drugs that have a long half-life and drugs that are metabolised rapidly. Both present potential problems. Drugs with shorter half-life require frequent administration if they are to maintain therapeutic plasma concentrations. Potentially they present more problems if adherence and compliance are unreliable. However, when it comes to longer half-life drugs, an increase in the development of resistance can occur if there are lengthy periods of low drug concentration [72,75].

Distribution of malaria in the world:♦ Elevated occurrence of chloroquine- or multi-resistant malaria ♦ Occurrence of chloroquine-resistant malaria ♦ No Plasmodium falciparum or chloroquine-resistance ♦ No malaria
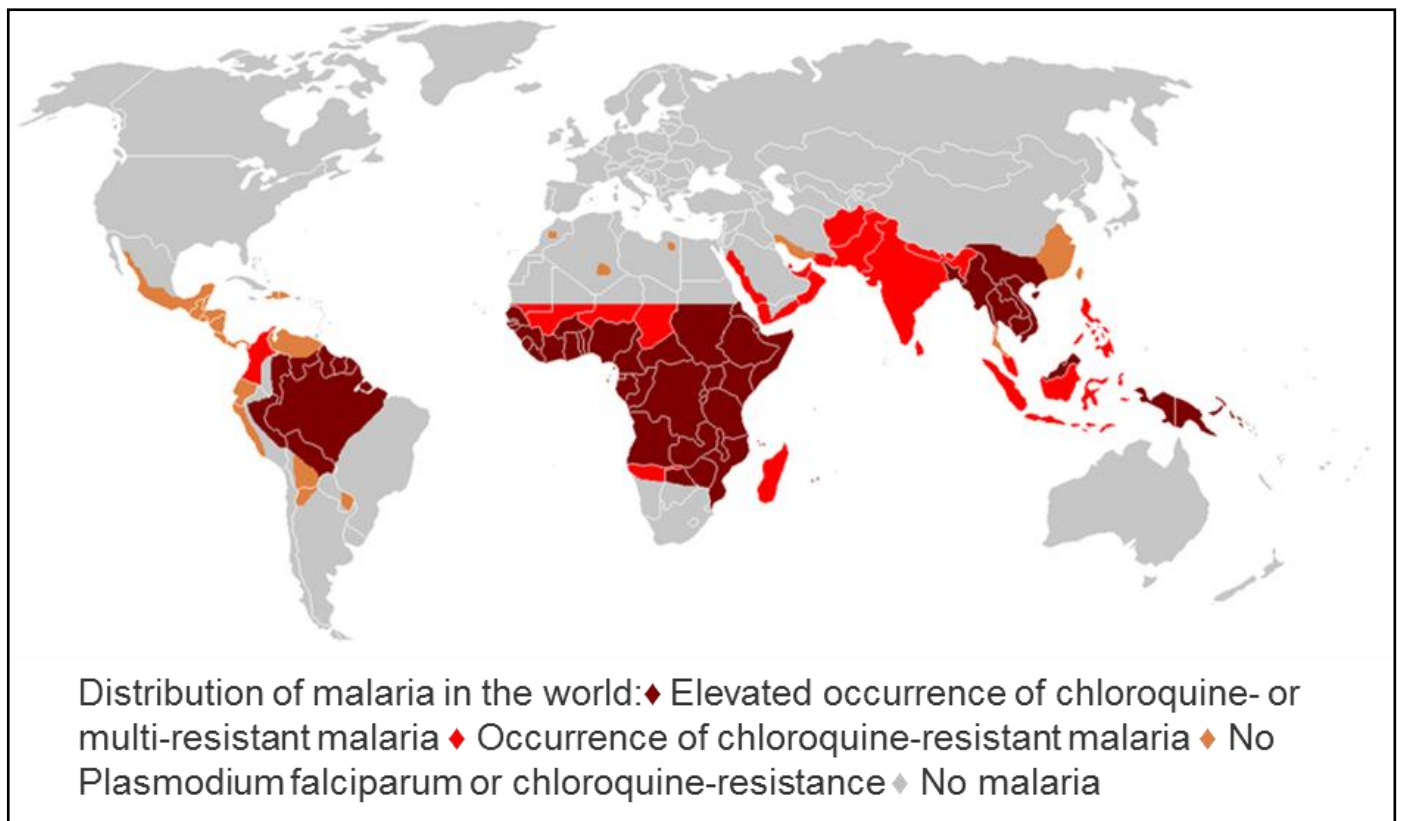
Figure 2.3: Map of places affected by malaria by type (Reprinted from Wongsrichanalai *et al,* [70] with permission from US Centre for Disease Control and Prevention).

3. The Pheroid®

The Pheroid® (Pheroid for simplification) system is an adapted fatty-acid centred delivery system basically entails a water, lipid and gas phase and includes the capacity to entrap, transport and deliver drugs of distinctly diverse chemical structures [78,79,80]. The delivery system is a colloidal system containing distinctive and stable structures called Pheroid, [23] which are uniformly scattered within a dispersion medium [81].
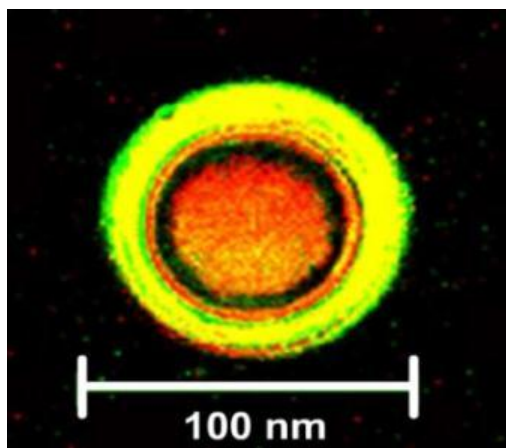
Figure 3.1: Confocal laser scanning micrographs of rifampicin entrapped in a Pheroid vesicles. The multiple layers of the multilamellar vesicle is visible in yellow as a result of fluorescent labelling with Nile red, while the red interior auto-fluorescence is that produced by rifampicin (Reprinted from Grobler, 2008:149 with permission from the author).

The unique composition of Pheroid and its characteristics may prove a useful modification to existing malaria treatment through the efficiant delivery of anti-malarial to the active site [23,80,82,83,84]. Advantages of the Pheroid formulations include: easy preparations, there is no use of any rigid nanomaterial or supporting polymer matrix and they are less expensive than most other drug delivery systems [23,85-90]. Listed below are some of these characteristics which could be useful in the treatment:

- Decreased cytotoxicity of therapeutic compounds and enhanced absorption of therapeutic compounds that was empirically determined in a previous study [86]
- Decreased drug resistance *in vitro,* with the potential for circumvent drug resistance *in vivo*
- Decreased minimal effective drug concentration (Combination therapy)
- Enhanced transport across physical barriers such as the erythrocytic cell membranes.

These characteristics could lead to a decrease in drug dosage, reduced side effects and shorter exposures to the drug owing to the shorter treatment time. All of these would lead to increased patient compliance and consequently enhanced treatment of malaria. A number of applications of this Pheroid technology have been patented in various countries.[85-90]

3.1 The history of Pheroid technology

The Pheroid was developed from Emzaloid™ technology, which was first used for the treatment of psoriasis and was originated by Piet Meyer at MeyerZall (Pty) Ltd. A research program was started with help from the South African Medical Research Council (MRC) after early observations that the Emzaloid™-based psoriasis product proved to be more effective than commercially available products for the condition. The conclusions were that the Emzaloid™ product contained micro-vesicles that were most likely responsible for the enhanced absorption of active ingredients. These findings were also reported in other studies [85,91,92]. The North-West University (NWU) acquired all the intellectual property with respect to the Emazaloid™ technology in 2003. Even though the two systems are similar in many ways, there are some differences between them. These differences include the manufacturing process, the role that α-tocopherol plays in the formation of the membranes and the gassing process. The gassing process for the Pheroid-based product is conducted under high pressure (200kPa) for four days, resulting in a saturated formulation, whereas the Emzaloid™-based products are gassed at low pressure gas (80 kPa) for four hours, yielding an under-saturated formulation.

3.2 Pheroid types

The Pheroid is very versatile, and can be grouped into three predominant morphological types:

- Lipid-bilayer Pheroid vesicles, which range in size from 80-300 nanometers
- Pheroid microsponges ranging between 0.5 and 5.0 micrometers
- Pro-Pheroid's, which can be regarded as a precursor for Pheroid vesicles [80].

Pheroid formulations can be modified to suit the specific needs of the drug that has to be entrapped, in terms of structure, size, morphology, function and whether it is hydrophilic or hydrophobic [81].

3.3 Pheroid component characteristics and function

The Pheroid basically consists of;

- Ethyl esters of essential fatty acids

- Kolliphor® EL

- Pegylated ricinoleic acid

- DL-α-Tocopherol

- Nitrous oxide

- Aqueous medium
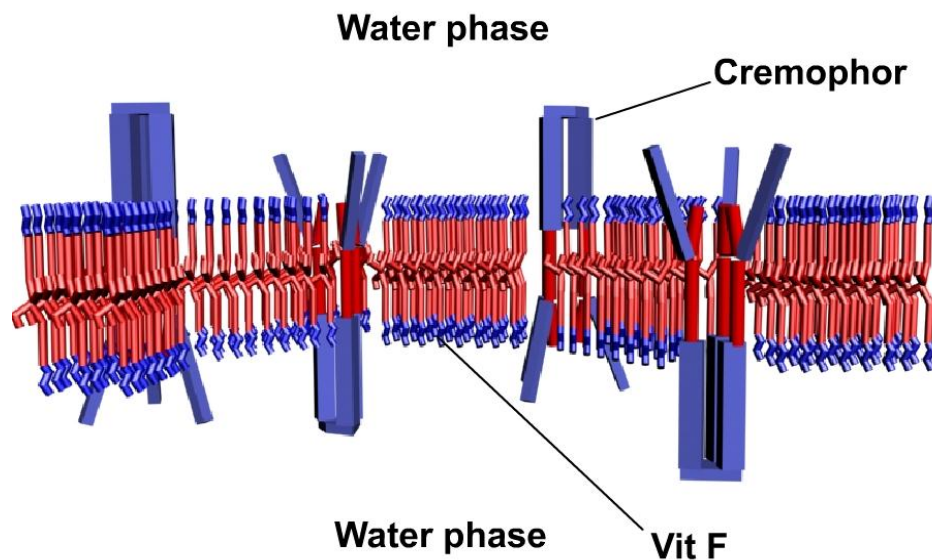
Figure 3.2: A graphic representation of the fatty acid constituent of the Pheroid vesicle. The hydrophilic domains are coloured blue and the hydrophobic domains red. The individual fatty acid ethyl ester is shown as a red hydrocarbon chain with a blue ethyl ester attached. (Reprinted from Grobler, 2008:194 with permission from the author).

3.3.1 Essential fatty acid component

The fatty acid element normally used in manufacturing Pheroid consists predominantly of ethylated and pegylated polyunsaturated fatty acids or esters, including linoleic acid and alpha-linolenic acid [23,85-90]. The essential fatty acid fragments are mainly responsible for the integral therapeutic qualities of the Pheroid system.

The purposes of this component are:

- Maintenance of membrane integrity of cells
- Modulation of the immune system
- Energy homeostasis [80].

3.3.2 Nitrous oxide component

The gas used in the formulation is laughing gas or nitrous oxide (N_2O), which is both water and lipid soluble [93]. Previous attributes are inhalation anaesthetic and analgesic [94]. Earlier molecular modelling studies proved that interactions between the fatty acids and the nitrous oxide exist, resulting in a nitrous oxide essential fatty acid matrix. This matrix delivers a practical model for transporting hydrophilic and hydrophobic drugs [80].

Other attributes of nitrous oxide are that it:

- Aids the miscibility of the essential fatty acids
- Conveys an ordered structure to the self-assembled vesicle
- Guarantees its stability.

Studies have shown that these vesicles maintain structural integrity for periods of more than two years at room temperature [79,83].

3.3.3 α-Tocopherol component

Vitamin E is the name given to four tocopherols (α-, β-, γ- and δ-) and four tocotrienols (α-, β-, γ- and δ-), of which α-tocopherol is the most important and contributes to the stability of the fatty acids by preventing oxidation and may also play the same role in drugs subject to oxidation. α- Tocopherol has been used extensively as a solvent for hydrophobic drugs and

is one of the components of Pheroids [95,96]. Another role of α-tocopherol is the anti-oxidative qualities it contains, and it contributes to the fatty acids of the Pheroid membranes. α- Tocopherol is also classified as a forager of lipid peroxyl radicals. These lipid peroxyl radicals prevent peroxidation of polyunsaturated fatty acids within cellular and sub-cellular membrane phospholipids [97].

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

MeyerZall Laboratories (Pty) Ltd., the North West University (NWU), the University of Cape Town (UCT) and the Swiss Tropical and Public Health Institute (STPHI) have all conducted studies on anti-malarial compounds entrapped in the Pheroid and various *in vitro* efficacy and *in vivo* bioavailability and efficacy results have been delivered. Numerous Pheroid technology pharmaceutical applications have been patented [88-90] and the subjects of these base patents can be divided into eight groups:

- Anti-infective agents
- Bio-agricultural
- Nucleic acids
- Topical
- Therapeutic proteins
- Vaccines
- Anti-inflammatory
- Central nervous system.

Further subdivisions in the anti-infective class can be made, i.e.; anti-virals, anti-bacterials and anti-protozoans. A number of studies were and are still being conducted on other classes of anti-malarials, including the artemisinins [78,79].

4. Conclusion

The development of a new formulation in which chloroquine would be entrapped in Pheroid vesicles may deliver an exciting new product with the possibility of aiding the treatment of malaria and resolving a respectable number of the current problems associated with malaria [12] and chloroquine.

The first notable problem with current malaria treatment is the high cost. Since malaria is far more prevalent and affects more people in developing and poor countries, [9,22] the high cost of effective chemotherapy causes many problems and greatly hampers the fight against the disease. Combining chloroquine and the Pheroid may deliver an inexpensive but still effective product.

Another increasingly problematic situation is patient adherence and compliance. Treatment regimens may be considered long by patients and the patients may experience side-effects. Patients may stop taking medication to soon after noticing an improvement in their health. In most developing countries, another reason for unsatisfactory compliance is 'fear of the unknown'. Because chloroquine is well-known after so many years of use, and the Pheroid would, in theory, decrease the side-effects and the treatment periods (a single or 2-day therapy would be beneficial), the combination should help with patient compliance.

Probably the most important cause for treatment failure is resistance [5,11]. However, the Pheroid/chloroquine combination should be helpful in overcoming or at least decreasing resistance [4,13,14]. This is because of the Pheroid's ability to increase and maintain chloroquine concentration at the site of action. This could possibly lead to programmed cell death, a mechanism described in recent studies whereby micro-molar concentrations of chloroquine instead of a nano-molar concentration at the active site lead to death of the parasite [98-102].

A further reason why this combination could be useful is that chloroquine was withdrawn from recommended treatment regimens in the early 90's, so resistance would most probably be absent from all the new malaria strains [72,73]. In combination with the added benefits of the Pheroid, it should lead to a very potent anti-malarial compound.

Due to the fact that high CQ concentrations are maintained, when CQ is entrapped in Pheroid, resistance should be decreased when the mechanism of resistance is decreased presence of CQ at the site of action [23,78,80].

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CHAPTER 3

MANUSCRIPT 2

TRACING OF THE PHEROID® BIO-DISTRIBUTION THROUGH THE USE OF RADIOACTIVE ^{99M}TC MDP

Tracing the Pheroid® bio-distribution through the use of radioactive ^{99m}Tc MDP

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Abstract

Studies have shown that the incorporation of an active pharmaceutical ingredient (API) into the Pheroid® delivery system enhances absorption of the API, and may contribute to an increase in the quantity of API present at the site of action. Increased drug levels at the active site should increase the effectiveness of a drug with a narrow therapeutic index (TI). This study aims to attain more clarity on the precise mechanism and bio-distribution of the Pheroid® system by using a radioactive tracer ^{99m}Technetium Methylene-Diphosphate (^{99m}Tc MDP). The duration of stay of the Pheroid® within the different organs and tissues and circulatory system was also examined. The Pheroid® delivery system consists of unique submicron emulsion-type formulations containing different types of Pheroid® structures, depending on the composition and method of manufacture. The Pheroid® was responsible for the absorption since statistically significant higher levels of the ^{99m}Tc MDP were reported in specific organs after it was entrapped in the Pheroid® system. The Pheroid® delivery system has therefore been shown to convey a drug, which is not absorbed in the intestinal tract under normal conditions, into the blood circulation through oral administration.

Key words: *Pheroid®, bio-distribution, radioactive tracer, Technetium, Methylene-Diphosphonate.*

1. Background

For a drug to function successfully, the active pharmaceutical ingredients (APIs) in the drug formulation have to reach the anticipated target or organ, be released from the formulation or delivery system and then elicit their effect at the target. One of the definitions of the bioavailability of an API is that it defines the fraction of the administered dose reaching the systematic circulation after administration.[1] The *in vivo* availability of an API can be significantly influenced by the formulation, as this could possibly play a role in redirecting the API to a different site, as well as influencing the place and rate of absorption, among other properties.[2] Enhanced absorption and efficacy would allow for lowering of the initial dose loadings, which has substantial benefits for drugs, particularly for those with a relatively narrow therapeutic index.

