The impact of Pheroid™ technology on the bioavailability and efficacy of anti-tuberculosis drugs in an animal model.

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ABSTRACT

Tuberculosis (TB) is a world wide pandemic with vast challenges in the fight against the disease. Challenges include the long and tedious treatment regimes and chemotherapy with adverse side effects. One approach in defeating TB is to improve patient compliance to the treatment regimes. The Pheroid™ drug delivery system possesses characteristics and abilities to make it a good candidate to lower the dosage of the anti-TB actives; ethambutol (EMB), rifampicin (RMP), pyrazinamide (PZA) and isoniazid (INH).

For the purpose of this study, the bioavailability of the anti-TB actives EMB, PZA, RMP and INH were determined in the mouse model. Plasma was collected from 8 mice per group over 4 hours and analysed by an independent laboratory. Area under the plasma curve, as a function of bioavailability, increased by 85.7% for EMB, 45.5% for INH and 33.2% for PZA with the Pheroid™ formulation when compared with the formulation currently prescribed. Although not significantly different, RMP also showed a 33.3% increase with the Pheroid™ formulation. Bioavailability decreased between 7% and 58% for EMB, INH, and PZA when the formulation currently prescribed is compared with the pro-Pheroid™ formulation, but RMP increased significantly by 84%.

Efficacy of a Pheroid™ based formulation was compared to the free actives in the mouse model. Mice were inoculated with *Mycobacterium tuberculosis* H37Rv reference strain. Treatment started two weeks after inoculation and continued for 12 weeks with a 3 month post treatment observation period. Methods used to assess treatment efficacy were the *Mycobacteria* Growth Indicator Tubes (MGIT) method in parallel with a method counting colony forming units (CFU) on agar plates. According to the MGIT method, the Pheroid™ treatment is statistically more effective at week 4 by 10% and at week 10 by 9%. Although not statistically more efficient, the Pheroid™ based treatment is 16.7% more effective at 3 months post treatment. According to the agar plate method, the Pheroid™ treatment is statistically 7% more effective than the Free drug treatment at week 2. Although the two methods gave slightly different results, a trend in efficacy could be observed. The mice treated with the Pheroid™ formulations showed a significant improvement from onset of treatment to about week 10 (with the exception of week 6) and again at 3 months post treatment.

The bioavailability and efficacy data accumulated in this study indicate that a new anti-tuberculosis treatment regime based on the Pheroid™ drug delivery system opens the opportunity for a new dosage form where APIs are either lowered, treatment time shortened or

treatment intervals increased. TB patient compliance might be improved with such a new treatment regime while assisting in the fight against TB.

Keywords: Tuberculosis, Ethambutol, Pyrazinamide, Isonaizid, Rifampicin, Pheroid™, Bioavailability, Murine model, *in vivo* efficacy, Pharmacokinetic parameters.

UITTREKSEL

Tuberkulose is 'n wêreldwye endemie met geweldige uitdagings in die stryd teen the siekte. Uitdagings sluit die lang en vermoeiende behandelings regime en chemoterapie wat ongunstige newe efekte het in. Een benadering vir die oorwining van TB is om patiënt medewerkendheid tot die behandeling regimes te verbeter. Die Pheroid™ geneesmiddel-afleweringsisteem besit eienskappe en vermoëns wat dit 'n goeie kanidaat maak om die dosis van die anti-TB aktiewes, etambitol (EMB), rifampisien (RMP), pirazinamied (PZA) en isoniasied (INH), te verlaag.

Vir die doel van hierdie studie is die biobeskikbaarheid van die anti-TB aktiewes EMB, PZA, RMP en INH in 'n muis model bepaal. Bloedplasma van 8 muise per groep oor 4 ure was vesamel en geanaliseer deur 'n onafhanklike laboratoruim. Wanneer die Pheroid™ formulering vergelyk word met die formulering wat tans voorgeskryf het die area onder die kurwe (AOK), wat 'n funksie van biobeskikbaarheid is, met 85,7% vir EMB, 45,5% vir INH en 33,2% vir PZA verhoog. Alhoewel statisties nie betekenisvol nie, het RMP ook in verhoging van 33,3% met die Pheroid™ formulering getoon. Biobeskikbaarheid het tussen 7% en 58% vir EMB, INH, en PZA gedaal wanneer die tans voorgeskryfde fomulering met die pro-Pheroid™ formulering vergelyk word, terwyl RMP betekenisvol verhoog het met 84%.

Die effektiwiteit van die Pheroid™ gebaseerde formulering was vergelyk met die Vry aktiewes in 'n muis model. Muise was ge-inokuleer met die *Mycobacterium tuberculosis* H37Rv verwysings stam. Behandeling het twee weke na inokulasie begin en vir 12 weke geduur met 'n 3 maande nabehandeling observasie periode. Die metodes gebruik om behandeling te evalueer was die *Mycobacteria* Groei Aanduidings Buise (MGAB) metode wat in parallel geloop het met 'n metode wat die kolonievormende eenhede (KVE) tel op agarplate. Na aanleiding van die MGAB metode was die Pheroid™ behandelings statisties betekenisvol meer effektief op week 4 met 10% en op week 10 met 9%. Alhoewel statisties nie betekenisvol nie, was die Pheroid™ gebaseerde behandeling 16,7% meer effektief teen 3 maande nabehandeling observasie. Na aanleiding van die agarplaat metode, was die Pheroid™ behandeling 7% meer statisties betekenisvol as die Vry geneesmiddel op week 2. Alhoewel die twee metodes geringe verskillende resultate verskaf, kan 'n patroon waargeneem word. Die muise wat met die Pheroid™ formulerings behandel was, wys 'n betekenisvolle verbetering vanaf die begin van behandeling tot week 10 (met die uitsluitsel van week 6) en dan week op 3 maande tydens die nabehandelings observasie periode. Alhoewel geen duidelike aanduidings vir eksperimentele fout op week 6 opgemerk kon word nie, bestaan die moontlikheid wel.

Die biobeskikbaarheid en effektiwiteits data vanuit hierdie studie dui daarop dat 'n nuwe anti-tuberkulose behandelings regime wat op die Pheroid™ geneesmiddel-afleweringsisteem gebaseer is, wel die geleentheid vir 'n nuwe doseerform waarvan die aktiewes verminder, behandelingstyd verkort of behandelings tydsintervalle verleng word. TB pasiënt medewerkendheid mag dan verbeter met so 'n nuwe behandelings riglyn en help in die stryd teen die siekte.

Keywords: Tuberkulose, Etambitol, Pirazinamied, Isoniasied, Rifampisien, Pheroid™, Biobeskikbaarheid, Muis model, *in vivo* effektiwiteit, Farmakokinetiese parameters.

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ABBERVIATIONS

ΔGI - Delta Growth Index

API(s) – Pharmaceutical Active Ingredient(s)

AUC - Area under the curve

BC – Before Christ

BCG -Bacillus of Calmette and Guerin

CFU – Colony forming units (of bacteria)

CLSM - Confocal Laser Scanning Microscope

C_{max} - Maximum plasma concentration

DNA – Deoxyribonuclei acid

DOTS - Directly Observed Treatment Short –Course

EMB – Ethambutol

GI- (Bacterial) Growth Index

GLP - Good Laboratory Practices

GMP - Good Manufacturing Practices

HIV – Human Immunodeficiency Virus

INH – Isoniazid

IU – International units

LD_{50} – Median lethal dose

M.tb – Microbacteruim tuberculosis

MDR-TB - multi drug resistant TB

MGIT - Mycobacteria Growth Indicator Tubes

MIC -Minimum Inhibitory Concentration

N_2O - nitrous oxide

NWU – North-West University

OADC -Oleic acid-albumin-dextrose-catalase

PBS - Phosphate Buffered Saline solution

PZA - Pyrazinamide

RMP – Rifampicin

RNA - Ribonucleic acid

SADOH - South African Department of Health

SAMCC - South African Medicine Control Council

SAMRC - South African Medicine Research Council

SANAS – South African National Accreditation System

TB – Tuberculosis

T_{max} - Time to reach the C_{max}

TTD - Time to detection

WHO – World Health Organization

XDR- TB - Extensively drug-resistant TB

CHAPTER 1

CHALLENGES IN THE FIGHT AGAINST TUBERCULOSIS

1.1 TB CHALLENGE

Tuberculosis (TB) is one of the leading killers world wide. According to estimates, in 2007 one third of the human population (about 1.86 billion people) was infected with *Mycobacterium tuberculosis* worldwide (WHO, 2007). The World Health Organisation (WHO) estimated that there were 8.8 million new cases of tuberculosis in 2004 and about 1.6 million deaths in 2005. As a result of factors such as Human Immunodeficiency Virus (HIV) co-infection, incomplete and inadequate treatment regimes, emerging drug resistant strains and persistent poverty, the global incidence rate of tuberculosis is now growing at approximately 1% per annum, with explosion in Sub-Saharan Africa (WHO, 2007).

TB is a progressive infection caused by the bacterium *Mycobacterium tuberculosis (M.tb)* with a period of latency following initial infection. It occurs most commonly in the lungs. Pulmonary symptoms include productive cough, chest pain, dyspnea, night sweats and weight loss. More than 80% of new tuberculosis cases result when small airborne droplet nuclei are inhaled by an individual after an infective individual coughed, sneezed or talked nearby. These small particles can remain airborne for a few hours due to its small diametre, thus resulting in a potentially high infection rate (Frieden *et al.,* 2003).

The WHO recommends the essential anti-tuberculosis drugs rifampicin, ethambutol, isoniazid, pyrazinamide, streptomycin and thioacetazone for treatment of patients diagnosed with TB for the first time. Incomplete, interrupted or inappropriate treatment of TB resulted in *M.tb* gaining resistance against these anti-bacterials. HIV co-infection also decreases efficacy of the essential anti-TB drugs and plays a role in elevating re-occurrence of tuberculosis (WHO 2007). Strains resistant to drugs evolved quickly and in January of 1987 the first case of multi-drug resistant (MDR) TB was diagnosed in the United States (Communicable Diseases 1, 2009). MDR-TB is defined as a TB strain resistant to at least isoniazid and rifampicin (Joshi & Gothi, 2006; WHO 2003). Now medical practitioners have to resort to alternative medication called "reserve drugs" such as amikacin, ciprofloxacin and kanamycin. Unfortunately, the prevalence of MDR-TB kept increasing and resistance evolved into extensively drug-resistant (XDR) strains. XDR-TB strains are resistant to two essential drugs and at least three of the reserve

drugs (PIH NEWS, 2007). Treatment of MDR-TB with reserve drugs is more expensive and more toxic than with the essential drugs (WHO, 2003) and is, therefore, less desirable.

In 1993 the WHO elevated TB to global emergency status and called upon researchers to pursue novel solutions with new fervor. According to Padma (2004), Rowan Gillies; International Council President of the Nobel-Prize Winning Doctors without Borders, felt that greater investment in research and development of new diagnostic tools, drugs and vaccines should be the cornerstone of future control of TB.