Universally, it is essential for a drug to diffuse out of a nanoparticle in order to have an effect.[2,5] In the case with the Pheroid, this is altered. Initial evidence acquired through fluorescent analysis of subcellular localization of Pheroid, shows that the Pheroid is taken up into the mitochondria of cells. Inside the mitochondria the fatty acid component of the Pheroid may be metabolised to produce energy and the active ingredient carried by the Pheroid is released due to this.[5]

As drug resistance may be due to the induction of drug efflux transporters in parasites,[3] the Pheroid® (Pheroid for simplification) may circumvent this problem in principle, given that the drug is entrapped in the Pheroid. The Pheroid also has the ability of protecting some drugs from metabolism and inactivation in the plasma, which otherwise may result in a sub-optimal drug concentrations reaching the target.[4-5] The use of Pheroid technology presents possibilities for using a lower drug dosage regimen over a shorter treatment period, thereby diminishing the side effects. As a drug delivery system, it has been shown in rodent, non-human primate and Phase 1 human studies to increase the bioavailability and efficacy of

several drugs.[4-8] The Pheroid delivery system consists of dissimilar types of Pheroid formulations conditional on the configuration and method of manufacture.[9] The Pheroid consists of lipid multi-layers that is dynamic and changes constantly while maintaining high stability.[10-11] It is known to remain stable and structurally integral for a period of 24 months at room temperature.[12-13]

However, deliberation on the exact mechanism of action and *in vivo* distribution continues. Further studies were undertaken to elucidate mechanistic aspects and internal delivery. The Pheroid can be manipulated for a variety of therapeutic applications, as both hydrophilic and hydrophobic drugs can be entrapped.[4,5,7,14,15] Furthermore, Pheroid preparations can be manipulated to alter release characteristics.[12,14,16] The boosted bioavailability of novel therapeutic compounds has been published.[4-7,14] However, there is still some uncertainty on the exact mechanism of action and *in vivo* distribution of the Pheroid and studies to elucidate mechanistic aspects and internal delivery are therefore necessary.

Technetium-99m (⁹⁹ᵐTc) is a pure gamma ray emitter with a 90%, 140 keV emission which is not attenuated by the body and ideal for detection on a gamma camera. Therefore, technetium compounds have been used for many years as imaging agents.[17-20] The radiation emitted by the isotope can therefore be used to reflect the distribution of ⁹⁹ᵐTc in different tissues. With its excellent physical characteristics and easy availability by means of a generator, technetium has become the most important nuclide for imaging in nuclear medicine.[21-24] Different tracers tend to concentrate in particular organs: ⁹⁹ᵐTc MDP accumulates in bone tissue and is not absorbed through the intestinal tract.[17] In this study the Pheroid system was labelled with ⁹⁹ᵐTechnetium Methylene-Diphosphate (⁹⁹ᵐTc MDP) to determine tissue distribution, physiological half-life through possible accumulation or deposition in organs and tissues. ⁹⁹ᵐTc MDP accumulates on the bone by chemical adsorption and is amalgamated into the hydroxyapatite structure. Clearance occurs via the kidneys.[17]

The fact that ^{99m}Tc MDP is not absorbed through the intestinal tract made it the tracer of choice for the work described herein; oral administration of entrapped drugs in the Pheroid system. The Pheroid system has been shown to increase oral absorption.[10,12,13,25,26] Any oral absorption, enhanced absorption levels or change in the bio-distribution (i.e. other than bone) can therefore be attributed to entrapment and delivery by Pheroid. This study aimed both to inspect the capacity of Pheroid technology to enhance the efficacy of a drug by improving its bioavailability and to measure the bio-distribution after it has been absorbed.

2. Methods

2.1 Materials

All the chemicals used were of analytical grade and were obtained from commercial sources. Vitamin F ethyl ester was obtained from Chemimpo, South Africa, and Kolliphor® EL was obtained from BASF, South Africa. DL-α-tocopherol was purchased from Chempure, South Africa. The technetium (^{99m}Tc) and MDP were provided by NTP Radioisotopes SOC Ltd, South Africa. The Sprague Dawley rats were sourced from the vivarium of the DST/NWU Preclinical Drug Development Platform.

2.2 Radiolabelling study

The radiolabelling is a spontaneous process whereby 1 ml of technetium (^{99m}Tc) in saline is added to a lyophilised vial of MDP. After five minutes at room temperature, labelling of ≥90% were quantified by Instant Thin Layer Chromatography Silica Gel (ITLC-SG) plates run in two mediums, acetone and saline. In saline the radioactive complex (^{99m}Tc MDP) is transported from the point of origin (bottom) to the top (front); and for acetone the radioactive complex (^{99m}Tc MDP) stays at the bottom as free ^{99m}Tc moves to the front. Both

of the above instances delivered the required results, which indicated that there was 90% or more labelling.

2.3 Reference formulations

The reference formulation was prepared by addition of ^{99m}Tc MDP (1 g) to deionised (DI) water (4 g) to deliver the required 5 ml.

2.4 Pheroid formulations

Preparation of the oil-phase of the Pheroid vesicle formulation

Pheroid vesicles were prepared by heating and mixing vitamin F ethyl ester (7.01 g) and Kolliphor® EL (2.58 g) to 70 °C. The mixture was cooled to 55 °C and DL-α-tocopherol (0.504 g) was added.

Preparation of the water-phase of the Pheroid vesicle formulation

The water-phase consists of the ^{99m}Tc MDP and nitrous oxide saturated water. The required amount of ^{99m}Tc MDP (1 g) was added to 3.8 g of the prepared nitrous oxide water and heated to 70 °C.

Pheroid vesicle formulation

The prepared water phase (4.8 g) was then combined with the oil-phase (0.2 g) to achieve a total volume of 5.0 ml. The mixture was homogenised with a Heidolph Diax 600 homogeniser (Labotec, South Africa) at 13 500 rpm for at least four minutes or until the temperature dropped below 40 °C.

2.5 Entrapment study

Because the product was radioactive, traditional methods in which the onset and efficiency of entrapment (of ^{99m}Tc into Pheroid vesicles) is tested, could not be used. An additional complication to this study is the relatively short half-life of ^{99m}Tc MDP (6 hours). For this reason, the shortest possible time had to be established in which the percentage of active ^{99m}Tc MDP was still sufficient to work with, and the entrapment of ^{99m}Tc MDP within the Pheroid was adequate. The hypothesis required the preparation of four identical formulations with different time periods for the entrapment of the product to take place. Formulation A was left on the magnetic stirrer for 1 hour (1 hour of entrapment time), B for 12 hours, C for 24 hours and D for 30 hours. Each of these formulations was prepared in the exact way as the Pheroid formulations was done in section 2.4. The formulations was then administered to the four test rats (A, B, C and D), which were sacrificed one hour later (after administration) and the distribution of the radiolabelled chloroquine was determined.

2.6 *In vitro* stability study

After the entrapment test had been completed, a stability/labelling test was carried out to determine integrity ^{99m}Tc labelled MDP after the 24 hour period. Pheroid entrapped ^{99m}Tc MDP formulations were placed onto ITLC plates and again run in acetone and saline mediums. In this case, please also refer back to Section 2.2, as the same technique was used. Through the above mentioned technique the resulting measured activity showed that there was still a 90% or higher ^{99m}Tc labelled MDP mixture present.

2.7 *In vivo* bio-distribution study

The bio-distribution study contained three study groups of twelve animals each. The first two groups received oral administration (*per os*, *po*) while the third group was intravenous. The following treatments were administered to the three respective groups:

- ^{99m}Tc-MDP alone in DI water *per os*;

- ^{99m}Tc-MDP entrapped in Pheroid vesicles *per os*;

- ^{99m}Tc-MDP alone in DI water intravenously.

Four animals from each of the treatment groups were sacrificed at three different time points, that is: after one, two and four hours. Each group contained four rats to reduce the number of rats sacrificed, while still giving statistically reliable results.

All the organs, including the femur, were harvested and the radioactivity was independently counted in order to determine the change in distribution due to Pheroid entrapment. Due to the radio active decay factor the exact time at which each radio active counting was done is stated and a back count formula is then used to dismiss the time differences between the readings. The radioactive counting was performed via a Capintec Beta Detector CRC®-Ultra Dose Calibrator and a Scintispec Well Counter.

2.8 *In vivo* imaging study

For *in vivo* imaging, four rats were used, for which ethical authorisation was granted by the Ethics Committee of the University of the Free State (Animal experiment number 25/2013). In three rats, the ^{99m}Tc-MDP, entrapped in Pheroid vesicles, was administered orally, the remaining rat serving as a control, where ^{99m}Tc-MDP was also administered orally, but not entrapped within the Pheroid system. Imaging was performed using a General Electric (GE) 400 AT gamma camera that is connected to a GE Genie acquisition station. For all four of the

rats, a static planar gamma scan was done at 0, 60, 120 and 240 minutes. After 240 minutes, the rats were sacrificed. The organs were harvested and counting of radioactivity was performed with a Capintec Beta Detector CRC®-Ultra Dose Calibrator and a Nal Well Counter.

2.9 Statistical analysis

The experimental data was evaluated in terms of the percentage of injected dose per gram of sample (%ID/g) versus time. When comparing the means of the groups, the nonparametric Mann-Whitney test was used. Due to the number of samples, the Mann-Whitney test was used, but the analysis can be expanded to include Kruskall Wallis as well, but the dergree of certainty would be lowered. The computer program Statistica[27] was used to arrive at the results.

3. Results

3.1 Entrapment results

In A (Formulation A and rat A) no distribution was measurable. With B, that had 12 hours of entrapment time, there was very little absorption and distribution of the ^{99m}Tc MDP possibly due to insufficient entrapment. Formulation C (24 hours) delivered adequate entrapment for detectable distribution of the ^{99m}Tc MDP. Measurements were not conducted for D (30 hours), owing to the decreasing activity of the ^{99m}Tc MDP. It was therefore concluded that the optimal time period for the purposes of this study (i.e. acceptable activity levels for ^{99m}Tc MDP, sufficient entrapment percentage) was 24 hours.

3.2 Bio-distribution

All *in vivo* experimental designs and protocols were approved by the Research Ethics Committee of the North-West University (NWU-00079-11-A5).

A number of animal models may be used, including guinea pigs, rabbits, rats, mice, and non-human primates. In all *in vivo* experiments performed in this study, a Sprague Dawley rat model was selected. Rats are relatively inexpensive, are available and are more easily handled than e.g. mice. It was also decided not to use mice, as the volume of labelled Pheroid that can be administered and the resultant level of radioactivity in the various organs could be too low for detection. Additionally a previous study has shown the rat model to be adequate.[6]
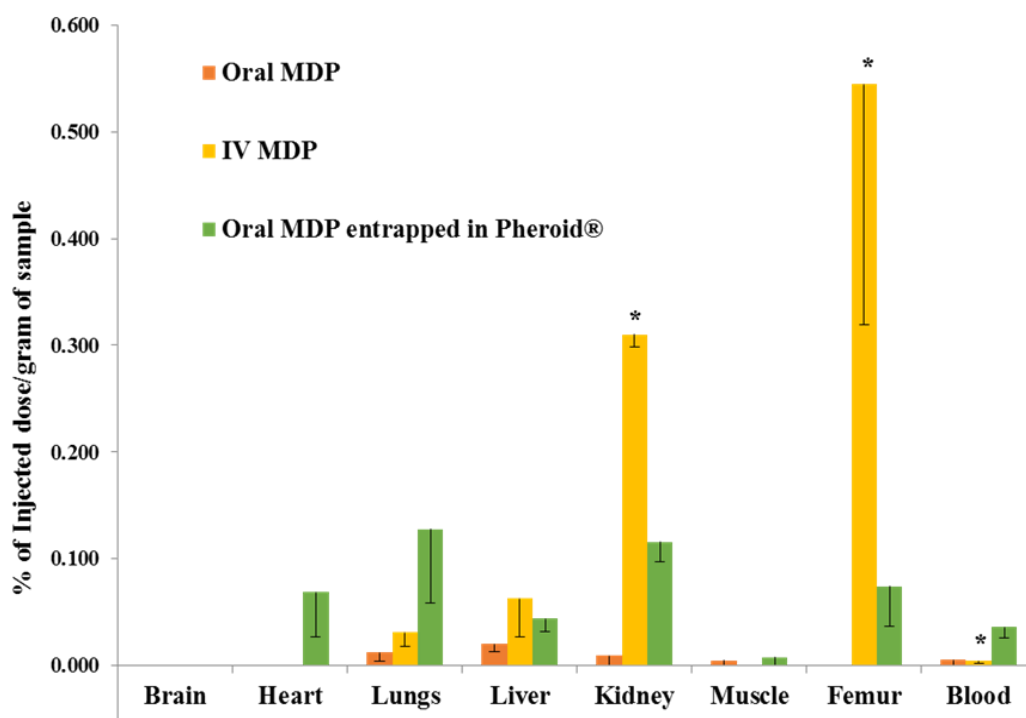
Figure 1: The differences in body accumulation and distribution of four hours after administration. The amounts are expressed as the percentage of injected dose per gram of sample, with the standard error (SE), after both oral and intravenous administration. * Statistically significant difference is observed between three test groups (p < 0.05).

Figure 1 illustrates the presence of the accumulated dose of ^{99m}Tc-MDP through the different modes of administration in the rats' respective organs after four hours. The bio-distribution was interesting, with marked differences in the three systems, where the percentage of the injected dose was plotted against routes of administration in the different organs. Enhanced oral absorption of the Pheroid entrapped ^{99m}Tc-MDP through the intestinal tract was observed, as opposed to the diminutive absorption levels of the control ^{99m}Tc-MDP (Figure 1).

Table 1. Comparisons of the different organs/body tissues four hours after oral and IV administration as well as the difference between the reference ^{99m}Tc MDP and ^{99m}Tc MDP entrapped in the Pheroid vesicles. The table also provides the analysis of variance p-values. Aggregate at the bottom is the sum of all the organs for a specific formulation.

Organ	Bio-distribution (%ID/g) after 4 hours			
	Pheroid/ ^{99m}Tc MDP Oral	^{99m}Tc MDP Oral	^{99m}Tc MDP IV	Analysis of Variance p-value
Brain	0	0	0	0
Heart	0.068±0.041	0	0	0
Lungs	0.127±0.069	0.012±0.008	0.030±0.012	5.6×10^{-5}
Liver	0.043±0.011	0.019±0.007	0.062±0.035	0.459
Kidney	0.115±0.018	0.008±0.008	0.309±0.011	6×10^{-6}
Muscle	0.007±0.007	0.004±0.004	0	0.525
Femur	0.073±0.036	0	0.544±0.225	0.053
Blood	0.035±0.009	0.005±0.005	0.004±0.002	0.009
Aggregate	0.468	0.048	0.949	-

When comparing the two *per os* administrations, the Pheroid entrapped ^{99m}Tc-MDP and the control ^{99m}Tc-MDP; it was found that at each measured time point the entrapped ^{99m}Tc-MDP levels were found to have improved, in contrast with the reference ^{99m}Tc-MDP (Table 1). After four hours, 9.75 times more of the drug was present in the organs noted. This was deduced after comparing the combined percentages in table 1 of the injected dose administered per gram of the sample. With this information, it is possible to argue that the Pheroid system makes it possible for a non-absorbable drug to be absorbed through the intestinal tract.

When comparing the intravenously administered ^{99m}Tc MDP to the entrapped ^{99m}Tc MDP, which was administered orally, it renders equally interesting results. After the four hours, twice as much of the drug was present in the specific organs after IV injection, but the Pheroid entrapped ^{99m}Tc-MDP compares relatively well, taking into account that IV administration always yields 100% bioavailability (*F), entrapment in Pheroid delivers up to 50% of the IV dosage orally. If the Pheroid could produce these kinds of results consistently it would have a profound effect on the medical field, especially for drugs where the IV route remains the only viable option or where oral administration renders a problem.

As ^{99m}Tc MDP is used as a bone-tracing agent, the femur results were investigated. The ^{99m}Tc MDP levels present in the femur after IV injection was seven times higher than when the Pheroid entrapped ^{99m}Tc MDP was administered orally. This is a substantial decrease, but the levels are still high enough for bone scans to be conducted. Comparing the femur results of the two oral routes of administration, the difference is clear, with zero ^{99m}Tc MDP present four hours after the control formulation was administered.

3.3 Blood results

Prolonged stay of the Pheroid entrapped ^{99m}Tc-MDP in the blood after four hours was apparent (Figure 2). The figure compares the amounts of administered formulation or compound left in the blood after four hours. High levels of the Pheroid-entrapped compound can be seen as opposed to very low levels after IV and oral administration without the help of the Pheroid vesicles.
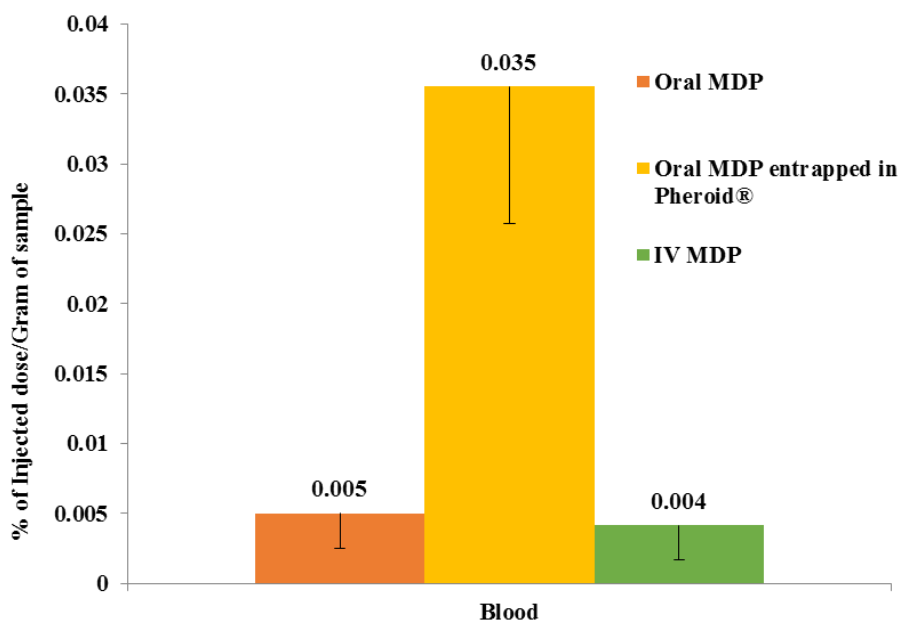
Figure 2: Difference between the amounts of ^{99m}Tc MDP present four hours after IV and oral administration and ^{99m}Tc MDP entrapped in Pheroid and ^{99m}Tc MDP on its own in blood. Standard error is also displayed.

The drug concentration levels in the blood after oral administration are nine times higher for the Pheroid-entrapped ^{99m}Tc-MDP (0.035 ± 0.009) than for the reference ^{99m}Tc-MDP (0.005 ± 0.005) after IV injection (0.004 ± 0.002) (Figure 2). The analysis of variance indicates a statistically significant p-value for the three blood samples. After four hours the higher levels point to a prolonged stay of the drug in the blood.

3.4 Enhanced absorption

Statistically significant higher levels of the ^{99m}Tc MDP were seen in specific organs after it was entrapped in the Pheroid system (Table 2). The Pheroid is directly responsible for the absorption.

Table 2. Comparisons of the different organs/body tissues at one, two and four hours after administration as well as the difference between the reference ^{99m}Tc MDP and ^{99m}Tc MDP entrapped in the Pheroid vesicles.

	Bio-distribution (%ID/g)								
	1 hour			**2 hours**			**4 hours**		
Organ	**Phe/ MDP**	**MDP**	**MWT**	**Phe/ MDP**	**MDP**	**MWT**	**Phe/ MDP**	**MDP**	**MWT**
Brain	0	0	-	0	0	-	0	0	-
Heart	0.021±0.021	0	0.376	0	0	-	0.068±0.042	0	0.215
Lungs	0.893±0.839	0.014±0.010	0.215	0.553±0.469	0.132±0.015	0.595	0.127±0.069	0.012±0.009	0.288
Liver	0.043±0.014	3.275±0.901	0.111	0.112±0.021	0.004±0.003	0.051	0.044±0.012	0.019±0.007	0.215
Kidney	0.080±0.074	0.013±0.008	0.376	0.293±0.115	0.006±0.004	0.051	0.116±0.019	0.008±0.008	0.051
Muscle	0	0.127±0.089	0.723	0.028±0.025	0	0.215	0.008±0.008	0.005±0.005	0.859
Femur	0.059±0.018	0.001±0.001	0.051	0.213±0.124	0	0.051	0.074±0.037	0	0.215
Blood	0.050±0.021	0.023±0.011	0.215	0.063±0.002	0	0.051	0.036±0.010	0.005±0.005	0.051

MWT = p-values of Mann-Whitney-test; Amounts given are the mean and standard deviation, Phe/MDP = ^{99m}Tc MDP entrapped in the Pheroid vesicles and MDP = reference ^{99m}Tc MDP.

A notable absence of accumulation is shown in the brain, as there were no ^{99m}Tc MDP traces in the brain. The amounts of ^{99m}Tc MDP in the intestine and stomach are not shown, as they were the point of origin on account of the *per os* administration. After one hour, the drug levels in the blood were three times higher for the Pheroid/^{99m}Tc MDP formulation (0.00564 ± 0.0013) than for the reference formulation (0.00193 ± 0.00078) (Table 2), which should lead to a quicker onset of the mechanism of action of the drug. However, while there is still some correlation between the two formulations after one hour, this changes completely after two hours and four hours with the difference between the two formulations increasing even further. These findings point to a prolonged stay of the radiotracer in the blood when it is entrapped in the Pheroid system (Table 2).

Also shown in Figure 3 (A, B and C) is that the accumulation in the liver is enhanced extensively. After one hour, the amount of Pheroid/^{99m}Tc MDP (0.043 ± 0.014) is 3.3 times higher than for the reference (0.013 ± 0.008), as illustrated in Table 2. After two hours, there was a 28 factor increase and 2.3 after four hours.

The results for the femur in Figure 3 are the same as for the liver, enhanced absorption and prolonged stay. At the one hour time point the drug levels are 2.6 times higher for the Pheroid/^{99m}Tc MDP (0.059 ± 0.018) in comparison with the reference (0.023 ± 0.011). The increases were 2.6, 213 and 74 times after one, two and four hours respectively.

Most of the ^{99m}Tc-MDP cleared through the kidneys in an hour for the reference formulation, compared to the consistent clearance during the four hour test period for the Pheroid/^{99m}Tc MDP formulation.
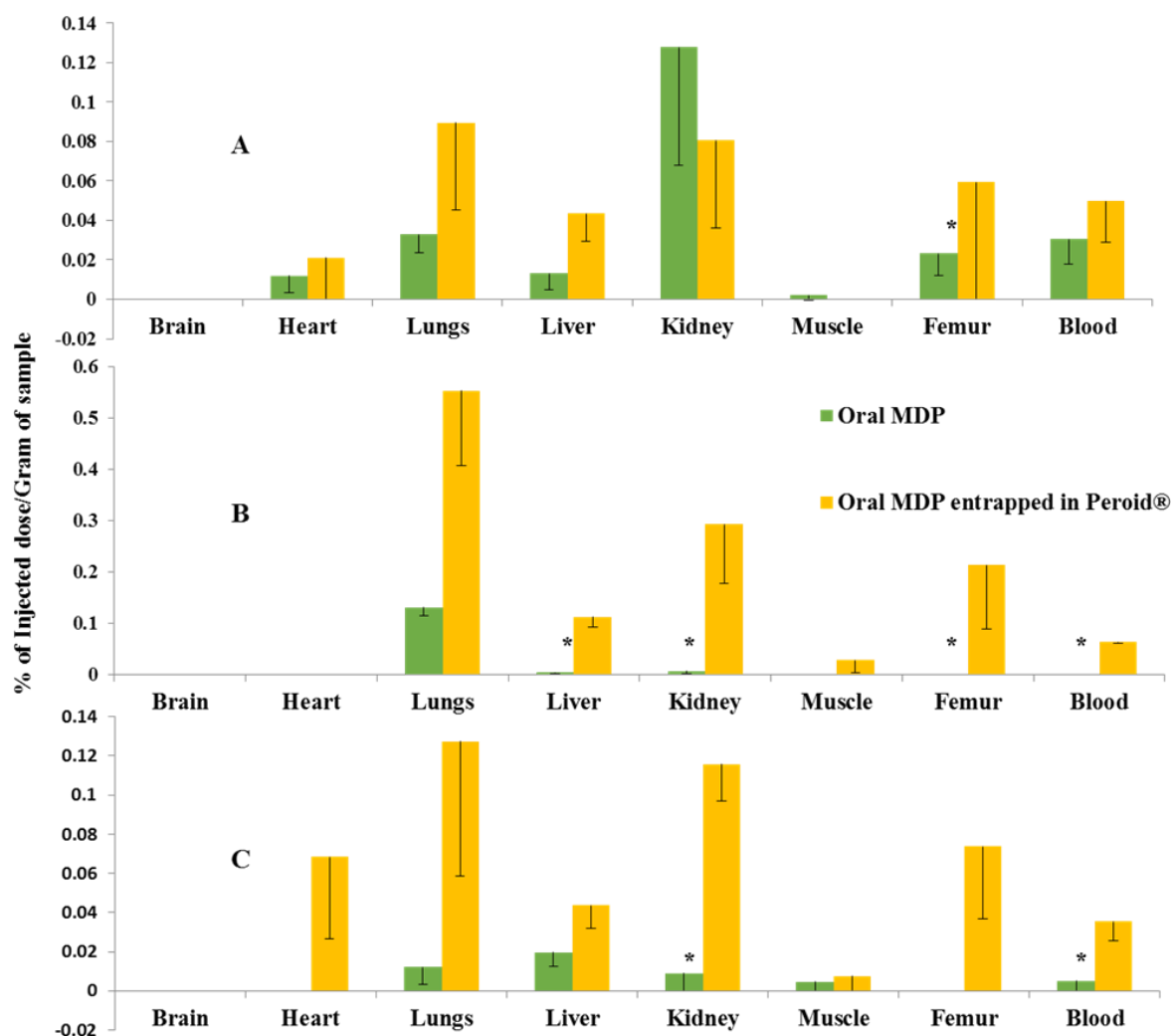
Figure 3. A graphic representation of the differences between reference ^{99m}Tc MDP (green) and Pheroid-entrapped ^{99m}Tc MDP (yellow) at the different time points (1, 2 and 4 h) as well as for the different organs. Evaluation is portrayed as percentage of injected dose per gram of sample. A was after 1 hour, B after 2 hours and C after 4 hours after formulation administration. *indicates that the values obtained were statistically significant ($p < 0.05$).

3.5 Imaging results

Directly after administration of ^{99m}Tc-MDP and Pheroid/^{99m}Tc-MDP, the PET images obtained were very similar. This is to be expected, as no time has elapsed to allow for absorption to take place. However, at the other time points, the difference in absorption and body distribution is pronounced (Figure 4). Where the ^{99m}Tc-MDP was administered on its own, there was no absorption from the intestinal tract, but where the ^{99m}Tc-MDP was entrapped in the Pheroid system, the absorption and body-distribution is clearly visible. In this case, the absorption is wide-spread throughout the body and its onset is also more rapid than had been expected.
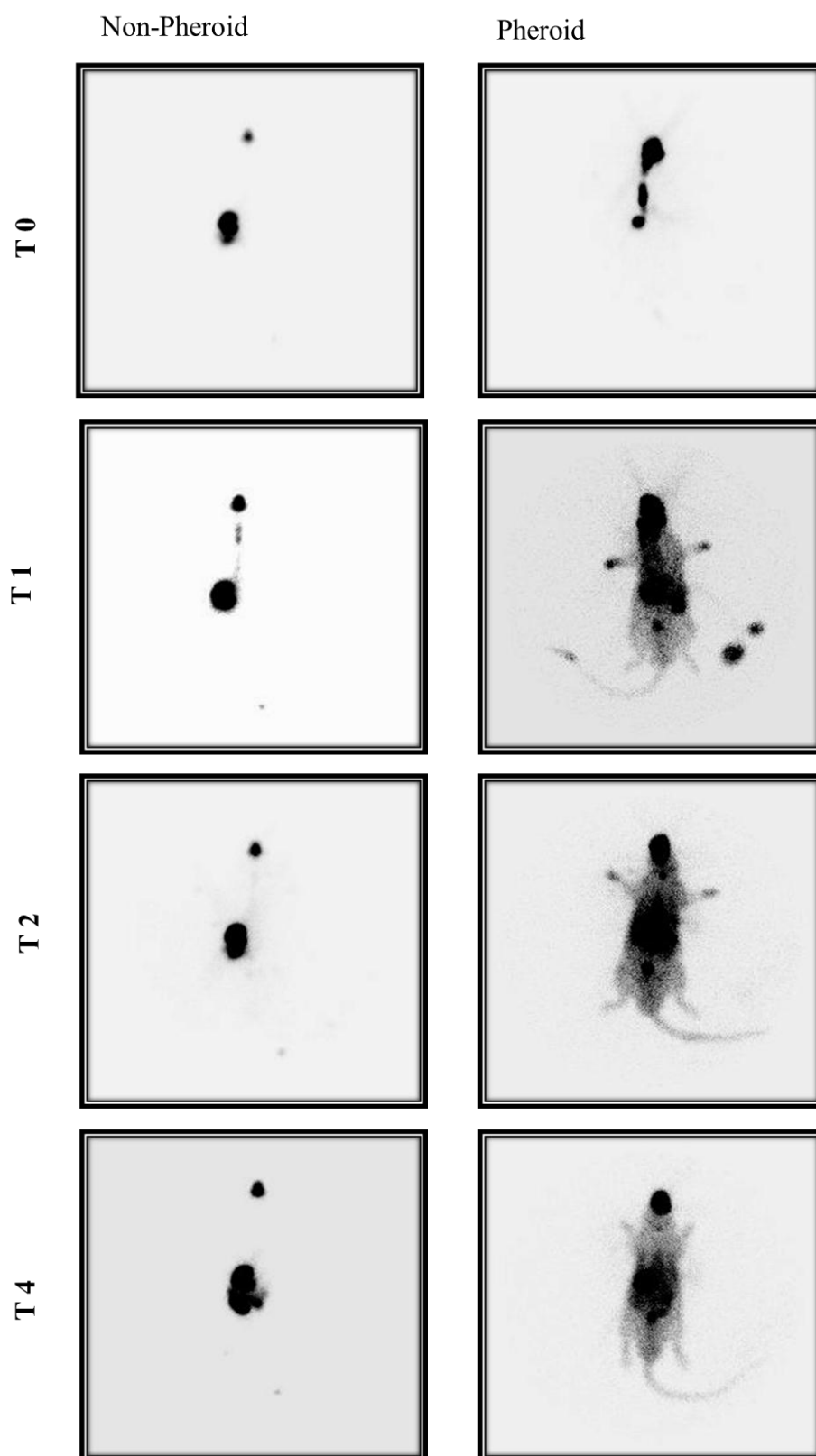
Figure 4. Static planar gamma images depicting the differences in absorption between Pheroid entrapped (right) and free ^{99m}**Tc MDP (left) at times zero, 1, 2 and 4 h.**

4. Discussion

[99mTc]-MDP is used as a bone-tracing agent, therefore tracing to the femur results were compared to examine whether [99mTc]-MDP could be used in a formulation that could potentially be administered *per os* (orally). The [99mTc]-MDP levels present in the femur after IV injection are seven times higher than when the Pheroid-entrapped [99mTc]-MDP was administered orally. This is a considerable difference, but the lower levels of [99mTc]-MDP in the femur are still high enough for the bone tracing procedures to be concluded. Although an intravenous injection of these radiotracers is the norm, there are some circumstances when the IV route is problematic, sometimes impossible, and consequently an oral alternative would provide a solution.

The capacity of the Pheroid system for conveying the drug, which is not absorbed in the intestinal tract under normal conditions, into the blood circulation through oral administration is exciting, as there are a number of drugs that can only be administered intravenously, owing to their lack of absorption through the gut. The Pheroid system will also be capable of aiding drugs that suffers from low absorption, which in turn could lead to lowered cost and fewer side-effects.

The prolonged stay of the Pheroid trapped drug in the blood circulation should increase the drug half-life. This will be beneficial for drug formulations targeting diseases within red blood cells.

Another area in which the Pheroid could be important is that of resistance. In most cases resistance occurs owing to inadequate drug levels or where the drug efflux is more rapid than its absorption into the target area. Theoretically, the Pheroid system should be able to bypass these effects, thus side-stepping resistance in certain cases.

The fact that there was zero brain absorption when [99mTc]-MDP was entrapped in the Pheroid is an encouraging sign and outcome for the Pheroid, as this could prevent the neurotoxicity of

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certain formulations or drugs; the Pheroid may have the ability to redirect drugs that normally cross the blood-brain barrier.

The Pheroid-associated increase in oral absorption has previously been noted[5] and, together with the above described properties of the Pheroid, could be of significant importance in the development of new medicines. From the results it can be deduced that the Pheroid system speeds up the whole absorption process and prolongs the stay of the drug in the body and organs, thereby decreasing the excretion of the drug and inhibiting the metabolism thereof. This inhibition of the metabolism of drugs has previously been reported.[2]

The Pheroid system produced much higher ^{99m}Tc-MDP organ and blood concentrations and also prolonged the stay in the organs and blood in comparison with the orally administered ^{99m}Tc-MDP.

Comparing the amount of ^{99m}Tc-MDP present in the noted organs obtained from the animal group injected intravenously after four hours to the amounts obtained in organs of rats after *per oral* administration of ^{99m}Tc-MDP entrapped in Pheroid, a 50% decrease was seen. However, given that IV injections result in 100% bioavailability, it can be concluded that the Pheroid system increases bioavailability for oral administration of compound(s) that usually show very little or zero oral absorption.