1.2 CURRENT TB TREATMENT REGIMES

Current TB treatment regimes have several deficits such as long, tedious treatment regimes and adverse side effects. Newly infected patients must follow an initial six month treatment program and stand the chance of extension if the patient continues to be smear positive. One hypothesis for the severe side effects is the long exposure to anti-bacterials such as rifampicin, ethambutol, pyrazinamide and isoniazid. The treatment regime shortfalls result in inappropriate use and aggravate the rapid spreading of the disease, the development of drug resistant TB strains, and often death of patients. An urgent need thus exists for shorter treatments and fewer side effects, or ultimately both.

The WHO implemented DOTS (Directly Observed Treatment, Short – Course) as part of a progressive Stop TB strategy. DOTS is an internationally recognised health care management system with a patient-centred approach. This system provides support by observing the patients during treatment, assisting in patient compliance and thus ensuring completion of the treatment regime. The DOTS programme also helps health care systems by identifying patients in the infectious phase, providing a suitable treatment regime and monitoring progress which all assist in successful treatment (WHO 2007).

The DOTS strategy, unfortunately, has flaws, implementation problems and logistic challenges. Due to the long treatment regimes, DOTS is labour intensive and relies on efficient national health care systems whereas the health care systems of countries suffering the most from this disease often lack in expertise and funds (Padma 2004).

1.3 FIGHTING TUBERCULOSIS WITH PHEROID™ TECHNOLOGY

Pheroid™ technology is a drug carrier system that has the potential to address some of the problems around TB treatment regimes. Bioavailability of anti-tuberculosis drugs in humans

increased significantly when using this technology (Grobler, 2006). *In vitro* studies showed that *M.tb* organisms ingest rifampicin loaded Pheroid™s and mycobacterial viability decreased in parallel (Grobler, 2006). Several other microbacterial studies such as zone inhibition, cell culture infection studies and bacterial culturing studies confirmed inhibition of bacterial growth with as little as $1/40^{th}$ of the usual dosage and even reversed drug resistance (Grobler, 2006). The Pheroid™ system has, furthermore, the ability to minimize cellular damage caused by active pharmaceutical ingredients (APIs) (Grobler 2006). It may, therefore, be possible to administer a lower concentration of drugs using Pheroid™ technology, thus possibly reducing side effects and shortening exposure to the drug due to a shorter treatment time. This will result in better patient compliance and possibly a higher success rate in the treatment of TB. Both governments and institutions such as the WHO will, therefore, benefit from having to employ less resources in order to make an action plan such as DOTS a global success.

1.4 DRUG REGISTRATION CHALLENGE

Commercialisation of TB medication containing Pheroid™ technology must be preceded by the registration of the medicine at each country's medical/drug regulatory body. The universal goal of such regulatory bodies is to ensure that a safe and effective product is made available to patients. Before proving safety and efficacy in human subjects, adequate data from non-human vertebrate tests must be presented. Permission for human trails will be only be granted when animal studies imply the treatment is safe for human use and that it will be effective as a treatment.

Registration, furthermore, requires proof of a stable and presentable product. The API and dosage form must have a commercially viable shelf life and be able to withstand a range of climate conditions.

1.5 ANIMAL MODEL CHALLENGE

Due to ethical importance, animal models are widely used as a human substitute for testing TB eradicating hypotheses. Ideally an animal model will mimic the exact human infection, pharmacokinetic and pharmacodynamic parameters and will reflect the true outcome as if the dosage form was tested in humans. The following have to be considered when choosing an appropriate model that will mimic human trials.

a) Correspondence to human systems.

b) Logistic matters such as ease of handling, housing requirements, availability and total cost.

c) Susceptibility to TB infection, the immune response and the inflammatory reaction after

infection.

d) Ethical considerations and acceptance of the model.

e) Existence of analytical methods and techniques.

f) Prior scientific knowledge about the animal such as genomics, enzyme reactions, immune and inflammatory responses, reproductive cycles etc. (Nuermberger, 1994; Kaufmann, 2003; Gupta & Katoch, 2005).

Choosing an animal model representative of human pharmacokinetics and pharmacodynamics must be done with great care weighing the pro's and con's of each model cautiously.

1.6 FORMULATION CHALLENGE

Shorter treatment regimes and less side effects from ingesting anti-bacterials is urgently needed in the fight against TB. The quantities of anti-tuberculosis drugs that must be consumed for effective treatment poses a formulation challenge. Recommendation by the South African Department of Health (SADOH) for a total of 4,275 g anti-tuberculosis medication per day for a ≥ 70 kg patient results in patient unfriendly dosage forms (SADOH, 2006; Matthee, 2007). A need also exists to reduce the amount of drugs that must be consumed daily.

Incompatibilities between anti-TB agents also need to be considered when such drugs are formulated together in a dosage form. Studied cases of rifamipicin – isoniazid interactions indicate that rifampicin hydrolise under acidic conditions to 3-formylrifampicin. The amine group of 3-formylrifampicin reacts with isoniazid to form isonicotinyl hydrazone in fixed dose combination dosage forms (Singh *et al.,* 2006). Ethambutol and pyrazinamide, furthermore, accelerate the rifampicin-ethambutol interaction (Singh *et al.,* 2006; Baldan *et al.,* 2007). The need, therefore, exists to separate isoniazid and rifampicin to ensure a stable shelf live of the anti-microbials.

1.7 AIM

This study will address the challenge of obtaining comparable data on bioavailability and efficacy in an appropriate animal model. Such data is required to obtain the approval of the regulatory authorities (South African Medicines Control Council) for the performance of a phase 2 human efficacy trial. This study also aims to address some of the challenges associated with TB dosage form development.

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CHAPTER 2

TUBERCULOSIS, CHEMOTHERAPY AND UTILISING ANIMAL MODELS IN THE FIGHT AGAINST THE DISEASE

2.1 TUBERCULOSIS

Mycobacterium tuberculosis (M.tb) is a relatively slow growing rod shaped gram positive, *strictly* aerobe bacterium. The cell wall of the bacterium has a high lipid constituency that allows the bacteria to survive in macrophages. Mainly humans are infected by this ubiquitous and highly contagious chronic bacterial infection. The disease is spread when an infected person speaks, coughs or sneezes via airborne dissemination of droplet size nuclei. The uninfected person inhales the nuclei where they embed in the alveoli and get indigested by macrophages. The body responds by forming granulomas as the body's first attempt to contain and eliminate infection (Frieden *et al.,* 2003). A latent period follows and if the macrophages fail to destroy the bacilli they multiply, spread and full blown TB develops. Various factors such as age, malnutrition, or a compromised immune system as with HIV, contribute to disease progression from latent to infection. Although infection stays mainly in the lungs, it can migrate to other organs such as liver, spleen and spinal cord. The TB bacilli can also lie dormant in the macrophages for years before onset occurs (WHO, 2007).

Symptoms for tuberculosis are similar to other respiratory illnesses, but a persisting cough for more than four weeks must be investigated. Other symptoms include hemoptysis, dyspnea, malaise, weight loss and night sweats (Medicine network, 2009).

Initial diagnosis is traditionally performed by one or a combination of four methods.

1: The tuberculin skin test involves intradermal injection of a tuberculin purified protein derivative into the inner forearm and evaluated 48 – 72 hours after injection. (Medicine network, 2009; Wood, 2007).

2: Staining of a sputum sample and positive identification using microscopy (Kumar *et al.,* 2007: 516; Wood, 2007).

3: Radiologically with a chest X-ray test visually identifying pulmonary lesions in the lung (Wood, 2007).

4: Microbiological culture tests of mostly sputum, but can also be from biopsy material. Culture tests are performed in laboratories and various include solid and liquid media (Wood, 2007; Kumar *et al.,* 2007: 516).

All the methods with the exception of the microbiological tests, are not 100% effective and are subject to opinion, training and experience of the health care workers, which are often luxuries in developing countries. One major negative aspect of the microbiological tests is the long waiting period to diagnosis which can take up to 56 days on some solid media (Wood, 2007).

Due to the problems experienced with TB diagnosis, a new generation of tests has been developed of which some are already implemented in health care systems around the world (Dinnes *et al.*, 2007;Wood, 2007). New TB tests are developed with the hope of offering cheap, fast and more accurate diagnosis. These tests include;

- Immune response tests such as interferon-gamma identifying *M.tb.* presence by antibody detection. A widely used rapid test that is relatively simple and inexpensive but not highly reliable (Wood, 2007; Dinnes *et al.*, 2007).
- Nucleic acid amplification tests such as polymerase chain reaction detects bacterial nuclei acids. This type of tests is time consuming and expensive thus impractical in the developing world (Reddy *et al.,* 2002).
- Antigen based tests recognising an array of *M.tb* cell wall molecules such as Liporabinomonnan (Wood, 2007) and mycolic acid (Verskoor, 2009).

Current anti-tuberculosis chemotherapy for newly diagnosed patients is a long-term treatment. A combination of four drugs is prescribed by various health authorities, including the WHO (2003) and the SADOH. The goal of treatment is to ensure cure without relapse. During the intensive initial phase the four anti-TB drugs rifampicin (RMP), pyrazinamide (PZA), ethambutol (EMB) and isoniazid (INH) are administered for 2 months. A continuous phase of rifampicin and isoniazid follows to ensure that all dormant bacilli are killed (Du Toit *et al.*, 2006). It is critical that treatment is not interrupted during the continuous phase since the result is the development of drug resistance (WHO, 2007).

There are several reasons for discontinuation of treatment: Patients have to ingest large amounts of tablets and the side effects from the drugs are severe. Poverty complicates logistics such as traveling and access to good medical care. The correct and accurate diagnostic tools in developing countries are also problematic (WHO, 2003). In 2006 the WHO launched the new "Stop TB" plan with the DOTS strategy at its core (WHO, 2007).

The six components of the "Stop TB" plan are:

a) Pursuing high-quality DOTS expansion and enhancement.
b) Addressing TB/HIV, MDR-TB and other clinical challenges.
c) Contributing to health system infrastructure.
d) Engaging all care providers.
e) Empowering people with TB, and communities.
f) Enabling and promoting research (WHO, 2007).

2.2 CHEMOTHERAPY TREATMENT AGAINST TB

Current chemotherapy involves the anti-tuberculosis drugs rifampicin (RMP), ethambutol (EMB), pyrazinamide (PZA) isoniazid (IHN), streptomycin and thioacetazone as approved by the WHO (2003) list of essential anti-tuberculosis drugs. Bactericidal activity, sterilizing activity and the ability to prevent resistance are the three main properties of these drugs. Isoniazid and rifampicin are the most powerful bactericidal drugs, active against all populations of TB bacilli (WHO, 2003). Rifampicin kills slow growing or non-replicating organisms (Du Toit *et al.*, 2006) and is the most potent sterilizing drug available to date. Streptomycin, isoniazid and ethambutol eradicate most of the rapidly replicating bacilli (Du Toit *et al.*, 2006; WHO, 2003). Ethambutol is generally used to prevent the emergence of resistant bacilli (WHO, 2003). Pyrazinamide is bactericidal against certain populations of TB bacilli (WHO, 2003) and plays an important role in the sterilisation of lesions by eradicating semi-dormant organisms that are not affected by any other anti-TB agents in sites hostile to the penetration and action of the other drugs (Du Toit *et al.*, 2006). These drugs are combined into fixed-dose combination formulations complying with the WHO's prescriptions and are commercially available.