The clearance of the ^{99m}Tc-MDP through the kidneys was consistent with the observations for the other organs. High amounts of the drug were reported throughout the body at all the time points for the Pheroid/^{99m}Tc MDP resulting in consistently higher excretion through the kidneys, while the reference formulation peaks in drug levels after one hour, therefore higher excretion at is observed at this point as opposed to zero or very little at the other two time points.

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CHAPTER 4

MANUSCRIPT 3

ENHANCED BIO-DISTRIBUTION OF RADIOLABELED

CHLOROQUINE ENTRAPPED IN THE PHEROID® SYSTEM

Enhanced bio-distribution of radiolabeled chloroquine entrapped in the Pheroid® system

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Abstract

This study compared the oral absorption and bio-distribution of ^{14}C-chloroquine entrapped in the Pheroid® system to that of ^{14}C-chloroquine on its own. The duration of stay of ^{14}C-chloroquine in the different organs and the drug concentrations within certain organs was also determined.

^{14}C-chloroquine was orally administered to Sprague Dawley rats (n=4 per group), which were sacrificed 1, 2 and 4 hours after administration and subjected to comprehensive macroscopic inspection. All organs were harvested and radioactivity was determined by liquid scintillation counting (LSC) after tissue solubilisation.

Much higher organ and blood concentrations and a prolonged stay within the organs and in blood was found for ^{14}C-chloroquine entrapped in Pheroid® when compared to that of the reference ^{14}C-chloroquine. The drug concentrations of the ^{14}C-chloroquine (administered as entrapped in Pheroid® vesicles) in the blood circulation were found to be 3 times higher and as much as 14 times higher in the liver.

Key words: *^{14}C-chloroquine, Pheroid®, malaria, absorption, bio-distribution, half life*

1. Introduction

Malaria is a major public health problem in more than 90 countries, which are inhabited by 40% of the world's population. The worldwide prevalence of the disease is estimated to be in the order of 135-287 million clinical cases each year.[1] Mortality due to malaria is in the range of 473 to 789 thousand deaths per year, the vast majority of which occurs among young children in Africa. More than 80% of all malaria cases are found in sub-Saharan Africa. This life-threatening disease is caused by infection by Plasmodium protozoa. The parasites are transmitted by the infective female Anopheles mosquito vector.[1] The five Plasmodium species known to cause malaria in humans are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale, Plasmodium knowlesi* and *Plasmodium malariae*.[1] *P. falciparum*[2,3] is responsible for the majority of malaria infections, and exhibits widespread resistance to standard chloroquine treatment.[4]

The emergence of multi-drug-resistant malaria resulted in earlier antimalarial drugs becoming ineffective, which became an major threat to the control of malaria.[4,5] This obstacle, combined with the high cost of conventional artemisinin-based therapy and the limited number of widely available chemo-prophylactic and chemotherapeutic agents, has contributed to the global number of malaria cases and resulting fatalities.[6] Drug resistance results in treatment failure and increased mortality, particularly among the elderly, pregnant women and children under the age of 5 years.[7,8] The development of cost-effective, anti-parasitic medicines geared for African conditions would contribute to the nation's health and that of the country's neighbours.

The drug development pipeline for antimalarial drugs is under severe stress.[9-11] Pheroid® (henceforth called Pheroid for simplification) technology is a drug delivery technology that has the ability to enhance the absorption of drugs, as has been demonstrated in both *in vitro*

and *in vivo* studies.[12, 13, 14] The most feasible answer to the drug development problem lies in reformulating and developing 'smart' formulations for known antimalarial drugs that may bypass resistance.[9, 10, 11]

The Pheroid is a patented delivery system that functions as a unique submicron emulsion-type formulation.[15,16] The Pheroid consists of lipid multi-layers that is dynamic and changes constantly while maintaining high stability. It is known to remain stable and structurally intact for a period of 24 months at room temperature.[13,17] The Pheroid formulations can differ, depending on the composition and method of manufacture.[18] The three predominant configurations are; Pheroid microsponges, Pheroid vesicles, Pheroid in depots or reservoirs.

Pheroid formulations are comprised of the ethyl esters of essential natural fatty acids, which, in conjunction with DL-α-tocopherol and pegylated ricinoleic acid, form discrete structures in a liquid saturated with nitrous oxide gas.[15,16,17]

The Pheroid system is used as a delivery system that entraps, transports and delivers the active pharmaceutical ingredient (API) to the desired site. These characteristics enhance the bioavailability of novel therapeutic compounds.[19-24] The Pheroid can be manipulated to suit a variety of therapeutic applications, as both hydrophilic and hydrophobic drugs can be entrapped in the drug delivery system.[19-24] Another advantage of the Pheroid is that preparations can be altered to change the release characteristics of the drug.[17,19,25] Numerous rodent drug studies, as well as studies in non-human primates and phase 1 human studies, have shown improvement in bioavailability and efficacy through the use of this drug delivery system.[20, 22-24]

Chloroquine (CQ) is a 4-aminoquinolone compound (Figure 1) with a complex and still relatively uncertain mechanism of action.[26,27] All forms of the schizonts (except chloroquine-resistant *P. falciparum* and *P. vivax* strains) and the gametocytes of *P. vivax, P. malariae,* and

P. ovale, as well as the immature gametocytes of *P. falciparum,* are targeted by chloroquine.[27] When *P. vivax* infections are treated with CQ, an added anti-pyretic and anti-inflammatory effect is observed and the drug could therefore remain useful, even with established resistance. When treating *P. knowlesi*, chloroquine is well-tolerated and very effective.[28]
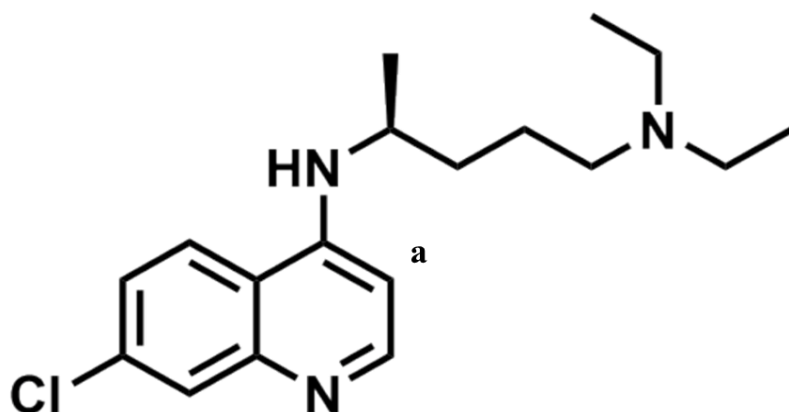
Figure 1. Chloroquine structure; [a]Location of the Carbon 14.

Until recently CQ was the most extensively used anti-malarial drug. CQ is also used as the model from which many novel techniques and treatments are derived. It is also the most inexpensive and broadly tested, as well as being the safest of all the currently available drugs.[27] However, the emergence of drug-resistant parasitic strains rapidly reduced its effectiveness.[29] The resistance has been linked to numerous polymorphisms in the PfCRT gene on chromosome 7.[30,31] New recommendations are that chloroquine should be used in combination with other antimalarial drugs.[31] Alternatively, the development of a chloroquine-containing smart formulation may extend its effective usage. Current research also points to the fact that there could be new mechanisms through which CQ could overcome resistance and be as effective in the fight against malaria as any of the current first-line choices.[31] Ch'ng J- et al. (2014) hypothesized that programmed cell death (PCD) may be the mechanism through which CQ is able to overcome resistance.[31-36] This mechanism requires higher doses

of CQ in the micromolar concentration range, as opposed to the nanomolar concentrations associated with the established antimalarial mechanisms.[31,34] Such micromolar concentrations may result in programmed cell death.

In this study, the combination of CQ and the Pheroid will be examined as a possible new smart formulation therapy against malaria.

2. Experimental

2.1 Materials

All the chemicals used were of analytical grade and were obtained from commercial sources. Vitamin F ethyl ester was obtained from Chemimpo, South Africa, and Kollophor® EL was obtained from BASF, South Africa. DL-α-tocopherol was procured from Chempure, South Africa. Nitrous oxide was supplied by Affrox. The ^{14}C-Chloroquine (^{14}C-CQ) was obtained from ViTrax, Inc., USA, and Biosol and Bioscint were purchased from National Diagnostics, Atlanta, USA. Hydrogen peroxide was sourced from Sigma-Aldrich, South Africa. The Sprague Dawley rats were sourced from the vivarium of the DST/NWU Preclinical Drug Development Platform.

2.2 Methods

2.2.1 Reference formulations

The reference formulation was prepared from ^{14}C-CQ (0.2 g) and deionised (DI) water (3.8 g) to deliver the 4 g needed.

2.2.2 Pheroid formulations

Pheroid vesicles were prepared by heating and mixing vitamin F ethyl ester (7.01 g), Kollophor® EL (2.58 g) up to 70°C. The mixture was cooled to 55°C and DL-α-tocopherol (0.504 g) was added. This mixture constituted the oil-phase of the Pheroid vesicle formulations. The water-phase was made up of the ^{14}C-CQ in nitrous oxide saturated water. The required amount of ^{14}C-CQ (0.2 g) was added to 3.6 g of the prepared nitrous oxide water and heated to 70°C. The prepared water phase (3.8 g) was then combined with the oil-phase (0.2 g) to achieve a total volume of 4.0 ml. The mixture was homogenised with a Heidolph Diax 600 homogeniser (Labotec, South Africa) at 13500 rpm for at least four minutes or until below 40°C.

2.2.3 Entrapment study

Traditional *in vitro* methods, in which the onset and conclusion of entrapment could be tested, could not be used on this occasion because of the radioactive nature of the product. Owing to the hydrophilic nature of CQ, the dynamic equilibrium processes of entrapment into Pheroid vesicles warranted further investigation, especially with regard to optimal entrapment time. Several formulations were prepared and each allowed a different time period for entrapment (1, 12, 24 and 30 hours).

The formulations were then administered to rats, which were sacrificed after one hour and the distribution of the radiolabelled chloroquine was determined.

2.2.4 *In vivo* bio-distribution study

A number of animal models may be used, including guinea pigs, rabbits, rats, mice and non-human primates. In this study the rat was the proposed model, as rats are relatively inexpensive, available and easy to handle. It was decided not to use mice, because the volume of labelled Pheroid that can be administered and the resultant level of radioactivity in the various organs may be too low for detection. A previous study has shown the rat model to be adequate.[37]

Twenty four Sprague Dawley rats were used in two groups of 12 rats each, with three different time points. Ethics approval for the experiments was gained from the North-West University Research Ethics Committee (NWU-000079-11-A5). The first group served as the control to which the ^{14}C-CQ was administered orally to establish a typical absorption profile through the intestinal tract. In the second group the ^{14}C-CQ was entrapped in the Pheroid vesicles and was also administered orally to show whether the system enhanced oral absorption and bio-distribution.

The following combinations were administered to the two respective treatment groups:

- ^{14}C-CQ alone in DI water *per os*;

- ^{14}C-CQ entrapped in Pheroid vesicles *per os*.

Four animals from each of the treatment groups were sacrificed at three different time points, after one hour, two hours and four hours. These intervals were determined during a previous study[37] and due to the fact that the Pheroid speeds up the absortion and distribution of drugs. The aim of the study was specifically to gaze at bio-distribution of Pheroid containing a model drug and was not directed at determining the change in circulating half-life. Each group contained four rats to decrease the number of rats that were sacrificed, while still giving good statistical results.

All the organs, including the skeleton, were harvested and the radioactivity counted in order to determine whether and how the Pheroid had altered the tissue localization. The radioactive counting was an extremely complex procedure, as extensive sample preparation was required and because carbon-14 as a beta-emitter is relatively weak in the context of radioactive emission. Because the literature on sample preparation was vague and inconclusive, a validation method had to be set up. Following the preparation, the radioactivity measurements were carried out using a triathler liquid scintillation counter (Hidex, Turku, Finland) or Perkin-Elmer Tri-Carb 3100 TR scintillation spectrophotometer.

2.2.5 Sample analysis

Certain parameters of the sample preparation technique were investigated and improved in order to increase measurement accuracy: (i) The amount of time and the processes needed to completely dissolve the tissue samples; (ii) The effect and concentration of H_2O_2 on the total counts as well as; (iii) the effect of the increased time before the measurement was to be performed.

These investigative findings can be seen in the annexures and led to the following optimised procedure, which was employed throughout the study. Weighed tissue samples (~0.2 g) were digested overnight in 1 mL of Biosol tissue solubilizer at 50 °C. Hemoglobin-coloured samples were decolourized by adding a maximum of 0.2 mL of 30% H_2O_2 for 60 minutes. A scintillation cocktail (Bioscint, National diagnostics laboratories, USA) was added to each sample. The radioactivity concentration was determined by counting the samples for 10 minutes, using a Perkin-Elmer Tri-Carb 3100 TR scintillation spectrophotometer. Standards of known activity were prepared by adding a ^{14}C-labeled compound of known activity to different organ samples, covering a range of spectral quench parameter of the isotope (SQPI)

values. The recovery of the known activity was 3% and provided credibility for the measured activities in the unknown samples. The samples were left for at least 30 minutes in the dark before the readings were taken.

2.2.6 Statistical analysis

The experimental data was assessed in terms of the percentage of injected dose per gram of the sample (%ID/g) versus time. To compare the means of the groups, the nonparametric Mann-Whitney test was used. The computer package *Statistica*[38] was used to analyse the results.

3. Results

3.1 Entrapment results

The results were as follows: after one and twelve hours there was very little absorption and distribution of the CQ within the body, owing to poor entrapment. The levels increased to an adequate amount after 24 hours, but remained the same at the 30-hour mark. These findings led to the conclusion that the optimal entrapment time was 24 hours. This was the shortest tested time in which the most or best CQ entrapment transpired.

3.2 Tissue distribution results

The drug levels of the Pheroid-entrapped ^{14}C-CQ in the blood circulation were enhanced at all the measured time points and often showed as much as double the intensity when compared with normal ^{14}C-CQ levels as shown in Table 1.

Table 1. Comparisons of the different organs/body tissues at 1, 2 and 4 hours after administration and also the difference between the references ^{14}C-CQ and ^{14}C-CQ entrapped within the Pheroid vesicles.

Organ	1 hour (%ID/g)[a]		2 hours (%ID/g)[a]		4 hours (%ID/g)[a]	
	Pheroid/ ^{14}C-CQ	^{14}C-CQ	Pheroid/ ^{14}C-CQ	^{14}C-CQ	Pheroid/ ^{14}C-CQ	^{14}C-CQ
Heart	2.783±1.046	0.535±0.091	2.426±0.950	1.829±0.844	0.376±0.284	0.067±0.025
MWT	0.03		0.193		0.03	
Lungs	0.299±0.094	0.162±0.053	0.541±0.075	0.134±0.025	1.577±0.534	0.088±0.019
MWT	0.112		0.03		0.03	
Liver	1.286±0.080	0.166±0.045	6.278±0.509	0.652±0.244	13.095±4.320	0.944±0.510
MWT	0.03		0.03		0.03	
Kidney	3.308±1.043	0.692±0.244	3.140±0.599	1.928±0.819	3.427±1.623	1.09±0.260
MWT	0.03		0.06		0.03	
Muscle	11.68±0.891	1.748±0.656	12.478±3.269	10.387±1.857	5.252±3.239	0.328±0.112
MWT	0.03		0.193		0.03	
Blood	0.564±0.136	0.193±0.079	0.464±0.186	0.179±0.072	0.414±0.193	0.257±0.050
MWT	0.03		0.03		0.665	
Total	19.921	3.498	25.330	15.112	24.143	2.776

[a]**Percentage of injected dose per gram biological sample x 10^2; MWT = p-values of Mann-Whitney-test; Amounts given are mean and standard deviation.**

Statistically significant enhanced oral absorption of the Pheroid entrapped ^{14}C-CQ through the intestinal tract was seen when compared with the normal absorption levels of ^{14}C-CQ.

At each measured time point, the entrapped CQ levels were found to have improved in contrast with the reference CQ. After one hour, there was 5.7 times more of the drug present in the noted organs. After two hours, the difference was closer at 1.7 but after four hours it increased again to 8.7 times more entrapped CQ present. These comparative drug levels were calculated from the combined percentages in Table 1 of the injected dose administered per gram of the sample. With this information, it could be argued that the Pheroid system speeds up the whole absorption process and also prolongs the stay of the drug within the body and organs, possibly through decreasing the metabolism and excretion of the drug. These high drug levels for prolonged time peroids could lead to side-effects, but this would be looked at in future studies.

Figure 2. A graphic representation of the differences between reference ^{14}C-CQ (green) and entrapped ^{14}C-CQ (blue) and the different time points (1h, 2h and 4h) as well as the different organs. Evaluation is done in percentage of injected dose per gram of sample (%ID/g). A was 1 hour, B after 2 hours and C after 4 hours after administration.

Figure 2 shows clear differences between the two administered formulations, as well as the faster absorption after one hour (A) and the prolonged stay of the drug within the organs after four hours (C) of the Pheroid-entrapped ^{14}C-CQ. It is noted that in both formulations, the highest accumulation of drugs combined in the organs occurs after two hours (B), but while the amount stays relatively the same after four hours for the entrapped ^{14}C-CQ, it drops

sharply for the ^{14}C-CQ administered on its own. Another noteworthy fact is that the Pheroid system does not change the tissue localization of the chloroquine, but only increases the levels within the different organs and prolongs the stay, as can been seen in Figure 2. This is also important, because a change in body distribution could result in the delivery system actually delivering the drug away from its target sites. A preliminary study displayed that the Pheroid is taken up into the mitochondria of cells, where the fatty acid component of the Pheroid may be metabolised to produce energy and the active ingredient is released due to this. Table 1 also shows that drug levels are much higher in the organs when compared with blood levels, which was consistent with results from a previous study in which the distribution of ^{14}C-CQ in rats was investigated.[39] A notable absence from the organs concerned is the brain; there were no ^{14}C-CQ traces in the brain. The amounts of ^{14}C-CQ in the intestine and stomach were left out, as they were the point of origin, owing to *per os* administration.

3.3 Organ comparison results

The presence of the ^{14}C-CQ in the blood, liver, lungs and muscles was prolonged after oral administration of the Pheroid-entrapped compound. Absorption of the entrapped ^{14}C-CQ was enhanced in all cases, indicating a possible faster onset of action. No brain absorption was seen in either of the two formulations, which was a good result for the Pheroid.
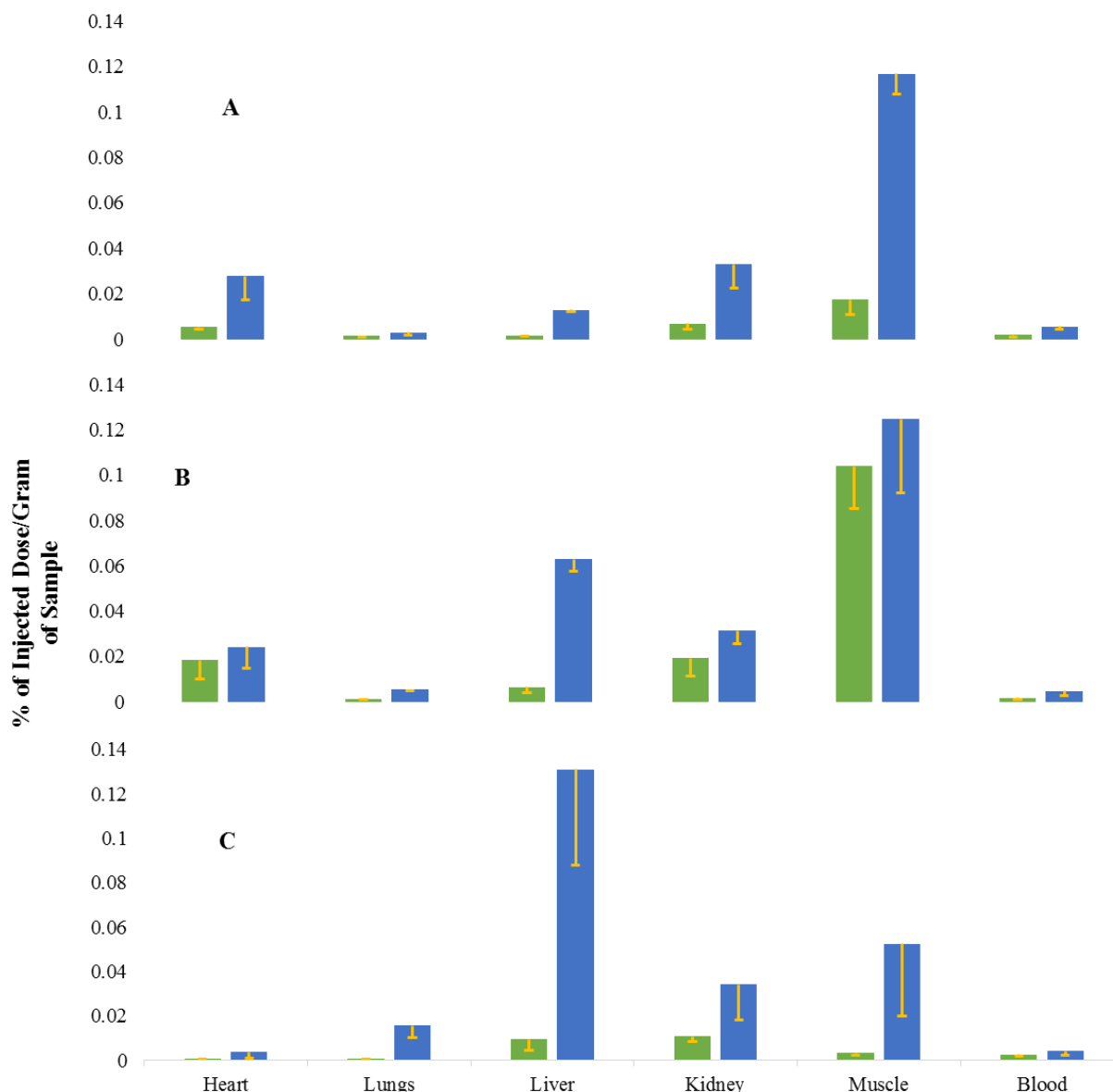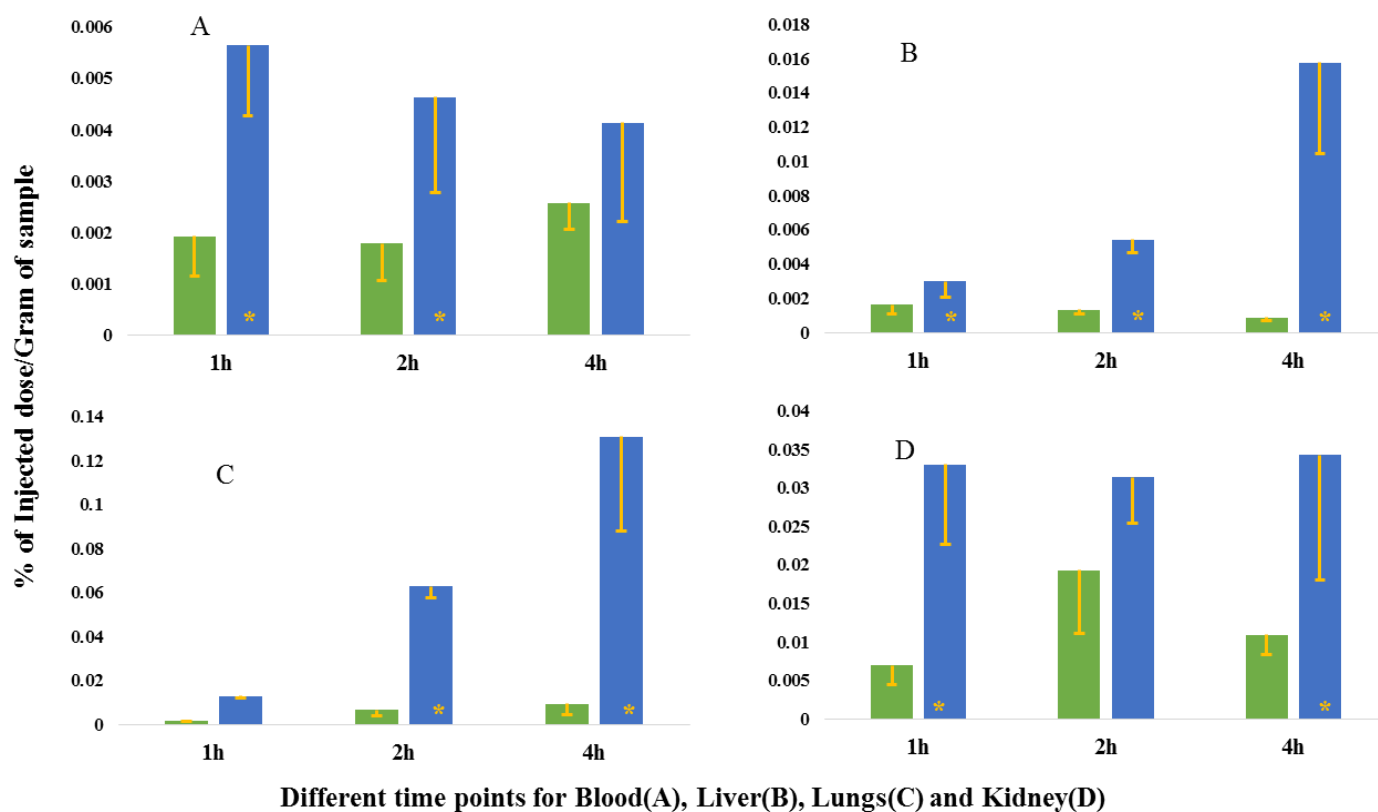
Figure 3. A graphic representation of the differences between reference ^{14}C-CQ (green) and entrapped ^{14}C-CQ (blue) in the noted different organs; Blood (A), Liver (B), Lungs (C) and Kidney (D) 1, 2 and 4 hours after administration. Evaluation is done in percentage of injected dose per gram of sample (%ID/g). * The values obtained was statistically significant (p < 0.05).

As shown in Figure 3A, after one hour the drug concentration levels within the blood are three times higher for the Pheroid-chloroquine (0.00564 ± 0.0013) than for the reference chloroquine (0.00193 ± 0.00078) and this should result in a quicker onset of action of the drug. After four hours, the levels are still higher, which points to the Pheroid prolonging the stay of the drug in the blood. Table 2 further illustrates the enhancement achieved with the use of the Pheroid system. A 2.9 factor increase was seen after one hour, 2.5 after two hours and 1.6 after four hours. These high levels are not toxic, although they are higher than

reference levels. This is due to the fact that in this study the pure base chloroquine was used. For the base form the absorption levels are very low when compaired to chloroquine sulphate and therefore toxic levels won't be reached. However if chloroquine sulphate is used in future studies, toxicity could be a problem.

Table 2. Illustrating enhanced blood distribution over the 4 hour time period.

Blood	%ID/g[a]	Enhancement Factor
[14]C-CQ 1h	0.193	-
Pheroid/^{14}C-CQ 1h	0.564	2.922
[14]C-CQ 2h	0.179	-
Pheroid/^{14}C-CQ 2h	0.464	2.592
[14]C-CQ 4h	0.257	-
Pheroid/^{14}C-CQ 4h	0.414	1.61

[a]Percentage of injected dose per gram biological sample x 10^2

Another aspect shown in Figure 3 B is that the absorption is extensively enhanced, as is its accumulation in the liver. After four hours, the amount of Pheroid-chloroquine (0.13095 ± 0.04320) is 13.8 times higher than for the reference chloroquine (0.00944 ± 0.00509), as illustrated in Table 3. After one hour there was a 7.7 factor increase, a 9.6 factor increase after two hours and 13.8 after four hours.

Table 3. Illustrating enhanced liver distribution over the 4 hour time period.

Liver	%ID/g[a]	Enhancement Factor
^{14}C-CQ 1h	0.166	-
Pheroid/^{14}C-CQ 1h	1.286	7.746
^{14}C-CQ 2h	0.652	-
Pheroid/^{14}C-CQ 2h	6.278	9.628
^{14}C-CQ 4h	0.944	-
Pheroid/^{14}C-CQ 4h	13.095	13.871

[a] **Percentage of injected dose per gram biological sample x 10^2**

The results for the lungs shown in Figure 3C compare well with the results for the liver, indicating enhanced absorption and prolonged stay as a result of Pheroid-entrapment. At the four-hour time point, the drug levels are 17.8 times higher for the test formulation (0.01577 ± 0.00533) compared with the reference (0.00088 ± 0.0001). The increase factors were 1.8, 4 and 17.9 after one, two and four hours respectively (Table 4).

Table 4. Illustrating enhanced lung distribution over the 4 hour time period.

Lungs	%ID/g[a]	Enhancement Factor
^{14}C-CQ 1h	0.162	-
Pheroid/^{14}C-CQ 1h	0.299	1.845
^{14}C-CQ 2h	0.541	-
Pheroid/^{14}C-CQ 2h	0.134	4.037
^{14}C-CQ 4h	1.577	-
Pheroid/^{14}C-CQ 4h	0.088	17.92

[a] **Percentage of injected dose per gram biological sample x 10^2**

The clearance via the kidneys remained consistent throughout the four-hour test period for the test formulation, in contrast with a hike in excretion at the two-hour time point for the reference ^{14}C-CQ , as can be seen in Figure 3D. This is consistent with the other results, as there are high amounts of the drug within the body at all the time points for the entrapped ^{14}C-CQ. This means that there is consistently higher excretion. For the reference compound, the peaks in drug levels are at the two-hour time point, so there is higher excretion at this point.

4. Discussion

The increased absorption of orally administered ^{14}C-CQ entrapped in Pheroid over ^{14}C-CQ alone could possibly lead to *in vivo* micromolar drug concentrations, as opposed to the nanomolar concentrations normally associated with CQ administered on its own from safe/normal dose regimens. This could allow for PCD to occur and could, in return, be responsible for the re-entry of CQ as a first-line drug against malaria infections.

The fact that there was zero brain absorption for CQ when entrapped in the Pheroid is an encouraging sign and a good outcome for the Pheroid, as drug crossing of the blood brain barrier could lead to neurotoxicity in other formulations or drugs.

Likewise, increased oral absorption of Pheroid-entrapped compounds has previously been noted[20,29] and, together with the potential of the formulation described here, could be of significant importance in the treatment of malaria, since CQ is inexpensive and widely available. Another crucial characteristic of the Pheroid formulation is that it could facilitate micromolar drug concentrations from normal, safe drug dosage regimes that usually produce only nanomolar levels.

One can deduce from the results that the Pheroid system speeds up the whole absorption procedure and also prolongs the stay of the drug within the body and organs, thereby decreasing excretion of the drug. This will lead to decreased dosage intervals, i.e. one tablet once a day, instead of two tablets three times a day. Consequently this should lead to superior patient compliance. Patient compliance failure is one of the main reasons for resistance occurring with drug regimens, so improved compliance would also, in turn, help stop or decrease resistance to malaria treatment.

One key aspect to be considered is that the sourced ^{14}C-CQ is the pure base form and not the phosphate salt combination, and that the concentration levels of both the entrapped and non-entrapped drug was significantly lower than it would be for chloroquine phosphate. However, the comparison between the two formulations in this study still holds significant importance and the relative results should be the same for chloroquine phosphate.

The intra-erythrocytic life cycle of *Plasmodium falciparum* that takes place in the blood is one of the main targets in the treatment of malaria, and is responsible for the disease-generating phase of the malaria parasite.[40,41] The faster absorption, more pronounced and prolonged stay of CQ in the blood will certainly be of advantage in the treatment of malaria. Since CQ targets mainly the intra-erythrocytic stages of the malaria parasite, the liver results reported do not have direct implications when it comes to the target areas of CQ. However, the findings should still be significant as many antimalarial and other drugs have been successfully entrapped in the Pheroid and these could possibly have targets in the liver, such as in *P.vivax* associated malaria. The lung results do not necessarily relate to the effect of CQ or malaria, but support the investigation of Pheroid combined with anti-tuberculosis drugs for pulmonary tuberculosis.

Higher levels were also seen in other organs, but since this study was fixated on malaria it was not discussed in detail. However it could prove to be useful in future studies.

5. Conclusion

The orally administered Pheroid system produced much higher ^{14}C-CQ organ and blood concentrations and also prolonged the stay within the organs and blood, as opposed to that of the reference ^{14}C-CQ orally administered. The drug concentrations of the ^{14}C-CQ entrapped in the Pheroid vesicles in the blood circulation are much higher than associated ^{14}C-CQ administered on its own, this may allow the programmed cell death (PCD) mechanism to complement the effectiveness of CQ against the malaria parasite.

From the increased blood concentrations levels, it is concluded that the use of Pheroid formulations is of possible significance in enhancing the effectiveness of CQ against the intra-erythrocytic stages of the malaria parasite.

This enhanced absorption and prolonged stay of the compound will also be very useful in future therapy regimens, as less of the drug could be given less often, assisting in patient compliance. A reduced amount of medication needs to be administered with longer time periods between administrations.

The lung concentrations results do not directly reflect on CQ or malaria, but bodes well for the Pheroid, as research is currently in progress in which the Pheroid is being combined with anti-tuberculosis drugs. There are many other possible combinations of formulations for which the lungs could be a target area. As far as tuberculosis is concerned, the main treatment target is the lungs.

The results certainly warrant future *in vivo* and *in vitro* studies into the effectiveness of a CQ-Pheroid formulation against malaria and the perfecting of this formulation.

6. Acknowledgements

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CHAPTER 5

SUMMARY AND FUTURE PROSPECTS

Although the Pheroid has shown the ability to significantly increase absorption and bio-distribution of drugs into the body through the gut (Steyn *et al.*, 2011, Aminakem *et al.,* 2012; Gibard, 2012; Grobler *et al.,* 2014), the exact mechanism is still open to debate.