The WHO recommends the use of fixed-dose drug combinations rather than individual drugs for the following reasons:

a) Prescription errors are less likely because dosage is straight forward and weight-dose adjustments easier.
b) Dosages and drug combinations are prescribed in the essential anti-TB drug list.
c) A smaller number of tablets to be ingested than with individual drugs.
d) Patients cannot be selective as to which drugs to ingest (WHO, 2003).

Rifafour® e-275 tablets (Aventis) is a fixed-dose combination regime containing RMP, INH, PZA and EMB drugs on the WHO essential drug list, but exempt streptomycin and thioacetazone. The WHO discourages the use of thioacetazone because of toxicity (WHO, 2003) while streptomycin was excluded due to a risk of damage to the cochlea in the case of overdosing (Gibbon, 2008:300). Rifafour® e-275 is recommended by the South African government to treat newly diagnosed tuberculosis.

Below are the accepted oral dosages, pharmacologic action, therapeutic effects, indications, side effects and special precautions needed when a patient is treated, for the four drugs in Rifafour® e-275. The ingredients of this commercial treatment are described because it was used as reference dosages in this study.

2.2.1 Rifampicin

The accepted oral dosage is 8-12 mg/kg per day to a maximum of 750 mg/day in humans. (Gibbon, 2008:296).

2.2.1.1 Pharmacological action and therapeutic effects

Rifampicin is a semi-synthetic, broad spectrum bactericidal antibiotic that suppresses the ribonucleic acid (RNA) synthesis pathway of *M. Tuberculosis,* resulting in bacterial death. It is readily absorbed following oral administration, with a wide distribution to most tissues *and fluids*, including cerebrospinal fluid (Gibbon, 2008:297).

2.2.1.2 Indications

Treatment of pulmonary and extra-pulmonary tuberculosis in the initial phase (Gibbon, 2008:297).

2.2.1.3 Side-effects in human

Some subjects may experience a cutaneous syndrome, i.e. facial flushing, itching, rash and eye irritation 2 to 3 hours after administration. After 3 to 6 months of irregular treatment and usually with doses of 20 mg/kg or more, flu-like syndromes including fever, chills, bone pain and malaise may occur. Gastrointestinal effects include nausea, vomiting, anorexia, diarrhoea and epi-gastric distress, which may be alleviated by administration with food. Pseudo membranous colitis has been reported. Hepatitis and symptoms of hepatitis including nausea, vomiting and unusual fatigue may occur. Haematological adverse effects include eosinophilia, leucopenia and haemolytic anaemia while intermittent regimens can cause thrombocytopenia and purpura.

Nervous system effects include headache, drowsiness, dizziness, ataxia, numbness, visual disturbances and muscular weakness. Alterations in kidney function and renal failure have occurred. Rifampicin may cause orange-red discoloration of urine and other body fluids. Menstrual disturbances have been reported (Gibbon, 2008:297).

2.2.1.4 Special precautions

If liver damage is present, the medicine should not be recommenced. Treatment should be discontinued permanently should thrombocytopenia, purpura, shock, eye degradation or renal failure occur. Periodic examinations during treatment are suggested. Special care must be taken not to administer more than the daily maximum dosage (Gibbon, 2008:297).

2.2.2 Isoniazid

The accepted dosage in humans is 4-6 mg/kg per day to a max of 300 mg/day (Gibbon, 2008:298).

2.2.2.1 Pharmacological action and therapeutic effects

Isoniazid is a synthetic prodrug that is activated by bacterial enzymes. Once activated isoniazid inhibits mycolic acid production and then acts as an antitubercular agent which is bacteriostatic against semi-dormant bacilli and bactericidal against actively dividing mycobacteria. Isoniazid is readily absorbed following oral administration with a wide distribution to most tissues and fluids, including the cerebrospinal fluid (Gibbon, 2008:298).

2.2.2.2 Indications

Treatment of pulmonary and extra-pulmonary tuberculosis in the initial phase (Gibbon, 2008:298).

2.2.2.3 Side-effects in human

Hepatitis and symptoms of hepatitis including nausea, vomiting and unusual fatigue may occur. Gastrointestinal effects include nausea, vomiting, anorexia, diarrhoea and pellagra. Hypersensitivity reactions such as skin eruptions including erythema multiforme, fever, lymphadenopathy and vasculitis may occur. Haematological effects that have been reported include various types of anaemia, agranulocytosis, thrombocytopenia, neutropenia and eosinophilia. Neurological effects include psychotic reactions, convulsions, peripheral neuropathy and optic neuritis. Pyridoxine supplementation is recommended in patients with compromised uptake such as pregnant women, malnourished patients, alcoholics, diabetes and

HIV-infected patients to prevent the development of peripheral neuritis, as well as most other nervous system dysfunctions. Other effects include: hyperglycaemia, metabolic acidosis, lupus-like syndrome, rheumatoid syndrome, urinary retention and gynaecomastia (Gibbon, 2008:298).

2.2.2.4 Special precautions

Treatment should be discontinued permanently should jaundice, rash and fever, hepatitis, visual impairment, thrombocytopenia, purpura, shock or renal failure occur. Periodic eye examinations during treatment are suggested. Special care must be taken not to administer more than the daily max dosage (Gibbon, 2008:298).

2.2.3 Pyrazinamide

The accepted dosage in humans is 20-30 mg/kg to a max of 2g/day (Gibbon, 2008:299).

2.2.3.1 Pharmacological action and therapeutic effects

Pyrazinamide may be bactericidal or bacteriostatic, depending on concentration and the susceptibility of the organism. Pyrazinamide is readily absorbed following oral administration with a wide distribution to most tissues and fluids, including the cerebrospinal fluid (Gibbon, 2008:299).

2.2.3.2 Indications

Treatment of pulmonary and extra-pulmonary tuberculosis in the initial phase (Gibbon, 2008:299).

2.2.3.3 Side-effects in human

The most serious side-effect is hepatotoxicity and its frequency appears to be dose-related. Hyperuricaemia commonly occurs, occasionally accompanied by arthralgia and may lead to attacks of gout. Gastrointestinal effects include nausea, vomiting and anorexia. Other side-effects that have been reported are malaise, fever, sideroblastic anaemia and dysuria. Some subjects may experience a cutaneous syndrome such as photosensitivity, skin rash and eye irritation (Gibbon, 2008:299).

2.2.3.4 Special precautions

If liver damage is present, this API should not be recommenced. Treatment should be discontinued permanently should jaundice, rash and fever, hepatitis and visual impairment

occur. Periodic eye examinations during treatment are suggested. Special care should be taken not to administer more than the daily max dosage (Gibbon, 2008:299).

2.2.4 Ethambutol

The accepted daily dosage in humans is 15-20 mg/kg/day to a maximum of 25 mg/kg/day for 2 months (Gibbon, 2008:299).

2.2.4.1 Pharmacological action and therapeutic effects

Ethambutol is a synthetic, bacteriostatic antitubercular agent because it inhibits both RNA synthesis and cell wall biosynthesis. It is readily absorbed following oral administration, with a wide distribution to most tissues and fluids, including cerebrospinal fluid (Gibbon, 2008:299).

2.2.4.2 Indications

Treatment of pulmonary and extra-pulmonary tuberculosis in the initial phase (Gibbon, 2008:299).

2.2.4.3 Side-effects in human

Optic side effects, affecting one or both eyes include retrobulbar neuritis with a reduction in visual acuity, constriction of visual field, central or peripheral scotoma, and green-red colour blindness. The degree appears to be dose dependent. Retinal haemorrhage occurs less frequently. Gastrointestinal effects include nausea, vomiting, anorexia, metallic taste and abdominal pain. Hypersensitivity reactions such as rash, pruritis, leucopenia, fever and joint pains may occur. Neurological effects include confusion, disorientation, hallucinations, headache, dizziness and depression. Renal clearance of urate may be reduced and acute gout has been precipitated. Other adverse effects: jaundice or transient liver dysfunction and peripheral neuritis (Gibbon, 2008:299).

2.2.4.4 Special precautions

If liver damage is present, the medicine should not be recommenced. Treatment should be discontinued permanently should jaundice, rash and fever, hepatitis, visual impairment thrombocytopenia, purpura, shock or renal failure occur. Periodic eye examinations during treatment are suggested. Special care should be taken not to administer more than the daily max dosage (Gibbon, 2008:299).

2.2.5 Rifafour® e-275

2.2.5.1 Pharmacological action and therapeutic effects

The combined pharmacological actions as well as the therapeutic effects of the API's in Rifafour® e-275 are equal to that of the individual API's.

2.2.5.2 Indications

These tablets are used as initial phase treatment of new adult patients and for re-treatment of adult patients with pulmonary and extra-pulmonary tuberculosis.

2.2.5.3 Special precautions

If liver damage is present, Rifafour® e-275 should not be prescribed and alternative anti-TB drug combinations should be considered as recommended in the WHO (2003) Tuberculosis Treatment Guidelines. Treatment with Rifafour® e-275 tablets should be discontinued permanently should jaundice, rash and fever, hepatitis, visual impairment thrombocytopenia, purpura, shock or renal failure occur. Periodic eye examinations during treatment are suggested. Special care should be taken not to administer more than the daily maximum dosage (Gibbon, 2008:300).

2.2.5.4 Contra-indications

Rifafour® e-275 tablets are contra-indicated in patients with hypersensitivity to rifamycins, isoniazid, pyrazinamide, ethambutol or other chemically related medication as well as in the presence of jaundice or active hepatic disease, and in patients with optic neuritis. Safety in pregnancy has not been established and all anti-bacterial agents are excreted in breast milk. Safety during lactation has not been established. Rifafour® e-275 is not recommended for treatment in children under 13 years of age (Gibbon, 2008:300).

2.3 TUBERCULOSIS RESEARCH USING ANIMAL MODELS

After *in vitro* studies the next step in formulation development is to investigate bioavailability, safety and efficacy in animals. In view of the chronic nature of TB treatment regimes and for the protection of the patient, animal experimentation must precede clinical trials during dosage determination as well as dosage form development. The literature provides several well described animal models suitable for TB research.

2.3.1 History of animal research in the fight against TB

Experimentation and vivisection on animals have been conducted as early as 400 BC by researchers such as the Greek scientists Aristotle and Erasistratus. Their experimentation focused on gaining knowledge on the anatomy and physiology of the experimental animal itself. Later, knowledge on human anatomy and physiology was expanded using animal parallels (McNeil, 2007).

It was not until the 19th century that animals were first used in disease research and drug testing. Emil von Behring was awarded the Nobel Prize in Physiology and Medicine in 1901 for the successful implementation of vaccination principles using animals. Behring developed a therapy for diphtheria and tetanus by injecting sterilised broth cultures into animals, which then produced antitoxins protecting the animal against infection (Anon, 1967). Robert Koch discovered the tubercle bacillus in 1882 and infected various animal species including cattle, chickens, cats, dogs, monkeys and a variety of rodents successfully with *M.tb.* (Kaufmann, 2003). Koch went further and developed a relatively easy diagnostic method for TB that involves an intradermal injection of a tuberculin purified protein. He developed the diagnostic method by comparing immune responses from guinea pigs with his own (Kaufmann, 2003). This method is still used today.