This study was conducted to shed some light on this uncertainty and possibly gather more information. The approach followed in this study was to use radiolabels (RL) entrapped in the Pheroid system for easy tracing of the detailed bio-distribution of these formulations in Sprague Dawley rats.

The selection of the RLs for this study, played an immense part in the accomplishment of the objectives for the study. The first RL chosen, ^{99m}Technetium Methylene Diphosphonate (^{99m}Tc MDP), was selected because it is relatively inexpensive and easy to work with. The previously-mentioned motives made for a less intimidating start to the study and also provided valuable practice and experience in working with RL and in a radioactive environment.

The second RL was chloroquine (CQ), labelled at the carbon 14 (^{14}C-CQ). Owing to costs, ^{14}C-CQ was used only in the second part of the study. It was hypothesized that the RL would provide a valuable insight into the bio-distribution of CQ entrapped in the Pheroid and would provide further support for a previous study in this study group (Gibard, 2012). A further point addressed in this selection was the worldwide problem of malaria.

Although there has been a steady decrease in the number of clinical cases and deaths since the year 2000, malaria is still responsible for between 135 and 287 million clinical cases each year and between 473 and 789 thousand deaths each year (WHO, 2013). In other words, it is still a major health problem and carries an extended financial burden. A further problem reported is the occurrence and spread of resistance to antimalarial drug therapy (WHO, 2013).

For these reasons, cheap and new formulations of existing drugs are crucial in the fight against malaria. This is why CQ and the Pheroid system are of particular relevance. Both are inexpensive and readily available.

Before resistance of *P. falciparum* to CQ became apparent in the 1960s, it was the backbone for anti-malarial therapy and was widely used (Trape *et al.*, 1998; O'Neil *et al.,* 2012; Kaur *et al.,* 2009). In the near future the re-introduction of CQ as a vital therapeutic possibility in combined therapies for malaria could be seen and the reasoning behind this is the following;

1. Predictions that the efficacy of CQ could return due to its withdrawal for such a long period (Kublin *et al.,* 2003; Laufer *et al.,* 2006).

2. The inexpensiveness of CQ.

3. New suggested mechanisms of action (Ch'ng *et al.,* 2014; Ch'ng *et al.,* 2012).

4. Smart formulations.

Developing new smart formulations of existing drugs with the help of carrier systems like the Pheroid will play a vital role in future malaria therapies. The Pheroid formulation consists mainly of nitrous oxide water and ethyl esters of essential fatty acids and has the capacity to entrap, transport and deliver hydrophilic and hydrophobic drugs of noticeably diverse pharmacological classes (Gibard, 2012; Grobler, 2008; Steyn et al., 2011; Grobler et al., 2009).

Pheroid technology has the capacity to increase the absorption of drugs, as has previously been established in both *in vitro* and *in vivo* studies (Aminakem *et al.,* 2012; Grobler *et al.,* 2014; Du Plessis *et al.,* 2012; Slabbert *et al.,* 2011; Du Plessis *et al.,* 2014) and will play an ever-increasing role in these new smart formulations.

^{99m}Tc MDP was selected as the ideal isotope for the following reasons:

- It is a gamma ray emitter, which is a penetrating form of radiation;

- Its detection is straightforward;

- It was used for many years as an imaging agent (Subramanian *et al.,* 1975; Wang *et al.,* 1979; Rudd *et al.,* 1977; Davis & Jones, 1976);

- It has excellent physical characteristics (labelling occurs spontaneously);

- It is available by means of a generator; and

- It is relatively inexpensive (Bouquot *et al.,* 2012; Schwochau, 1994; Rey, 2010; Weber *et al.,* 1976).

Another property of ^{99m}Tc MDP is that it accumulates in bone tissue and is not absorbed through the intestinal tract (Subramanian *et al.,* 1975). The above details further added to the rationale behind its selection as the choice isotope with which to work. Any oral absorption, enhanced absorption levels or change in the bio-distribution (i.e. other than bone) can therefore be attributed to entrapment and delivery by Pheroid.

Chapter 3 defines the bio-distribution and bioavailability studies (Figure 1) conducted on Sprague Dawley rats (NWU-00079-11-A5).
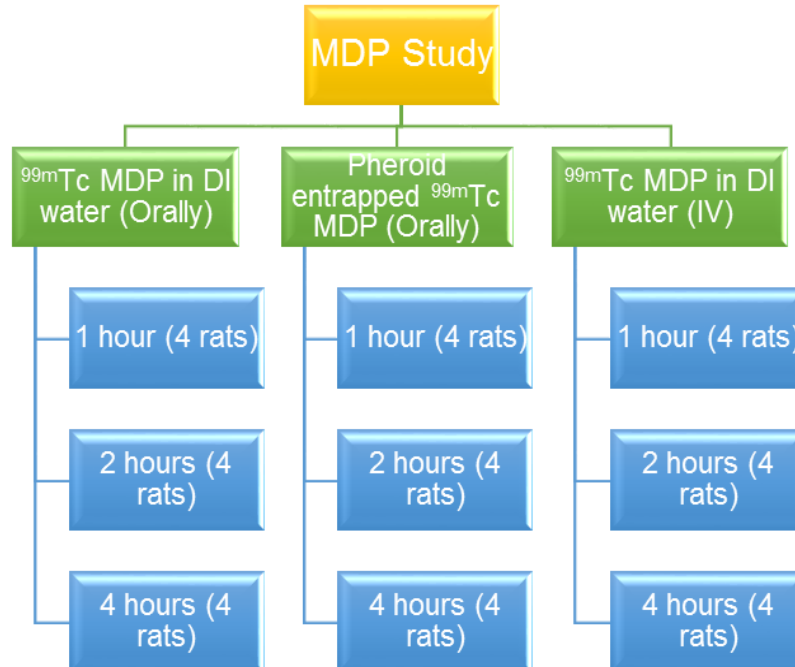
Figure 1: Design layout for the ^{99m}Tc MDP study.

In addition to this, an imaging study was also done to track bio-distribution visually (Figure 2).
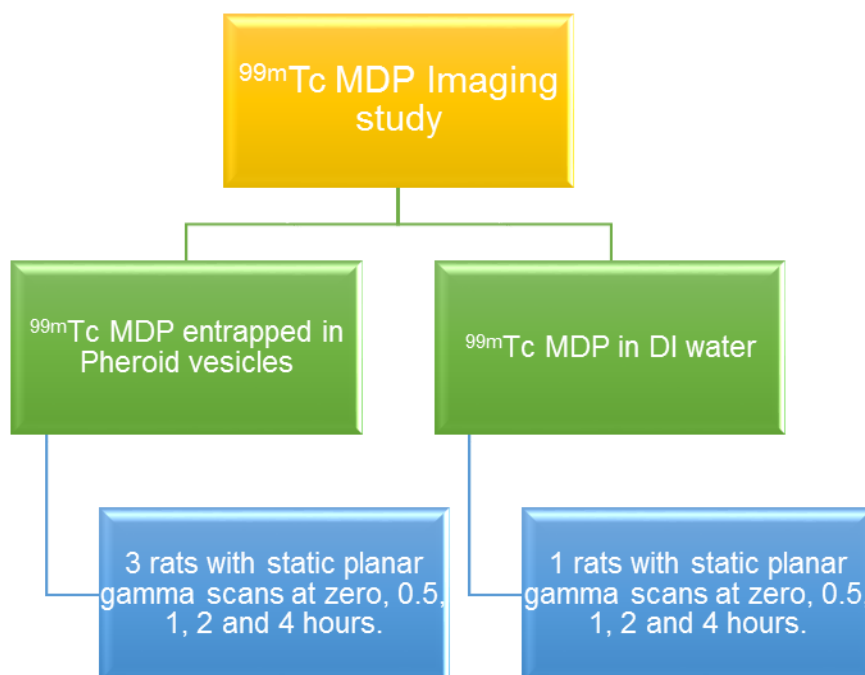
Figure 2: ^{99m}Tc MDP imaging study.

After each time point, there was a marked difference between the two *per os* formulations. The Pheroid was responsible for all these increases. An increase of a factor of 9.75 was noted in the organs after comparing the collective percentages of the injected dose administered to the two *per os* groups, per gram of the sample. The conclusion that, the Pheroid system could lead to the absorption of a non-absorbable drug through the intestinal tract was made.

A comparison of the IV reference formulation with that of the Pheroid entrapped ^{99m}Tc MDP also yielded interesting results. The four-hour time period resulted in double the drug concentrations present in the specific organs (heart, lungs, liver, kidney, muscle, femur and blood) after the IV injection as for the Pheroid entrapped ^{99m}Tc MDP. However the Pheroid system still relates comparatively well, considering that IV administration always yields 100% bioavailability. The conclusion is that, after *per os* administration of Pheroid entrapped ^{99m}Tc MDP, as high as 50% bioavailability can therefore be expected.

The results of the most important organ in terms of ^{99m}Tc MDP accumulation and the most relevant biological sample, the femur and the blood respectively, were studied further. In both cases there were higher drug concentrations for the entrapped ^{99m}Tc MDP. A quicker onset of action and prolonged stay of the drug after being entrapped in the Pheroid system was also reported.

The results of the imaging study are clearly portrayed in Figure 3. The images match the results given above.

Non-entrapped vs Entrapped
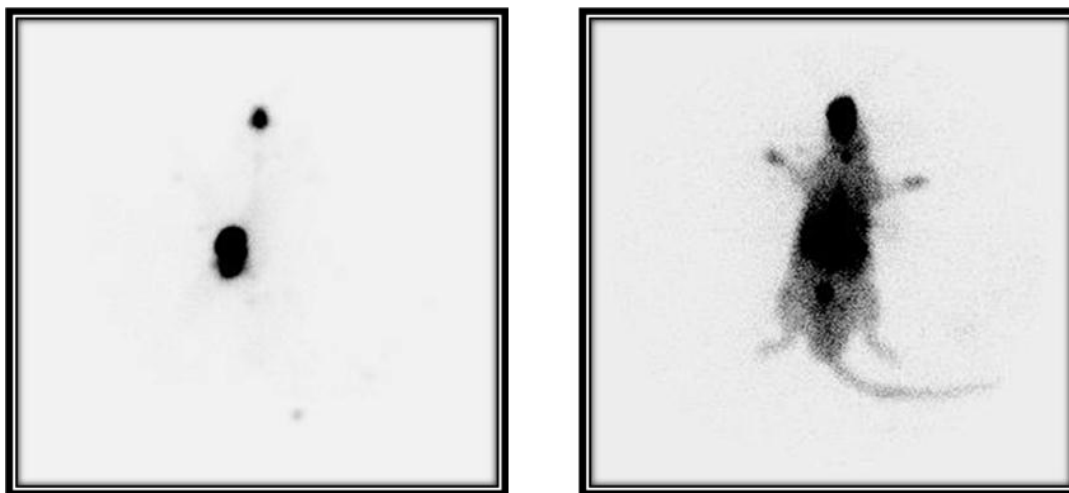
Figure 3: Images depicting the differences in absorption between entrapped and non-entrapped ^{99m}Tc MDP after 4 hours.

The tissue distribution results gathered from the ^{14}C-CQ tests from Chapter 4 were very similar to those of ^{99m}Tc MDP in Chapter 3. Boosted absorption and a prolonged stay were also reported, which was consistent with previous studies (Gibard, 2012).

The layout of this study was very similar to the previous studies mentioned above. The only exceptions were the omission of the IV group and the manner in which the samples were analyzed, that is, liquid scintillation.

The amount of ^{14}C-CQ present in the noted organs was compared for the two different formulations, namely reference and test. At all three time points there was significantly more ^{14}C-CQ present in the organs, for the formulation that was entrapped in the Pheroid. Following this, it could be debated that the Pheroid system speeds up the entire absorption process and also prolongs the stay of the drug in the body and organs, possibly through reducing the metabolism and excretion of the drug.

As blood plays an essential role in malaria disease progression, the results are promising. One hour after administration, the ^{14}C-CQ concentration in the blood was three times higher for the test formulation (0.00564 ± 0.0013) than for the reference formulation (0.00193 ± 0.00078). Furthermore, a 2.6 factor increase was seen after two hours and 1.6 after four hours. The fact that the intra-erythrocytic life cycle of *P. falciparum* takes place in the blood makes it the main target in the treatment of malaria (Bergqvist & Domeij-Nyberg, 1983; Fitch, 1969). For this reason, the improved absorption and extended stay of CQ in the blood may be of benefit in the treatment of malaria.

Another vital organ in malaria, the liver, also produced interesting results. Absorption was expansively improved, as was its accumulation and stay in the liver. After four hours, the amount of test formulation (0.13095 ± 0.04320) was 13.8 times higher than for the reference formulation (0.00944 ± 0.00509). A 7.7 factor increase was seen after one hour and there was a 9.6 factor increase after two hours. The fact that CQ mainly targets the intra-erythrocytic stages of the malaria parasite in the blood, the liver results described have no direct implications for CQ or its associated targets. Nevertheless, the outcomes are still of importance in the other antimalarial drugs that specifically target the liver stages of the malaria parasite.

These increases in oral absorption have been described in previous studies (Awasthi & Das, 2013; Grobler, 2014) and, together with the potential of these new formulations, they could prove useful in the treatment of malaria, as CQ is inexpensive and widely available.

Judging by the increased blood concentration levels, it can be concluded that the use of Pheroid formulations could play a role in enhancing the effectiveness of CQ against the intra-erythrocytic stages of the malaria parasite.

The enhanced absorption and prolonged stay of the formulation should also lead to forthcoming therapy regimens in which a lowered concentration of the drug could be administered less often. Enhanced patient compliance should result, as a reduced amount of drug would have to be administered with longer intervals between administrations.

Recommendations to consider for future studies on ^{99m}Tc MDP and Pheroid technology may include the following:

- Enhancement and development of the ^{99m}Tc MDP-Pheroid formulation;

- Further trials using larger and more relevant animal models;

- Human clinical trials on the effectiveness and necessity of the availability of orally administered ^{99m}Tc MDP.

The ^{14}C-CQ results lends it self to future *in vivo* and *in vitro* studies on the effectiveness of a CQ-Pheroid formulation against malaria and the perfecting of this formulation.

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Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Highlights

Highlights are a short collection of bullet points that convey the core findings of the article. Highlights are optional and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

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Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

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As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

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The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

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• E-mail address

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• Phone numbers

• Disk is enclosed

• The electronic version and the hardcopy of the manuscript are identical

• Disk has been labelled with article details (first author, first words of title) file name(s) media format (e.g., PC, Mac) file format (e.g., Word, LaTeX)

• All text pages

• Keywords

• Original artwork (high-quality prints)

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

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AUTHOR GUIDELINES: JOURNAL OF NANOMEDICINE:

NANOTECHNOLOGY, BIOLOGY, AND MEDICINE

GENERAL REQUIREMENTS

1) The manuscript must be of INTERDISCIPLINARY nature, representing the overlapping fields of biology, and medicine with nanotechnology.

2) NOVELTY and ORIGINALITY are of primary importance. The results presented must significantly advance the field and improve scientific knowledge. Manuscripts with similar approaches that have already been undertaken by other groups should be submitted to appropriate journals. Originality of submissions is routinely checked by the editors using professional software.

3) SIGNIFICANCE: Accounts of research must appeal to a broad readership. In the cover letter, authors should provide a paragraph explaining how the work differs from the knowledge available in the literature and describe how it improves or has the potential to improve medicine.

Experimental studies must include at least in vitro results, although in vivo is preferred. Accordingly, synthesis and characterization of nanotechnology-based medicines (i.e., substances that promote healing) must accompany bioavailability and toxicity data and their comprehensive evaluation.

Manuscripts that do not satisfy these general requirements will not be sent out for peer review and will be returned to the authors.

ORIGINAL ARTICLES: Full-length articles describe a full account about hypothesis-based research or theory in nanomedicine. Length should not exceed 5,000 words (including body text, and figure legends), and the article should have no more than 6 figures in the main article. No more than 50 references should be cited. Include an Abstract of 150 words or less without internal subheadings. The body text should include the four separate headings: Background, Methods, Results, and Discussion. Use of supplementary materials is recommended for detailed descriptions (see below).

TITLE PAGE: The title page should contain these elements: full title, all authors' names, academic degrees, and affiliations; short title (not to exceed 50 characters, including spaces); name and complete address for corresponding author and address for reprints if different from correspondence; fax and telephone number and e-mail address; word count for abstract; complete manuscript word count (including body text and figure legends, but excluding abstract, title page, and references), and number of figures/tables. There is no length limit on Supplementary Material. **Manuscripts with over-the-limit word counts will be returned to the authors without further review.** The title page must include statements

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TEXT: For Communications and Original Articles, the text should include the headings Background, Methods, Results, and Discussion. Only essential data and descriptions should be provided in the Methods and Results. All experimental details including synthetic and analytical procedures must be provided as SUPPLEMENTARY MATERIALS (see details below). All pages must be numbered. Abbreviations must be parenthetically notated at first mention in the text. Each table and figure must be mentioned in the text. Reports of studies on humans and animals must indicate that each study has been approved by an institutional review committee and the procedures followed are in accordance with institutional guidelines. Provide generic rather than trademarked names of drugs.

ACKNOWLEDGMENTS: The acknowledgments section recognizes substantive contributions of individuals who do not meet the criteria for authorship (see below). The Editorial Office must receive written, signed consent from each person recognized in the acknowledgments to be mentioned in the article because acknowledgment can imply endorsement of data and conclusions. (See a sample of an Acknowledgement.) Upload each permission separately in the online system. Do not include statements of funding, conflicts, or other disclosures in the Acknowledgments; these must appear on the title page.

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References can be in any style or format as long as the style is consistent. Author(s) name(s), journal title/book title, article title, year of publication, volume and issue or book chapter and the pagination must be present. The reference style required by the journal will be applied to the published version by Elsevier.

For those who wish to format the references, if using EndNote software, the journal reference style is Embellished Vancouver (Style 3a). Reference format should conform to the examples shown below, and journal abbreviations should conform to the style used in the Cumulated Index Medicus.

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Journals: authors' last names and initials; title of article; *journal name;* date; **volume number**, and inclusive pages (list all authors when six or fewer; when seven or more, list six and add et al):

1. Graeber GM, Gupta NC, Murray GF. Positron emission tomographic imaging with fluorodeoxyglucose is efficacious in evaluating malignant pulmonary disease. *J Thorac Cardiovasc Surg* 1999; **117:719**-27.

2. Lytle BW, Blackstone EH, Loop FD, Houghtaling PL, Arnold JH, Akhrass R, et al. Two internal thoracic artery grafts are better than one. *J Thorac Cardiovasc Surg* 1999; **117:855**-72.

Books: authors' last names and initials; chapter title, editor's name, book title, edition, city, publisher, date, and pages:

1. Mollnes TE. Analysis of in vivo complement activation: In: Herzenberg LA, Weir DM, Herzenberg LA, Blackwell C, editors. *Weir's handbook of experimental immunology.* Volume 78, 5th ed. Boston: Blackwell Science; 1997, p. 1-8.

FIGURES: Figures must be of professional quality and suitable for evaluation purposes. Be sure all font is at a readable size. When possible, please use first-generation artwork. If a revision is requested, source files must be provided: Figures must be submitted in electronic figure file format:.tif, .eps, or .jpg format. Figures may be black and white line art, graphs, halftones (photographs), or color. Line art (black lines on a white background) must have a minimum resolution of 1,000 ppi. Combination line art (eg, line art with gray fill patterns) must be created at 500 ppi. Black and white or color photographs must have a minimum resolution of 300 ppi.

Illustrations should be saved at the recommended resolution setting and sized as close to a column width (3 to 4 inches) as possible. Create figures in scale with each other to the extent possible. Avoid background gridlines and other formatting that do not convey information (eg, superfluous use of 3-dimensional formatting, background shadings). Use uniform lettering and sizing of all original artwork. As a general rule the lettering on the artwork should have a finished, printed size of 7 points. Smaller lettering will yield barely legible text. Recommended font choices include Arial, Helvetica, or Symbol. Labeling of multipart figures (eg, A, B, C...) should use capital letters only and should be done consistently, preferably using uppercase type (Arial, Helvetica or Universe, 11 or 12 points) in the lower-right corner of the figure. Avoid headings on the figure when possible. Additional

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SUPPLEMENTARY MATERIALS: *Nanomedicine: NBM* accepts supplementary data files to accompany the online article, allowing authors to support and enhance their papers. Supplementary materials may include experimental details, additional images, background datasets, video clips, etc. Experiments should be described in such detail that someone trained in the art could repeat the experiment or measurement. Please provide text-based data as a separate PDF file when submitting your manuscript. All supplementary materials are subject to peer review but will not be edited by *Nanomedicine: NBM* and will be posted as provided by the authors. There are no limitations for length, figures, and references in Supporting Materials.

RAW DATA RESULTS FROM SECTION 3 OF CHAPTER 3 FROM

MANUSCRIPT 2

Ref: Oral 1 hour

Organ	%ID/g	STD
Brain	0	0
Thyroid	1.205838	0.317752
Heart	0.011839	0.008568
Lungs	3.275795	0.901112
Stomach	1.801854	2.077541
Small Intestine	4.472381	2.416062
Large Intestine	4.228486	1.628251
Liver	0.01316	0.008411
Kidney	0.127618	0.089529
Bladder	1.59586	0.505831
Muscle	0.001958	0.002261
Femur	0.023245	0.011324
Tail	0.011386	0.003697
Blood	0.030466	0.012779

Test: Oral 1 hour

Organ	%ID/g	STD
Brain	0	0
Thyroid	1.551565	0.860267
Heart	0.021135	0.021135
Lungs	0.893364	0.839111
Stomach	7.907888	0.65936
Small Intestine	1.481912	0.663602
Large Intestine	3.316488	2.71737
Liver	0.043424	0.014071
Kidney	0.080457	0.074175
Bladder	0.305306	0.305306
Muscle	0	0
Femur	0.059284	0.059284
Tail	0.061235	0.034665
Blood	0.049625	0.020624

Ref: Oral 2 hour

Organ	%ID/g	STD
Brain	0	0
Thyroid	0.772246	0.310281
Heart	0	0
Lungs	0.130217	0.016411
Stomach	3.850621	4.01853
Small Intestine	2.12546	0.827936
Large Intestine	6.851824	1.206696
Liver	0.004089	0.003311
Kidney	0.006229	0.004235
Bladder	0.247484	0.161555
Muscle	0	0
Femur	0	0
Tail	0.009866	0.003851

Test: Oral 2 hour

Organ	%ID/g	STD
Brain	0	0
Thyroid	1.481836	0.952616
Heart	0	0
Lungs	0.55321	0.468761
Stomach	4.009488	0.946095
Small Intestine	1.653591	1.344223
Large Intestine	6.803284	2.581736
Liver	0.112154	0.020631
Kidney	0.292842	0.114597
Bladder	5.168411	1.132068
Muscle	0.027548	0.0248
Femur	0.212575	0.123762
Tail	0.173173	0.152726

Blood	0	0	Blood	0.062685	0.002395

Ref: Oral 4 hour			**Test: Oral 4 hour**			**Ref: IV 4 hour**		
Organ	**%ID/g**	**STD**	**Organ**	**%ID/g**	**STD**	**Organ**	**%ID/g**	**STD**
Brain	0	0	**Brain**	0	0	**Brain**	0	0
Thyroid	0	0	**Thyroid**	1.027944	0.238265	**Thyroid**	0	0
Heart	0	0	**Heart**	0.068484	0.041831	**Heart**	0	0
Lungs	0.003896	0.003896	**Lungs**	0.127441	0.069072	**Lungs**	0.03081	0.012681
Stomach	8.311218	2.094859	**Stomach**	6.122935	0.992618	**Stomach**	0.012223	0.012223
Small Intestine	4.877375	1.729243	**Small Intestine**	1.968099	0.433215	**Small Intestine**	0.014594	0.012454
Large Intestine	4.554165	1.186027	**Large Intestine**	4.111506	2.409576	**Large Intestine**	0.022441	0.012957
Liver	0.01969	0.007286	**Liver**	0.043662	0.011758	**Liver**	0.062323	0.035975
Kidney	0	0.208924	**Kidney**	0.115782	0.018717	**Kidney**	0.309884	0.011414
Bladder	0.05405	0.034257	**Bladder**	0.488259	0.213089	**Bladder**	3.815433	2.658159
Muscle	0.00451	0.00451	**Muscle**	0.007629	0.007629	**Muscle**	0	0
Femur	0	0	**Femur**	0.073721	0.036861	**Femur**	0.54454	0.225466
Tail	0.041261	0.024471	**Tail**	0.088167	0.031306	**Tail**	2.118493	0.495518
Blood	0.005007	0.005007	**Blood**	0.035527	0.009767	**Blood**	0.004151	0.0025

%ID/g: percentage of injected dose per gram of organ

Oral: Oral administration

IV: Injected

Ref: MDP injected alone

Test: MDP entrapped in Pheroid

AUTHOR GUIDELINES: JOURNAL OF LABELLED COMPOUNDS

AND RADIOPHARMACEUTICALS

Manuscript style. Use a standard font of the 12-point type: Times, Helvetica, or Courier is preferred. It is not necessary to double-line space your manuscript.

Tables must be on separate pages after the reference list, and not be incorporated into the main text. Figures should be uploaded as separate figure files.

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- Include the name(s) of any **sponsor(s)** of the research contained in the paper, along with **grant number(s).**
- Enter an **abstract** of no more than 250 words for all articles. Please see the guidance below on acceptable abstract writing for JLCR.
- **Keywords.** Authors should prepare no more than 10 keywords for their manuscript.

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An abstract is a concise summary of the whole paper, not just the conclusions. The abstract should be no more than 250 words and convey the following:

1. An introduction to the work. This should be accessible by scientists in any field and express the necessity of the experiments executed

2. Some scientific detail regarding the background to the problem

3. A summary of the main result

4. The implications of the result

5. A broader perspective of the results, once again understandable across scientific disciplines

It is crucial that the abstract convey the importance of the work and be understandable without reference to the rest of the manuscript to a multidisciplinary audience. Abstracts should not contain any citation to other published works.

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Nomenclature, abbreviations and symbols should follow the latest rules of the International Union of Pure and Applied Chemistry (IUPAC). In IUPAC nomenclature, isotopic substitution is indicated by a prefix consisting of (1) any necessary locant numeral or numerals; (2) the atomic symbol with the mass number as a superscript to the left of the symbol; and (3) a subscript (other than the unity) indicating the number of such atoms. Trivial names should be avoided unless the compound is to be mentioned often in the text, in which case a systematic name should also be given the first time the trivial name is used.

Journal of Labelled Compounds and Radiopharmaceuticals also touches on biology and medicine, and therefore a wide range of nomenclature rules apply./p>

For biological and biochemical nomenclature authors should conform to the (identical) guidelines published in J Biol Chem 260:16-42 (1985) and Eur J Biochem 138:9-37 (1984).

For biomedical nomenclature authors should conform to rules given in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals prepared by the International Steering Committee of Medical Editors. Reprints of this document are available from the Editor, Annals of Internal Medicine.

For questions of style, usage and punctuation, refer to The Chicago Manual of Style, which will be the final authority.

Reference Style

References should be cited by superior numbers in square brackets and listed at the end of the paper in the order in which they appear in the text. Authors should cite available published work. If necessary, cite unpublished or personal work in the text but do not include them in the references list. Journal titles should be italicized and abbreviated in accordance with the "Chemical Abstracts Service Source Index" (CASSI; no commas appear in the journal names).

Examples for Journals

[1] R. K. Harris, A. Nordon, K. D. M. Harris, *Rapid. Commun. Mass Spec.* **2007** ; *21* , 15.

Examples for Books

[2] K. Schmidt-Rohr, H.W. Spiess, *Multidimensional Solid-State NMR and Polymers,* Academic Press, London, **1994** .

[3] V. Sklenar, in *NMR Applications in Biopolymers* , (Eds: J.W. Finley, S. J. Schmidt, A. S. Serianni), Plenum, New York, **1990** , pp. 63-70.

Examples for Websites

[4] The Oncology Website. http://www.mit.co/oncology/ [24 April 1999].

Graphical Table of Contents

JLCR's table of contents will be presented in graphical form with a brief abstract.

The table of contents entry must include the article title, the authors' names (with the corresponding author indicated by an asterisk), no more than 80 words or 3 sentences of text summarising the key findings presented in the paper and a figure that best represents the scope of the paper. (see the section on abstract writing for more guidance).

Table of contents entries should be submitted to Manuscript Central in one of the generic file formats and uploaded as 'Supplementary material for review' during the initial manuscript submission process.

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Examples for arranging the text and figures as well as paper title and authors' names are shown below.

Supporting Information

Data that are (i) not amenable to presentation in a traditional print format, (ii) of interest primarily to specialists and do not require Journal page space, or (iii) particularly useful to the community in electronic (downloadable) form can be published online as supplementary material hosted within Wiley Online Library.

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All human and animal studies must be approved by an appropriate ethics committee or review board (depending on local arrangements), and a statement to this effect should be included in the methods section, or the reasons why it was not necessary if this is the case. All clinical investigations must have been conducted according to the principles expressed in the Declaration of Helsinki (http://www.wma.net).

Article formats published in JLCR

Research Articles must either be of current general interest or of great significance to a more specialized readership. They are peer-reviewed and report details of studies that have not been published previously, except in the form of a preliminary communication (reprint requested). Manuscripts should be between 15 and 20 pages in length (double spaced), and be divided into sections in the following order: Introduction, Experimental, Results and Discussion, Conclusions, Acknowledgements, References.

RAW DATA RESULTS FROM SECTION 3 OF CHAPTER 4 FROM

MANUSCRIPT 3

Test: Oral 1 hour

Organ	%ID/g	STD
Heart	0.027836	0.010463
Lungs	0.002993	0.000939
Liver	0.012861	0.000801
Kidney	0.033086	0.010435
Muscle	0.1168	0.008906
Blood	0.005644	0.001363

Ref: Oral 1 hour

Organ	%ID/g	STD
Heart	0.005352	0.000908
Lungs	0.001623	0.000535
Liver	0.001663	0.000449
Kidney	0.006927	0.002441
Muscle	0.017482	0.006556
Blood	0.001934	0.000787

Test: Oral 2 hour

Organ	%ID/g	STD
Heart	0.024268	0.009501
Lungs	0.005418	0.000753
Liver	0.062789	0.005087
Kidney	0.031405	0.005989
Muscle	0.124782	0.032688
Blood	0.004644	0.001858

Ref: Oral 2 hour

Organ	%ID/g	STD
Heart	0.018296	0.008442
Lungs	0.001343	0.000247
Liver	0.006529	0.002443
Kidney	0.019288	0.008191
Muscle	0.103877	0.018567
Blood	0.001791	0.000718

Test: Oral 4 hour

Organ	%ID/g	STD
Heart	0.003768	0.002839
Lungs	0.015775	0.005337
Liver	0.13095	0.043205

Ref: Oral 4 hour

Organ	%ID/g	STD
Heart	0.000673	0.000246
Lungs	0.000884	0.000188
Liver	0.009443	0.005096

Kidney	0.034279	0.016231	**Kidney**	0.0109	0.002599
Muscle	0.05252	0.032394	**Muscle**	0.003289	0.001123
Blood	0.004145	0.001935	**Blood**	0.002576	0.000503

%ID/g: percentage of injected dose per gram of organ

Oral: Oral administration

IV: Injected

Ref: MDP injected alone

Test: MDP entrapped in Pheroid

SAMPLE ANALYSIS RESULTS FROM SECTION 2.2.5 OF CHAPTER

4 FROM MANUSCRIPT 3

1. **The effect that H_2O_2 has on the reading as well as time passed before the reading is done.**

Sample	Time 0 hours	Time 24 hours	Time 48 hours
1	1848	1497	1458
2	1737.5	1408	1491
3	1669.5	1404	1397
4	2018	1643	1682
5	1881.5	1640	1635
6	1929.5	1602	1617
Agv	1847.3	1532.3	1546.6
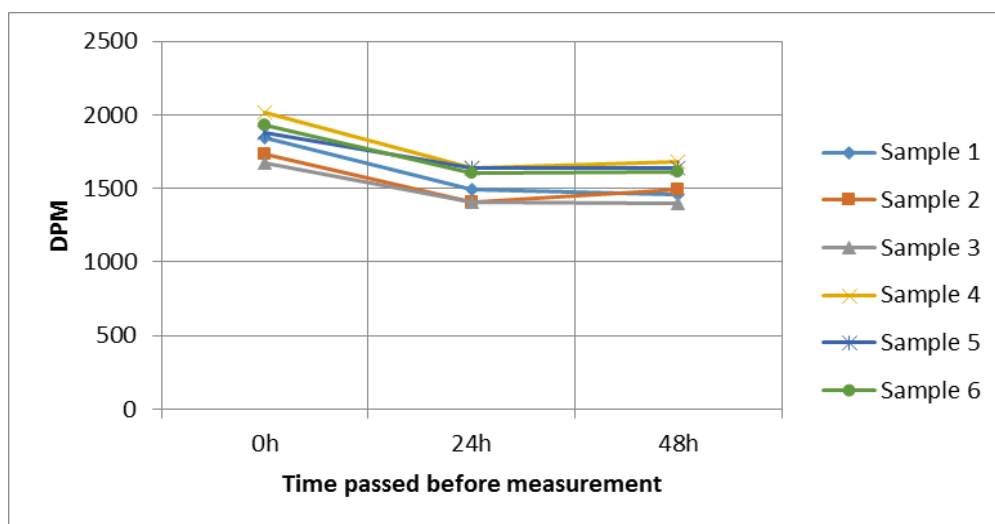
The H_2O_2 has a quenching effect called *transformed* Spectral Index of the external standard (tSIE) at the start but after 24 hours the effect is reduced and the 48 hours it stays the same. This leads to the conclusion that the reading should only be done 24 hours after the H_2O_2 has been added.