Also in 1882, a Liverpool physician, James Carson conducted some of the first investigations into TB treatment by observations of experimental animals. Carson noted medical reports of Italian soldiers surviving tuberculosis after receiving a deep chest wound resulting in the lung to collapse. He opened the thoratic cavity of rabbits and noticed that one lung of an animal may be reduced to a state of collapse without the other being affected. This suggested to him that the treatment of some pulmonary diseases such as TB can be achieved by opening the chest on the side and inducing collapse of the infected lung (Dubos & Dubos, 1953). Although this practice became popular for a while, it proved for reasons known today not to be very successful.

Chemotherapy research and anti-TB drug discovery increased after the then popular surgical method's low success rates. Convincing reports using animals to test the therapeutic effect of anti-tuberculosis candidates were first documented in the 1930's. Although the drugs tested in those reports turned out to be ineffective against human TB, the demonstration of their effectiveness in animals acted as a great motivation for further research and soon led to the discovery of several substances which can be used in humans (Dubos & Dubos, 1953).

In January 1944 three microbiologists simultaneously reported their discovery of streptomycin and it's effectiveness against an array of micro-organisms including *M. tuberculosis*. The efficacy of streptomycin as an anti-tuberculosis chemo agent was tested in guinea pigs in the following year. The treatment results after tuberculosis infection of the animals showed a striking difference between the controls and the treated animals. The results were so promising that although further animal trials were underway, treatment of the first human began immediately (Murray, 2004).

The discovery of other anti-TB drugs soon followed and TB was treated successfully using combinations by the 1950's (Murray, 2004)

2.3.2 Current animal TB models

Presently several animal models are used in TB research, each with its own strengths and weaknesses as summarised in Table 2.1. The most popular animal models are mice (Orme, 2003; Mustafa *et al.,* 1999; Phyu, 1998; Orme, 1999; Nuermberger 1994), guinea pig (Karlson & Feldman, 1949; Kaufmann, 2003), rabbit and several monkey models such as the macaque, rhesus and Philippine cynomolgus monkeys (Flynn *et al.,* 2003; Gupta & Katoch, 2005).

Table 2.1: A summary of the advantages and disadvantages of each animal model used in tuberculosis drug research.

Animal model	Advantages	Disadvantages
Guinea Pig	• Abundant prior knowledge (such as median lethal dose - LD_{50}) available[3]. • Experimental methods available[3]. • Large body advantages such as more blood and the ease of performing experimental procedures (such as surgical)[1]. • Highly susceptible to TB[2]. • Cellular response to initial aerosol infection most similar to humans[1,2,7,8]. • Immunology close to human[2]. • Inbred strains commercially available[2].	• Five times as expensive as mice[8]. • Larger animal results in more specialized housing and space requirements[6].
Rabbit	• Cellular response to initial aerosol infection very similar to humans[1].	• Immunologic reagents scarce[1]. • Inbred strains non-existant[1].

	• Highly susceptible to TB[2]. • Relatively large size makes the performance of experimental procedures (such as surgery) easier.	• Larger animal results in more specialized housing and space requirements.
Monkey	• Pathology and immunology very similar to humans[1,4]. • Immunologic reagents commercially available[1,4]. • Easy procedural manipulations[1]. • Large size makes the performance of experimental procedures (such as surgery) easy[1]. • Possible to infect with simian immunodeficiency virus simulating HIV co-infection[1]. • Very susceptible to TB [1].	• A biohazard to humans and interspecies cross contamination is a possibility[1]. • Housing problematic[1]. • Expensive[1]. • Animal rights and ethical issues[1]. • Very susceptible to TB, which makes possible outbreaks in natural colonies problematic[1].
Mouse	• **Most cost effective model**[4,7,8]. • Small size makes housing easier • Small size more space economical and large numbers can thus be used at once[7]. • Model for latent[9] and slowly progressing TB exists[5]. • Extensive similarities to human immune response to TB[7]. • Vast database of reagents commercially available[4,7]. • Extensive gene knowledge. • Genetically homogenous mice commercially available[7,8]. • Experimental methods available[3].	• Small size – uncomplex organs i.e. bronchial tree and lymphatics[7]. • Generally resistant to tuberculosis infection[8]. • Most effective infection method is intra-venous injection[8]. • Pathology least similar to humans[1].

References
1. Flynn, *et al.*, 2003. 2. Gupta & Katoch, 2005. 3. Karlson & Feldman, 1949.
4. Kaufmann, 2003. 5. Mustafa *et al.*, 1999. 6. Nuermberger, 1994 7. Orme, 2003. 8. Orme, 1999. 9. Phyu *et al.*, 1998.

Motivated by the advantages and disadvantages of the various animal models, the mouse was preferred for this study because of the following reasons:

a) small size makes housing more space economical and large numbers of animals can thus be used at once,

b) most cost effective model,

c) similarities to the human immune response to TB are adequate,
d) genetically homogenous mice are commercially available,
e) experimental methods are well defined and easy available,
f) there are less animal rights and ethical issues than with monkeys for example,
g) the only known TB animal model presently available in South Africa, and
h) both the bioavailability and efficacy experiments can be done in the mouse model, facilitating comparability.

An inbred strain of mouse, the BALB/c was chosen to minimize inter-subject variability. The number of mice needed to illustrate statistical differences between tests formulations could, therefore, be reduced. The BALB/c, furthermore, has an intermediate susceptibility to *M.Tb* and its immune system will allow the bacilli to multiply after infection to a constant plateau (Nuermberger, 1994).

2.4 CONCLUSION

Treating TB effectively is problematic due to various reasons such as unaccurate diagnosis, poverty, logistics and patience non-compliance. This study will focus the overcoming the problem of patience non-compliance by developing a new dosage form with reduced amount of rifampicin, pyrazinamide, ethambutol and isoniazid compared to the current fix dose dosage form currently prescribed in South Africa. The Pheroid™ drug delivery system has the ability to increase bioavailability and efficacy of some drugs *in vivo* and will be discussed in Chapter 3. This ability of the Pheroid™ will assist in a binary approach in reducing API's. An increase in bioavailability will allow a directly proportional decrease in the amount of API's or increased dosage frequency. An increase in efficacy will also allow a directly proportional decrease in the amount of API's. Increased bioavailability and efficacy have to be balanced with decreased dosage amount and frequency thus reducing side effects. A decrease in API's will hopefully reduce side effects, thus overcoming patient non-compliance currently experienced by TB treatments. Increased bioavailability and efficacy using Pheroid™ will be investigated in this study utilizing non-human vertebrates. The most popular animal models currently used for tuberculosis research are mice, guinea pig, rabbit and monkeys and each model has strengths and weaknesses and the BALB/c mouse model was chosen for investigations here presented.

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CHAPTER 3

USING PHEROID™ TECHNOLOGY IN THE FIGHT AGAINST TB

3.1 INTRODUCTION

Various colloidal drug delivery systems have been investigated for use in the fight against TB, the most popular of which are liposomes (Khuller *et al.,* 2004: Rastogi *et al.*, 2006), microspheres (Rastogi *et al.*, 2006) and nanoparticles (Gelperina *et al.*, 2005: Rastogi *et al.*, 2006). Each system holds advantages and disadvantages but have in common that they present a potentially more effective method for anti-tuberculosis drug delivery. The advantages of liposomes as a drug delivery system include having the ability to deliver drugs selectively, incorporation of both hydrophilic and hydrophobic API's, generally non-toxic and biocompatible (Khuller *et al.,* 2004). Nanoparticles have the advantages of high stability, high entrapment capacity, Various routes of administration are possible, sustained drug release and also incorporation of both hydrophilic and hydrophobic API's (Gelperina *et al.*, 2005). The communal aim for using drug delivery systems are to sustain effective *in vivo* drug levels using lower dosages or decreasing dosing frequency and, therefore, improving patient compliance and resulting in a decrease in side effects (Rastogi *et al.*, 2006). Pheroid™ technology is a novel drug delivery system that may contribute to this communal aim. This chapter focuses on the proven advantages that Pheroid™ technology have over the other drug delivery systems, making it a potentially successful candidate in the fight against TB.

3.2 PHEROID™ TECHNOLOGY

Pheroid™technology entails a method of drug delivery comprising of hollow hydrocolloid with vehicles of stable submicron and micron sizes. Hydrocolloid is defined as particles homogenously dispersed in water. Pheroid™ consists of essential fatty acids and nitrous oxide spheres dispersed in water and is stable when it maintains shape and size. Other molecules such as API's, proteins and even deoxyribonucleic acid (DNA) can either be embedded/entrapped into the fatty acids plus nitrous oxide (N_2O) fraction or in the water fraction (Meyer, 2004).

The Pheroid™ drug delivery vehicle can be manipulated in terms of morphology, structure, size, function and drug loading capacity depending on the needs of the treatment regime. The

unique composition and structure of the Pheroid™ results in characteristics useful as an anti-tuberculosis drug delivery system.

These special characteristics include:

- Components are an essential ingredient of the body and non-toxic.
- Move rapidly across physical barriers such as bacterial cell membranes.
- Deliver drugs quickly and release API's from the delivery vehicle.
- Increase bioavailability and enhance pharmacokinetic parameters such as C_{max} (Maximum plasma concentration) and T_{max} (time to reach C_{max}).
- Entrap soluble, insoluble, hydrophilic and hydrophobic drugs.
- High drug entrapment efficiency.
- Protect the drug from metabolism.
- Minor leakage of drug prior to reaching the target with optimal drug dosage delivery.
- No immune response in humans.
- Target site of delivery on sub-cellular level.
- Eliminate and/or reduce drug resistance.
- Stable with a consistent size and elasticity (Grobler, 2006).

These characteristics lead to the possibility of administering a lower amount or dosage of drugs using Pheroid™ technology, thus possibly reducing side effects and shortening exposure to the drug due to a shorter treatment time.

Pheroid™s can be separated into different types namely vesicles, sponges and depots according to size and function. Table 3.1 summarises the Pheroid™ vehicle types and sizes.

Table 3.1: Pheroid™ types and sizes. Adapted from Grobler (2008).

Pheroid type	Nano-vesicles	Micro-vesicles	Multi laminar vesicles	Micro-sponges	Natural depots
Size	10-200 nm	1-5 µm	5-50 µm	1-10 µm	30-100 µm

Pheroid™ nano-vesicles and micro-vesicles are usually collectively referred to as vesicles and include both types.

3.2 PRO-PHEROID™

Pro-Pheroid™ entails the oil phase consisting of the fatty acid plus N_2O fraction of the Pheroid™ but excluding the fluid in which the vehicles are dispersed. The various Pheroid™ vehicles will form once pro-Pheroid™ come into contact with a fluid.

3.3 PRECEDING TB BASED PHEROID™ STUDIES

Prior to this study the results of several studies indicate why Pheroid™ technology is a good candidate for a better and more efficient treatment regime in the fight against TB. Previous *in vitro* studies proved that anti-TB drugs entrapped in Pheroid™s inhibit *M.tb* growth and surmount drug resistance. Previous *in vivo* studies included drug bioavailability in humans and on pilot scale in mice. This study aims to supplement previous bioavailability and efficacy data in mice with the aim of compiling a dossier that can be submitted to the regulatory authorities for drug registration.

3.3.1 *In vitro* studies

In vitro studies showed the ability of the Pheroid™ to increase the antibacterial effect of isoniazid, rifampicin, pyrazinamide and ethambutol on *M. tuberculosis* considerably. The following studies proved that the Pheroid™ with these entrapped actives inhibit bacterial growth at much lower concentrations than with the free drug, while the Pheroid™ drug delivery system even eliminated growth of several drug resistant strains.

3.3.1.1 Bacterial growth inhibition of *M.tb*

Growth of reference strain H37Rv was determined with the use of radio-active labels in a BACTEC system.

The H37Rv reference strain of *M.tb* used in most of these experiments has been isolated from a human patient in 1905 by E.R. Baldwin, Trudeau Sanatorium, New York. This strain has since been used extensively in biomedical research worldwide and has been designated as the neotype of *Mycobacterium Tuberculosis* in 1972 (Kubica *et al,.* 1972). Unlike some clinical isolates, this strain displays typical species growth characteristics and retains full virulence *in vitro* and *in vivo*. The H37Rv strain also shows typical drug susceptibility and biochemical activity (Kubica *et al.,* 1972).

M.tb. strain cultures were inoculated into BACTEC vials. Cultures in BACTEC were grown until a growth index (GI) of 500(±50) was reached. BACTEC growth of cultures was monitored over a

period of days and the Delta Growth Index (ΔGI) value for every 24-hour doubling period determined.

Pheroid™ entrapped isoniazid exhibited effective growth inhibition at a concentration of 0.005 mg/ml whereas the free drug only showed growth inhibition at 0.02 mg/ml (Nieuwoudt *et al.,* 2008) as illustrated in figure 3.1 below. Entrapment of rifampicin and ethambutol in Pheroid™ showed similar enhancement in *in vitro* efficacies (Grobler, 2008)

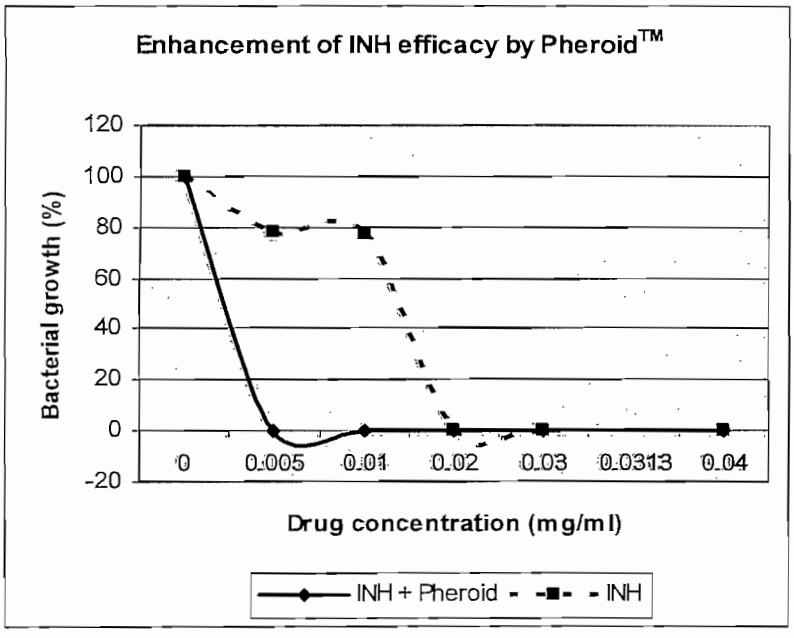


Figure 3.1: The growth of reference strain H37Rv at various concentrations of Pheroid™ entrapped vs. free isoniazid. The solid line indicates Pheroid™ entrapped isoniazid and broken line free isoniazid (Grobler, 2008). Reprinted with permission.

3.3.1.2 Reduction and suggested elimination of drug resistance to anti-TB drugs

The Pheroid™ has been shown to reduce and/or eliminate drug resistance to rifampicin, isoniazid, ethambutol and pyrazinamide *in vitro.*

3.3.1.2.1 Elimination of rifampicin (RMP) drug resistance

M.tuberculosis rifampicin resistant patient isolates, hosted in the sample bank of the University of Stellenboch, Centre for Molecular and Cellular Biology at Tygerberg were cultured for this study (Meyer 2004). The Pheroid™ entrapped rifampicin resulted in a complete bactericidal effect on the resistant strain, whereas free rifampicin showed no inhibition as Figure 3.2 illustrates (Grobler, 2006).

It was, therefore, found that Pheroid™ entrapped rifampicin at concentrations of 1 µg/ml kills the resistant strain to a much greater extent than rifampicin alone. It may be concluded that the rifampicin does not stay enentrapped in the Pheroid™, but is released from the vesicles into the bacilli's internal environment for bactericidal activity (Meyer, 2004).

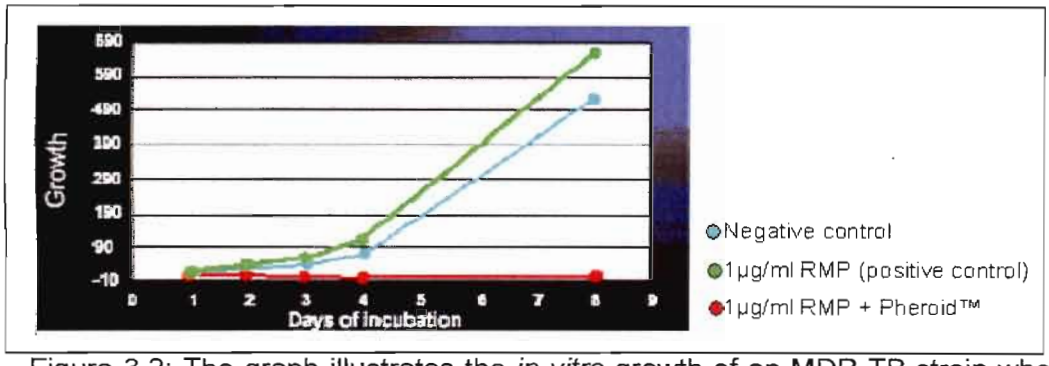


Figure 3.2: The graph illustrates the *in vitro* growth of an MDR-TB strain when challenged with both entrapped and free rifampicin (Grobler, 2008). Reprinted with permission.

3.3.1.2.2 Elimination of isoniazid (INH) drug resistance

Strain TV25, acquired from the strain bank at the University of Stellenboch, Centre for Molecular and Cellular Biology (Tygerberg, South Africa), was determined to be isoniazid resistant. This study comprised of two sections. First, Pheroid™ vesicles were diluted in the ratios of 1:2, 1:8, and 1:32, and isoniazid added to the dilutions to give a final concentration of 0.1 µg INH/ml Pheroid™ in a 4 ml BACTEC vial. Secondly, isoniazid was entrapped in undiluted Pheroid™ vesicles to give a final concentration of 0.1 µg INH per ml and then diluted in the ratios of 1:2, 1:8, and 1:32 afterwards. The viability of drug resistant strain TV25 was evaluated in the presence of the various Pheroid™ plus INH combinations in BACTEC. The ΔGI was calculated for each combination after 6 days of incubation (Grobler, 2009; Meyer, 2004). The ΔGI is an indicator of mycobacterial growth over a period of time and is determined: ΔGI = GI (day+1) - GI (day).

The results set out in Figure 3.3 show that the ΔGI for *M.tTb.* strain TV25 was still below 10 in the presence of the priorly entrapped isoniazid in the undiluted Pheroid™, whereas the sample subjected to the formulation of the isoniazid added to the diluted Pheroid™ shows positive growth with a ΔGI = 28 (Meyer, 2004). Both the isoniazid added into 1:2 diluted Pheroid™ and the priorly entrapped INH in the undiluted Pheroid™ showed a 7 fold and a 2½ growth inhibition respectively when compared with the negative control. Figure 3.4 also illustrates that isoniazid

in Pheroid™ kills the drug resistant strains of *M.tuberculosis* to a much greater extent than free isoniazid at dilutions as low as 1:32 for both the drug and Pheroid™.

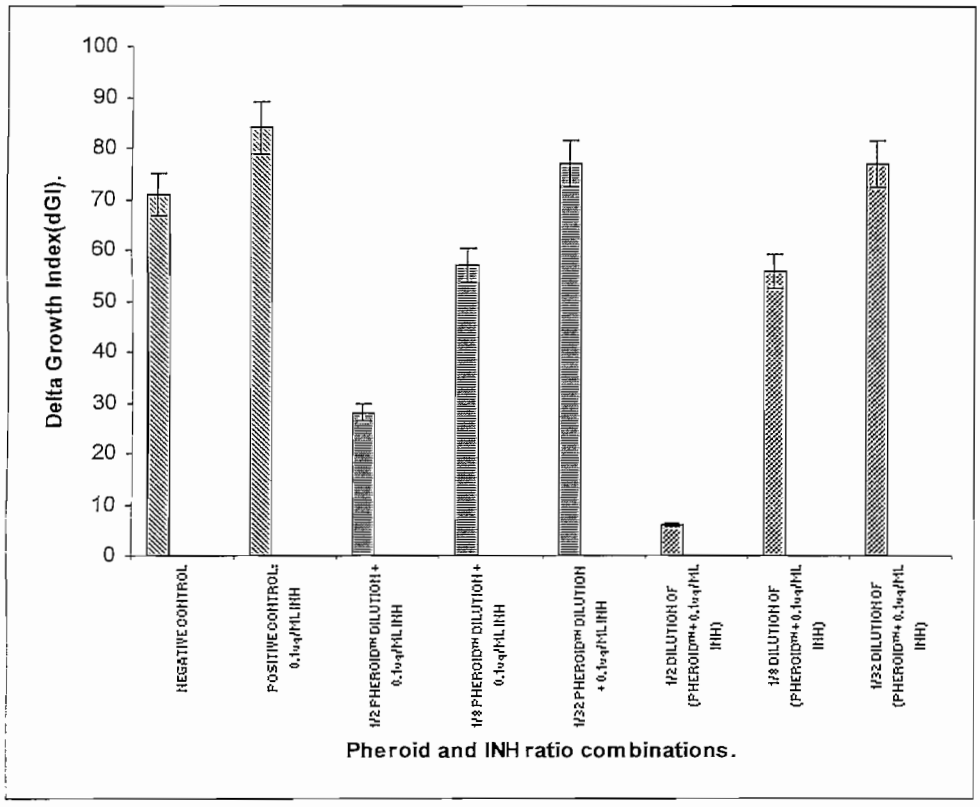


Figure 3.3: *M.tb* strain TV25 cultivated for 6 days in BACTEC and treated with various Pheroid™ and INH dilution ratios. The horizontal filled bars represent the INH added to diluted Pheroid™ and the checker bars represent the priorly entrapped INH in the undiluted Pheroid™ (Adapted from Meyer, 2004).

3.3.1.2.3 Elimination of Ethambutol (EMB) drug resistance

Ethambutol was prepared in sterile deionized water and dissolved in Pheroid™ at different concentrations. The mixtures were then sonicated in a cup-horn sonicator at frequency setting 7 for one minute. It was added to a culture of *M.tuberculosis* H37Rv (known to be resistant to ethambutol) in a BACTEC system. GI was determined every 24 hours ± one hour (Meyer, 2004; Grobler, 2009) for 9 days. GI values were calculated relative to the control value of 415 to give % inhibition of growth. The results on day 6 after calculations are set out in Figure 3.4.

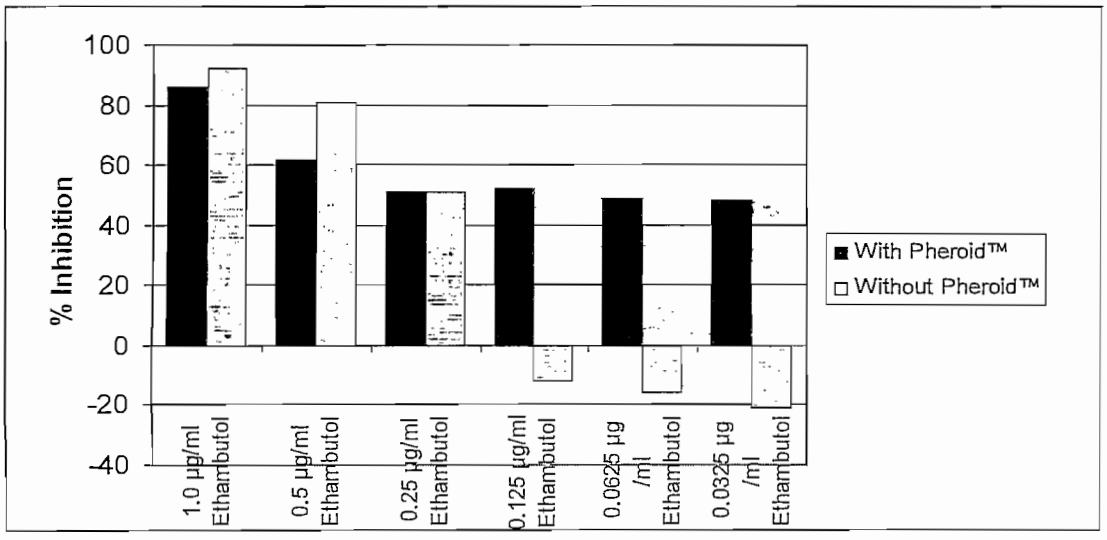
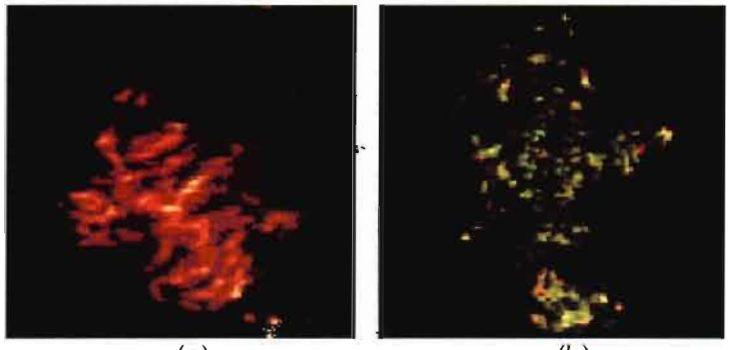


Figure 3.4: Comparison of growth inhibition of *M.tb* reference strain H37Rv after 6 days of treatment with ethambutol with and without the Pheroid™ (Meyer, 2009).

The results show that Pheroid™ entrapped ethambutol inhibit *M.tuberculosis* (H37Rv strain) growth at a concentration as low as 1.0 µg/ml *in vitro*. As from ≤ 0.125 µg/ml ethambutol, Pheroid™ presence maintains a strong inhibitory effect on *M.tb* growth compared to Pheroid™ untreated bacteria, whereas a slight growth increase (negative values) is observed. Not much difference in growth inhibition occurs between Pheroid™ vs. non Pheroid™ treatments at concentrations 0.25-1.0 µg/ml. The very high concentrations appear to have a better effect in the Pheroid™ untreated *M.tb* while inhibition at the lower concentrations is steadily maintained by EMB entrapped Pheroid™ (approx 50% inhibition over the range of 0.03125 µg/ml-0.125 µg/ml). This strongly supports the possibility of an effective dosage form at lower concentrations of ethambutol.

3.3.1.2.4 Elimination of pyrazinamide (PYR) drug resistance

This investigation was performed by confocal laser scanning microscopy (CLSM), using a Nikon PCM 2000 with a 60x ApoPlanar oil objective and a small pinhole. A live/dead fluorescent viability stain known as Baclight was used to stain live bacteria green and dead bacteria red. All Bacillus of Calmette and Guerin (BCG) strains are resistant to pyrazinamide. General viability of the BCG's was determined by the green/red ratio of the bacteria. The effect of equal amounts and concentrations of free and Pheroid™ entrapped pyrazinamide on BCG viability was investigated (Grobler, 2009; Meyer, 2004).



(a) (b)

Figure 3.5: The CLSM micrographs show the effect of (a) Pheroid™ entrapped pyrazinamide and (b) free pyrazinamide on cell viability, showing that BCG was sensitive to Pheroid™ entrapped pyrazinamide, but not to free pyrazinamide at the same concentration and within the same time period of 4 hours (Grobler, 2009). Reprinted with permission.

The general viability of the BCG's before any addition of antibiotics was between 85 – 95%. The BCG-viability after a two-hour incubation of the applicable dosage of free pyrazinamide was 68 –72% while mobile live bacteria was observed. Pyrazinamide entrapped in Pheroid™ vesicles led to a 65-75% decrease in BCG viability within one hour of incubation. No moving BCG was observed after two hours (Grobler, 2009; Meyer, 2004). It can therefore be concluded that pyrazinamide entrapped in Pheroid™ kills pyrazinamide resistant strains of *M.tuberculosis* to a much greater extent than free pyrazinamide.

The study was then extended to determine whether entrapment of pyrazinamide into Pheroid™ vesicles could overcome resistance of intracellular bacteria, using infection of human macrophages THP1 by BCG in culture as an *in vitro* infection model. The THP1 macrophage cells were cultured and infected with pre-treated labelled BCG bacteria. Treatment consisted of equal concentrations of 0.075 µg/ml of free and Pheroid™ entrapped pyrazinamide. CLSM was used to determine infection and survival of the bacteria (Meyer, 2004). The viability of BCG's after infection in macrophages reflected that BCG's inside macrophages treated with pyrazinamide entrapped in Pheroid™ were effectively killed (Meyer, 2004). Pyrazinamide entrapped in Pheroid™ therefore kills the resistant strains of *M.tuberculosis* to a much greater extent inside human macrophages than free pyrazinamide when challenged at a concentration of 0.075 µg/ml.

3.3.2 *In vivo* bioavailability studies

The main parameters that are generally investigated during bioavailability studies are the maximum plasma concentration (C_{max}) the API attains, the time it takes to reach the maximum concentration (T_{max}) and the area under the curve (AUC), i.e. the period that the active is detectable in the plasma. Therapeutic efficacy can be relayed to the AUC and is determined by the amount of the drug absorbed and transported to the plasma of the patient and the time the API is present in the plasma. The longer time and higher concentration the APIs are present in plasma, the more effective the treatment (Holford, 2004:42) The following bioavailability studies show why the Pheroid™ may lead to the possibility of administering a lower concentration of drugs, shortening exposure to the drugs and reducing side effects.

3.3.2.1 *In vivo* pilot mouse study on the bioavailability of Pheroid™

Four CD57 inbred mice per time point were divided into two groups; group A acted as positive control and received Rifafour® e-275 (dissolved in water) and group B received a Pheroid™ formulation (Pyriftol) with the anti-TB drugs. Rifafour® e-275 is a commercially available treatment containing the WHO prescribed anti-TB drug dosages. 200 µl of both the test formulations were administered via oral gavage on a daily basis for four days. All dosages were administered at the same time each morning. The mice were sacrificed at 20, 90, 120, 180 and 240 minutes after administration on the fourth day (Matthee, 2007) and whole blood was collected from the heart into microfuge tubes. Plasma was separated and transferred to cryotubes for storage in liquid nitrogen. The frozen cryotubes were transported on dry ice to the University of Cape Town (South Africa) where the samples were assayed using an LC/MS/MS (Matthee, 2007). The laboratory that executed the analysis is accredited as a WHO Reference Centre for the analysis of Tuberculostatics.

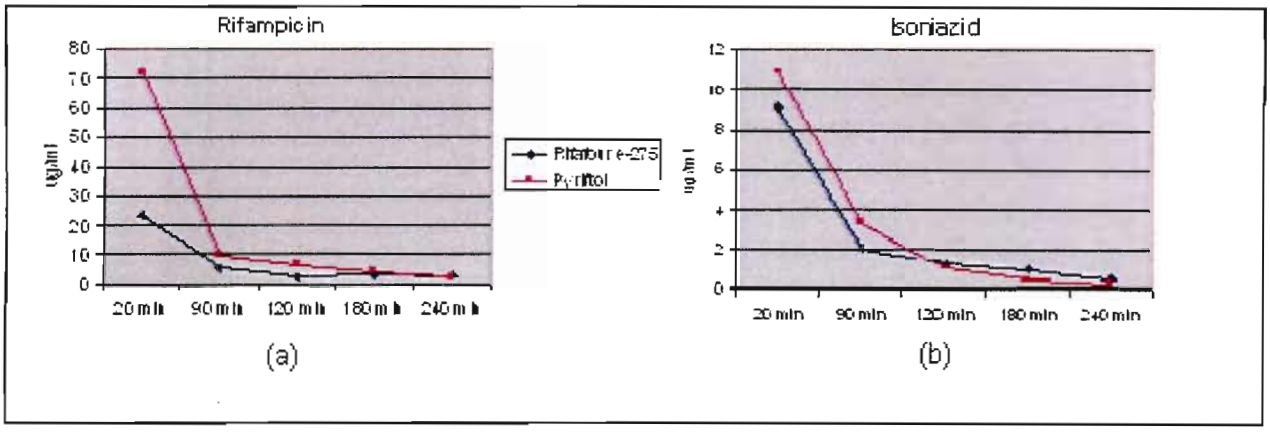


Figure 3.6: Comparative graphs of plasma concentrations of (a) rifampicin and (b) isoniazid formulated in the Pheroid™ formulation (Pyriftol) and found in Rifafour e-275® (Matthee, 2007). Reprinted with permission.

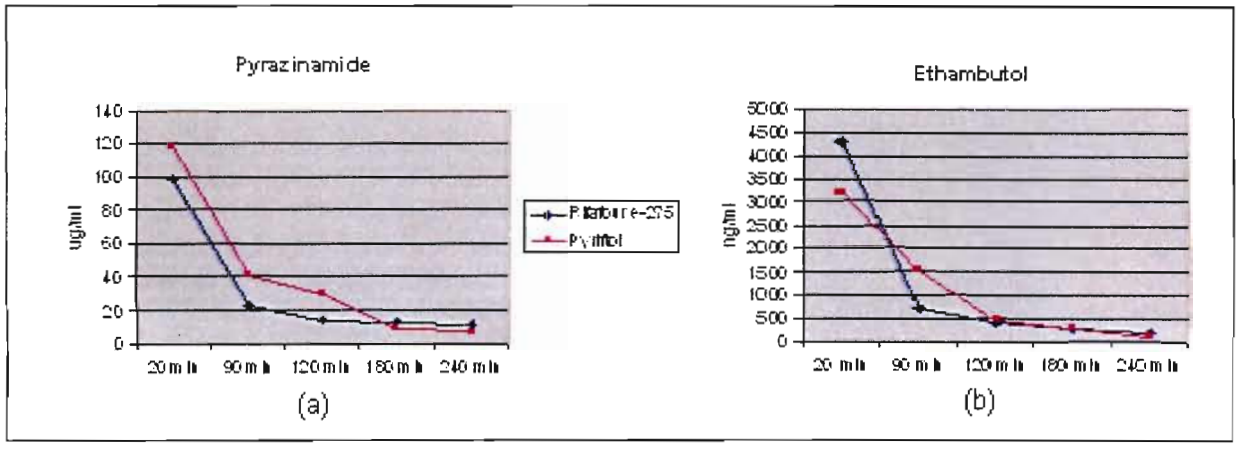


Figure 3.7: Comparative graph of plasma concentrations of (a) pyrazinamide and (b) ethambutol between the Pheroid™ formulation (Pyriftol) and Rifafour e-275® (Matthee, 2007). Reprinted with permission.

Plasma levels at 20 min were read from Figures 3.6 and 3.7, and tabled in Table 3.2. Increase in bioavailability (%) was determined using the following equation:

$$\%Bioavailability = \frac{t_{20}(\text{Pheroid}^{TM}) - t_{20}(\text{Rifafour e-275}^{\circledR})}{\text{Pheroid}^{TM}} \times 100$$

Table 3.2: Mean Drug Plasma concentration (µg/ml) at t=20 min				
Active ingredient	Negative Control	Rifafour e-275® (t_{20})	Pheroid™ (t_{20})	Increase in bioavailability (%)
Rifampicin	0	23.49	71.6	305%
Isoniazid	0	9.15	10.98	120%
Ethambutol	0	4.3	3.18	74%
Pyrazinamide	0	99.37	118.3	119%

The results of the pilot study revealed that Pheroid™ technology increased the bioavailability of rifampicin at 20 min after administration three times in mice plasma (as shown in Table 3.2). Isoniazid and pyrazinamide plasma levels were also elevated considerably. During the execution of this pilot study most if not all problems were resolved and methods were developed that will give accurate and repeatable results. These methods make the mouse model physically and metabolically suitable for determining anti-Tb drug pharmacokinetics.

This pilot study served as the basis for determining the number of experimental groups, number of mice per group and time intervals in planning a statistically accurate full-scale mouse PK study (Chapter 4).

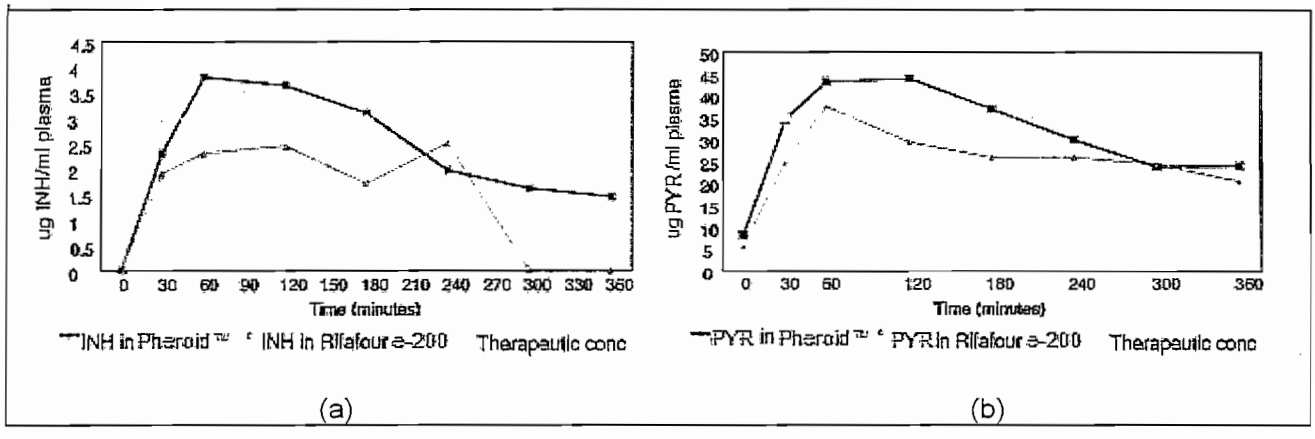
3.3.2.2 Comparative *in vivo* bioavailability in human volunteers

3.3.2.2.1 Case study

This study was a double open crossover study with a two week wash out period in one healthy volunteer. The bioavailability of the generally used anti-tuberculosis treatment agents, i.e. rifampicin, isoniazid, ethambutol and pyrazinamide were determined when a) entrapped into the Pheroid™ drug delivery system in the form of pro-Pheroid Soft Gel capsules, and b) the commercially available treatment regime, which was Rifafour® e-200 at the time of the study. Dosages were administered as set out in Table 3.3. The study was conducted over two periods of 4 days each, interrupted by a two week wash out period. During the last day (day 4) of each period, blood samples (10 ml each) were taken at 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours and 9 hours after administration of the drugs to determine several pharmacokinetic parameters (Meyer, 2004). All side effects were noted.

Table 3.3: Dosing of bioavailability case study (adapted from Meyer, 2004).		
		mg/day
Rifafour® e-200	RMP/INH/PZA/EMB	600/300/1500/1000
pro-Pheroid™ soft gel capsules	RMP/INH/PZA/EMB	400/200/1000/660

Plasma was separated and transferred to cryotubes for storage in liquid nitrogen. The frozen cryotubes were transported on dry ice to the University of Cape Town (South Africa). The laboratory that executed the analysis is accredited as a WHO Reference Centre for the analysis of Tuberculostatics. The plasma concentrations of rifampicin, isoniazid, ethambutol, pyrazinamide and their active metabolites were determined by high performance liquid chromatography.



(a) (b)

Figure 3.8: Bioavailability graphs comparing plasma levels of (a) isoniazid and (b) pyrazinamide in a single healthy volunteer after administration of Pheroid™ entrapped and commercially available dosage forms over time (Meyer, 2004). Reprinted with permission.

Figure 3.8a shows the enhanced bioavailability of INH when entrapped in the Pheroid™ delivery system, even though the daily dosages were only two thirds of that of the comparator, Rifafour® e-200 (Meyer, 2004). The highest plasma levels (C_{max}) reached were 3.81 and 2.45 µg INH/ml for the Pheroid™ and for Rifafour® e-200 respectively. This indicates an increase of peak plasma concentrations of between 45% and 55%, which resulted in a projected increase of between 73% and 83%, were dosages equal. The T_{max} was obtained 30 minutes earlier for Pheroid™ entrapped isoniazid. The obvious difference between the two dosage forms is the fact that the effective therapeutic concentration of 3 µg/ml plasma (Moffat *et al.,* 2004) was never reached in this volunteer when the isoniazid was administered as Rifafour® e-200,

whereas the therapeutic effective concentration was maintained for 120 minutes after administration of Pheroid™ formulation (Meyer, 2004).

Figure 3.8b shows the enhanced bioavailability of pyrazinamide when entrapped in the Pheroid™ delivery system, even though the daily dosages were only two thirds of that of the comparator, Rifafour® e-200 (Meyer, 2004). A comparison of the blood profiles shows that administration of pyrazinamide in the Pheroid™ formulation increased the C_{max} by between 15 and 17%, with a projected increase of 24% if the dosages were to be equal. Although the T_{max} for the two products were both at 30 minutes, the period during which the effective plasma concentration of 35 µg/ml plasma was sustained, increased dramatically in the Pheroid™ formulation i.e. 130 min for Pheroid™ vs. 15 min for Rifafour® e-200, effectively an 866% increase in coverage (Meyer, 2004).

The study produced similar results for rifampicin and ethambutol (Meyer, 2004), results not shown.

No significant liver function side effects were found with the Pheroid™ formulation. The S-unconjugated bilirubin, which did show levels elevated above the normal range, almost returned back to normal with in 8 hours post drug administration, and was normal the next day. None of the liver enzymes showed levels higher than the normal range (Meyer, 2004). The only adverse reaction after administration of the Pheroid™ based formulations was nausea, whereas the comparator Rifafour® e-200 led to serious headache, jitters and nausea (Meyer, 2004). The lesser side effects indicate that the Pheroid™ based formulation may result in better patient compliance.

3.3.2.2.2 Double blind crossover study on sixteen healthy volunteers

A bio-equivalence study was performed to determine whether the Pheroid™ delivery system would attain drug plasma levels that would support therapeutic efficacy. This Phase 1 trial was approved by the South African Medicine Control Council (SAMCC) and the South African Medical Association's Research Ethical Committee (Meyer, 2004).

Bioavailability of the generally used anti-tuberculosis treatment agents, i.e. rifampicin, isoniazid, ethambutol and pyrazinamide were determined when a) entrapped into the Pheroid™ drug delivery system in the form of pro-Pheroid™ Soft Gel capsules, and b) the commercially available treatment regime, namely Rifafour® e-200. The Pheroid™ based products, Pyriftol C

and P, contained only 60% of the active ingredients of that in Rifafour® e-200 (Grobler, 2008). The study was conducted over two periods of 4 days each, interrupted by a two week wash out period. During the last day (day 4) of each period, blood samples were taken at 0 minutes, 30 minutes, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 6 hours and 9 hours after administration of the drugs (Grobler, 2008). All side effects were noted. Plasma was separated and transferred to cryotubes for storage in liquid nitrogen. The frozen cryotubes were transported on dry ice to the University of Cape Town (South Africa). The laboratory that executed the analysis is accredited as a WHO Reference Centre for the analysis of tuberculostatics. The plasma concentrations of rifampicin, isoniazid, ethambutol, pyrazinamide and their active metabolites were determined by high performance liquid chromatography and the mean average plasma profiles are illustrated in Figures 3.9 and 3.10.

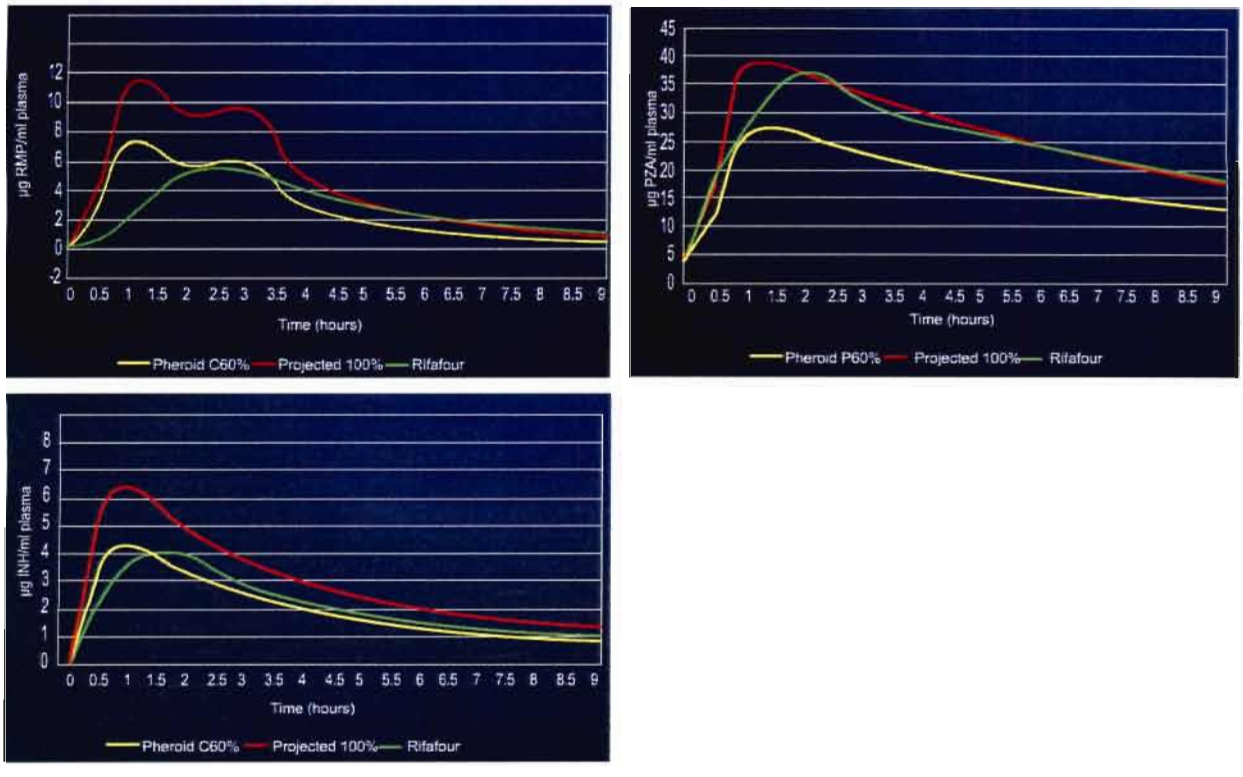


Figure 3.9: Bio-availability profile of RMP, PZA and INH calculated from blood plasma levels of 16 healthy human volunteers. The green line illustrates plasma levels after administration of Rifafour® e-200. The yellow line illustrates plasma levels after administration of the Pheroid™ based dosage form containing only 60% API of that of Rifafour® e-200. The red line is a projection of what the plasma levels could be after administration of the Pheroid™ based dosage form containing the same dosage as Rifafour® e-200 (Grobler, 2008). Reprinted with permission.

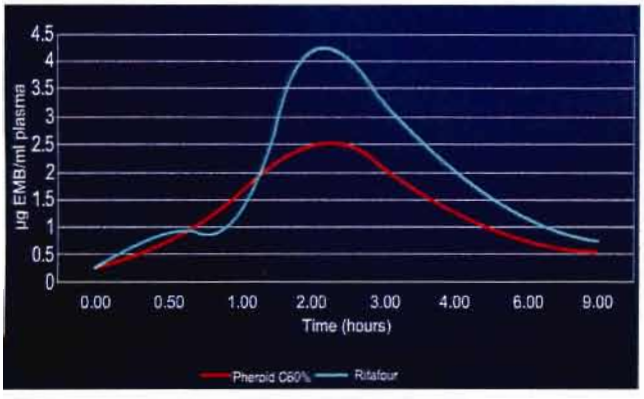


Figure 3.10: Bio-availability profile of EMB calculated from blood plasma levels of 16 healthy human volunteers. The blue line illustrates plasma levels after administration of Rifafour® e-200. The red line illustrates levels after administration of the Pheroid™ based dosage form containing only 60% EMB of that of Rifafour® e-200 (Grobler, 2008). Reprinted with permission.

The entrapment of rifampicin, isoniazid, pyrazinamide and ethambutol into Pheroid™ led to an increase in absorption with a resultant increase in the plasma levels of these antimicrobials. The C_{max} for rifampicin in Pyriftol C was increased by 27% using only 60% of the Rifafour® e-200 dosage and represents a statistically significant difference. In the case of the isoniazid in Pyriftol C, the C_{max} is higher by 49% (Grobler, 2006). The entrapment of the antimicrobials led to a much quicker absorption when compared to Rifafour® e-200, with T_{max} decreased by nearly half for pyrazinamide, rifampicin and isoniazid which represent significant differences.

The therapeutic concentrations are maintained for longer and the circulatory time of the drugs are extended, resulting in the increased exposure of the bacteria to the antimicrobials. The AUC of all the Pheroid™ based formulas are consequently higher even with a 60% dosage (Grobler, 2006). A lower dosage than currently available on the market can, therefore, be used to obtain similar therapeutic concentrations suggesting that it may be possible to shorten the treatment period of 6 months to 2 or 3 months using Pheroid™ technology.

The adverse events of the phase 1 trial were analysed by Dr H McIlleron, Division of Pharmacology, University of Cape Town. Of the 45 events reported, the only significant differences were for drowsiness (7 Rifafour ® e-200, 0 Pyriftol). No vomiting occurred after Pyriftol administration. No changes in the chemical pathology or haematology findings were regarded by the investigator as adverse events (Grobler, 2006). The lesser side effects in this study again indicate that the Pheroid™ based formulation might result in better patient compliance, with less chance for the development of multi-drug resistance.

3.4 CONCLUSION

Pheroid™ technology is a novel drug delivery system that possesses characteristics and abilities that makes it a good candidate to lower anti-tuberculostatic quantities in a new dosage formulation. Pheroid™ inhibits *M.tb* bacterial growth at much lower dosages than the free drug alone. Previous studies proved that Pheroid™ formulations reduce and eliminate drug resistance to RMP, INH, EMB and PYR *in vitro.* Bioavailability studies *in vivo* also showed a significant increase in plasma levels in mice and human studies. This increase could contribute in lowering anti-tuberculostatic dosages in a new formulation.

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CHAPTER 4

BIOAVALIBILITY OF THE PHEROID™-ENTRAPPED ANTI-TUBERCULOSIS DRUGS IN THE MOUSE MODEL

4.1 INTRODUCTION

Proving *in vivo* availability of pharmaceutical active ingredients (APIs) is an imperative step in drug development. Bioavailability of an API can be defined as the fraction of active unchanged drug reaching the systematic circulation after administration (Holford, 2004:41). *In vivo* availability of any API can be greatly influenced by the dosage form since the formulation plays a role in the stability of the API as well as the place of absorption and absorption rate. Preceding studies on anti-TB APIs rifampicin, isoniazid, ethambutol and pyrazinamide in a Pheroid™ based dosage form indicated elevated bioavailability for at least three of the four APIs. Although an *in vivo* study involving 16 humans volunteers had previously been done (Chapter 3), a need exists for a non-human mammalian model that will allow an investigation into both pharmacokinetics and efficacy. The inbred mouse model is preferred over other mammalian models due to practical reasons discussed in Chapter 2. A study with the title: *In vivo* pharmacokinetic evaluation of anti-tuberculosis drugs entrapped in the Pheroid™ drug delivery system has, therefore, been conducted. This study has been approved and monitored by the NWU ethics committee - approval number: NWU-0031-08-S5.

4.2 AIM

To obtain a comparative pharmacokinetic model of the anti-TB drugs: rifampicin, ethambutol, pyrazinamide and isoniazid when delivered by the Pheroid™ drug delivery system vs. both a commercially available treatment and the pure pharmaceutical actives.

4.3 METHODS

4.3.1 Subjects

Female BALB/c mice at an average mass of 20 g were purchased from the South African Vaccine Producers (Sandringham) and certified as pathogen free. Animals were housed at the Animal Centre at the Potchefstroom Campus of the North-West University, under normal temperature of 21 ± 2°C, humidity of 55 ± 15% and light intensity of 350- 400 lux one metre

above floor level with 12 hours light and 12 hours dark, and a ventilation rate of 18 changes per minute. The animals were fed a standard pellet diet and water was supplied *ad libitum.*

4.3.2 Anti-tuberculosis drugs

Rifampicin (RMP/NC-020/06) and pyrazinamide (PYR/P-228/06) were obtained from Lanaria Chemicals Limited (Thailand). Isoniazid (IF070501) and ethambutol (IF070608) were obtained from Iffect Chemphar (HK) Company Limited (China) through BD Fine Chemicals (Pty) Limited (South Africa).

All pharmaceutical active compounds were used as received. Particle sizes of powders were determined by the method of laser diffraction using a Malvern Mastersizer Micro 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The method incorporated the Hydro 2000 SM cell at a pump speed of 1500 rpm until an obscuration of 14- 22 % was obtained. Cyclohexane (Merck Germany, analytical grade batch no: 1032247) was preferred as dispersion medium over H_2O and ethanol for all four powders because all the powders illustrated least solubility and no obvious coagulation when using cyclohexane. Samples of each raw material were measured in quadruple. The average particle sizes are set out in Table 4.1.

Table 4.1: Particle size analysis of anti-tuberculosis raw material powders.

Size distribution	Rifampicin	Pyrazinamide	Isoniazid	Ethambutol
d(0.1)	11.0 µm	18.1 µm	45.0 µm	13.7 µm
d(0.5)	73.2 µm	53.0 µm	88.8 µm	40.0 µm
d(0.9)	194.2 µm	155.3 µm	163.9 µm	111.7 µm

Particle size of the raw material powders may play a role in physical characteristics of the dosage form. In this case the particle size will influence the viscosity of the formulation and as discussed in more detail below, a fluid formulation that flows freely through a very thin needle will be preferred. The particle size distribution defines the size range for 10 % (d 0.1), 50% (d 0.5) and 90% (d 0.9) of the particle sample examined. The sizes specified indicate the largest crystal for that particular fraction of crystals. When one looks at the size distribution of rifampicin for example, 10% of the crystals were 11 µm or smaller, 50% at 73.2 µm or smaller and 90 % at 194.2µm or smaller. Large crystals might obstruct the fluidity of the formulation and smaller crystal sizes are preferred.

4.3.3 Test formulations

4.3.3.1 Pheroid™ technology based formulation development

Pheroid™ based formulations were developed and manufactured by the Unit for Drug Research and Development, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. The pro-Pheroid™ concept was used to develop a suitable formulation. Pro-Pheroid™ is deemed the best choice due to volume constraints and API instability of especially rifampicin and isoniazid in the presence of liquid suspensions (Seifart *et al.,* 1991). The pro-Pheroid concept was discussed in Chapter 3. The main considerations when developing the Pro-Pheroid™ formulation were a) physical characteristics of the actives; b) daily dosage per weight of the formulation in comparison with Rifafour® e-275; and c) future dosage form development for human trials/ consumption. Viscosity was the determining physical characteristic and the viscosity of the final formulation had to be low enough to enable an easy flow through the gavage needle used to administer test formulations in the mouse model. The doses administered were guided by Rifafour® e-275 recommended quantities with rifampicin at 10 mg/kg, isoniazid at 5 mg/kg; ethambutol at 18.3 mg/kg and pyrazinamide at 26.6 mg/kg (Gibbon, 2008:300). The rational behind testing the anti-tuberculoststics in a four dose combination is to include the API interactions *in situ (*Singh *et al.