**2. The effect of the amount of H$_2$O$_2$ was also looked at.**

Sample	Amount of H$_2$O$_2$	DPM average
4 (a,b,c) Liver	200 µl	3697.1
5 (a,b,c) Liver	400 µl	3769.8
6 (a,b,c) Liver	300 µl	3575.5
13 (a,b,c) Muscle	200 µl	5981.6
14 (a,b,c) Muscle	400 µl	5906.3
15 (a,b,c) Muscle	300 µl	5707
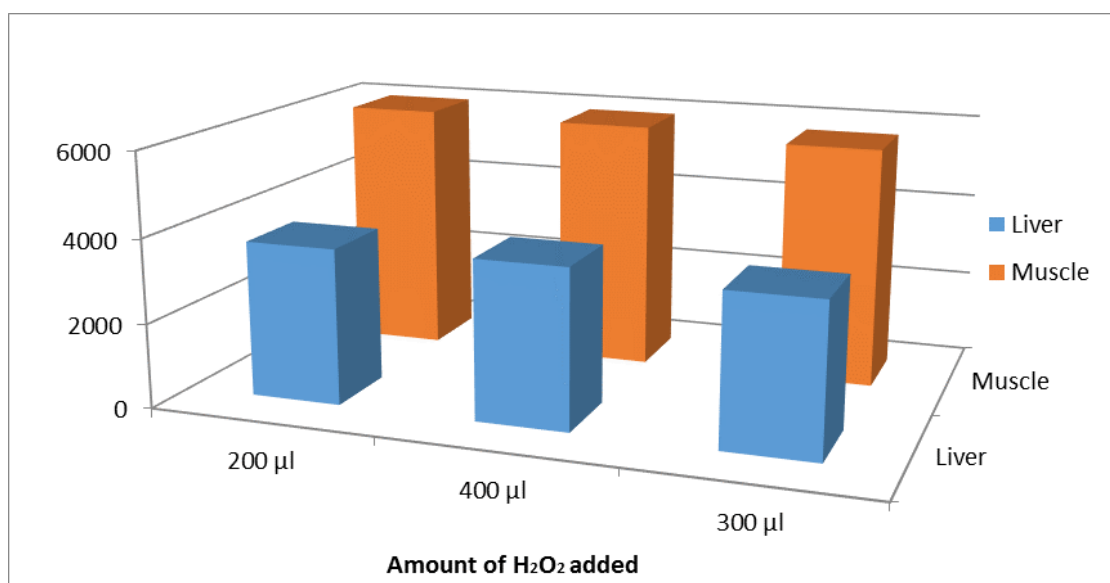
The different amounts of H$_2$O$_2$ does not seem to have an effect on the reading and since we know it takes time for the H$_2$O$_2$ reaction to work out the smallest amount will be best suited to this procedure.

3. The effect of the amount of liquid was also tested, in other words whether the dilution had an effect on the DPM.

Sample	Amount of Biosinct	DPM
A1	15ml	7344.5
A2	15ml	7339.5
A3	15ml	6955.5
B1	17ml	6949.5
B2	17ml	7202
B3	17ml	7132
C1	20ml	7006.5
C2	20ml	6999
C3	20ml	6721.5
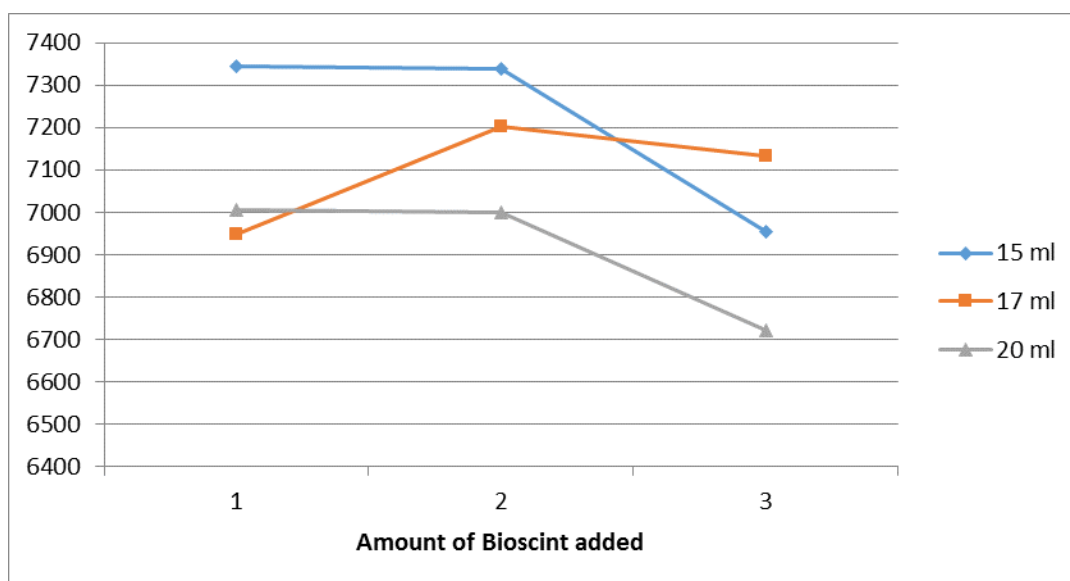
As can be seen, all the readings are relatively the same and thus the conclusion can be drawn that the amount of liquid does not play a role and the readings are not quantitive.

4. **The readings of clean vials that were spiked with C^{14} was then compared to vials containing muscle and liver and also spiked with C^{14}.**

Sample	Organ	Amount of C^{14}	DPM
7(a,b,c)	Liver	40	10885.1
16(a,b,c)	Muscle	40	13977.3
D(1,2,3)	None	40	15170.8
8(a,b,c)	Liver	80	25601
17(a,b,c)	Muscle	80	29122.6
E(1,2,3)	None	80	30773.5
9(a,b,c)	Liver	160	54578.6
18(a,b,c)	Muscle	160	58881
F(1,2,3)	None	160	60214.3
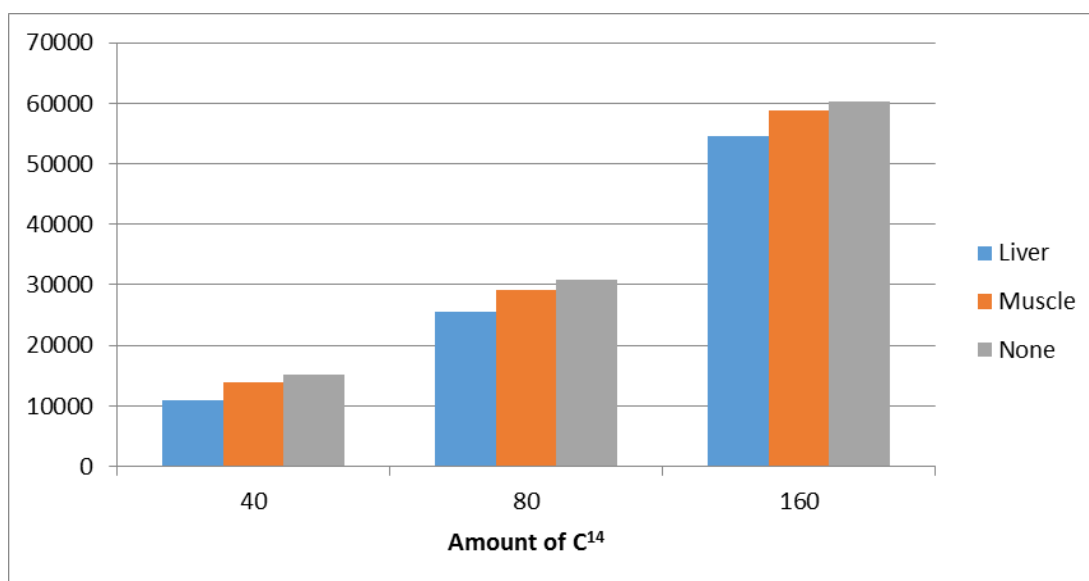
The rise in DPM of vials without any organ is consistent with the rise in amount of C^{14} and if there was no interference due to the colour quenching from the organs. The organs DPMs should have been the same. In the case of the Liver the quenching is the most because it has the darkest colouring. Thus leading to the conclusion that, the darker the colour of the organ or sample, the greater the quenching.

5. The need to use H$_2$O$_2$ was also investigated, due to the fact that a 24 hour time period is needed to nullify the effect it has on the readings.

Sample	Amount of H$_2$O$_2$	DPM
G1	200µL	373
G2	200µL	411.5
G3	200µL	385
H1	0µL	284
H2	0µL	307.5
H3	0µL	295
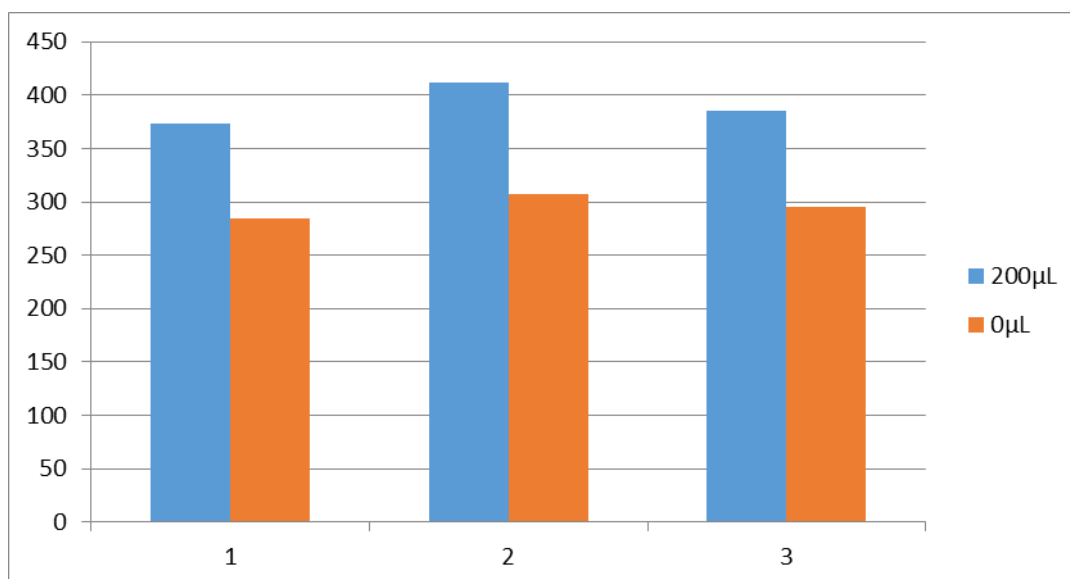
As can be seen the samples without H$_2$O$_2$ has got a lower DPM and this is due to quenching. Therefore H$_2$O$_2$ is needed to decrease the effect that colour quenching has on the readings.

PROOF OF EDITING

Dr Karen Batley

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(Academic and language practitioner)

2014-11-25

<u>To whom it may concern</u>

I edited Mr Braam Swanepoel's thesis in my professional capacity. If any further

assistance is required, I can be contacted.

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SPECIFICATIONS AND STATISTICS OF ^{99M}Tc MDP

[99M]TECHNETIUM METHYLENE DIPHOSPHONATE

A [99M]Tc generator (maximum 30 mCi or 1110 MBq) will be used. It contains the isotope Mo-99 en [99M]Tc. The Mo-99 is attached to the alumina column inside the lead protection plate. Tc-99m is in the form of pertechnetate in saline solution.

Dangers due to radioactive exposure are very low. Tests on people are carried out daily with injections of 1110 MBq Tc-99m and it only delivers a total dose of 5 mSv. CT scans delivers a total dose 18 mSv and an X-ray photo 0.1 mSv. The rats will only receive 20-40 MBq.

The generator will be handled in a separate flow cabinet. The [99M]Tc containing formulations will be stored in lead containers and where possible, will be kept in the containers during handling. All the radioactive waste will be kept in the lead containers at NECSA. I will be trained as a radiochemical worker and will be examined during a formal exam at NECSA. Electronic dose meters will be worn during the studies to monitor accumulated radiation doses. All of the staff involved in the handling of the radio-actives are male. A contamination monitor will be positioned at the exit of the lab to ensure that there is no radioactive contamination outside the lab.

Radiation type	Unit	Abbreviation
Radiation dose (Effective dose)	Sievert	Sv (Radiation sickness = 250 mSv)
Activity	Becquerel	Bq
Activity (Older unit)	Curie	Ci (1 Ci = 37000 MBq)

^{99m}Tc-MDP will be used in this study. The following are points of note:

POTENTIAL HEALTH EFFECTS

The hazardous ingredients found in ^{99m}Tc-MDP are skin and eye irritants, but due to the small quantities present in the container, no adverse health effects are expected to occur from exposure.

Eye Contact: Not expected to be a health hazard.

Skin Contact: Not expected to be a health hazard.

Inhalation: Not expected to be a health hazard.

Ingestion: Not expected to be a health hazard.

Chronic Exposure: Not expected to be a health hazard.

Aggravation of Pre-existing Conditions: No information found.

CARCINOGENICITY

None of the components present in this material at concentrations equal to or greater than 0.1 % is listed by IARC, NTP, OSHA, or ACGIH as a carcinogen.

Composition

Chemical Ingredients

Medronic Acid 10 mg

p-Aminobenzoic Acid 2 mg

Stannous Chloride Dihydrate 1.1 mg

First Aid Measures

Eye Exposure: Wash thoroughly with running water for at least 15 minutes. Get medical advice if irritation develops.

Skin Exposure: Wash exposed area with soap and water. Get medical advice if irritation develops.

Inhalation: Not expected to require first aid measure; remove to fresh air, support breathing by usual methods if necessary.

Ingestion: Not expected to require first aid measure; call physician if necessary.

Handling and Storage

The radioactive solutions are stable between 2 ⁰C and 25 ⁰C. Radio actives should be discarded after twelve (12) hours from the time of preparation. Handling devices such as syringe shields and tongs should be used. Storage and disposal of the reconstituted, radioactive product should be controlled in a manner that is in compliance with the appropriate regulations of the government agency authorised to license the use of this radionuclide.

Exposure Controls

Respiratory Protection: Not expected to require personal respirator usage.

Skin Protection: Wear protective gloves and clean body-covering clothing.

Eye/Face Protection: Safety glasses.

Physical and Chemical Properties

Appearance: Small, dry, white plug or crystals clinging to inside of 10 mL glass vial.

Odour: Odourless.

Solubility: Soluble in water.

Boiling Point: ca. 100 ºC (212 ºF) reconstituted.

Melting Point: ca. 0 ºC (32 ºF) reconstituted

Stability and Reactivity

Stability: Stable under ordinary conditions of use and storage.

Hazardous Decomposition Products: When heated to decomposition, substance may emit oxides of carbon and corrosive fumes of hydrochloric acid.

Hazardous Polymerisation: Will not occur.

Incompatibilities with other Materials: None reasonably foreseeable.


Disposal Considerations

MDP reconstituted with sodium pertechnetate Tc-99m is classified as radioactive waste until the activity has decayed to non-detectable levels. Radioactive waste must be handled in accordance with procedures established by the Radiation Safety Officer, NRC, CNSC, and other applicable regulations. If medical waste is involved, such as blood, blood products, or sharps, the waste must be handled as a biohazard and disposed of accordingly. If not radioactive or a biohazard, MDP is considered non-hazardous. If and as required, local, provincial or state, regulations will be consulted for proper disposal.

CERTIFICATE OF ¹⁴C-CQ TECHNICAL DATA SHEET

ViTrax

Technical Data Sheet

VC 505

Chloroquine, [3-14C]

mw 319.88
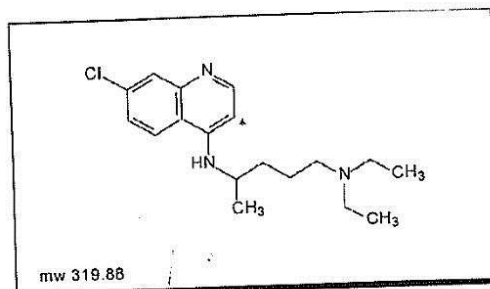
Lot Number: 147-027-000

Specific Activity: 45 mCi/mmol by Mass Spec.

Packaged: 250 µCi, Solid

Primary Container: B5

Total Batch Activity: 250 µCi
Reserve: 0 µCi

Radiochemical Purity: >99% by HPLC on 6/12/2012
Chemical Purity: >97% by HPLC on 6/12/2012

HPLC
Zorbax SB-C18, 5µm , 4.6 x 150mm
Mobile Phase A: 0.05% TFA/H_2O
Mobile Phase B: CH_3CN
Flow Rate: 1.0 mL / min., RC Flow Det.
time program
  0 min 10%B
  10 min 90%B
  12 min 10%B

RC Profile attached

TLC
N/A

Stability and Storage Recommendation: The exact rate of decomposition is unknown. However, it can be assumed that the product may decompose at a rate of approximately 2% per month when stored at -20º C in the original container.

Caution: Not for use in Humans or Clinical Diagnosis. This product is intended for investigational or manufacturing use only. It is pharmaceutically unrefined and is not intended for use in humans. Responsibility for its use in humans, as a diagnostic reagent, and compliance with federal laws rests solely with the purchaser.

Document Issue Date: June 14, 2012

Approved By: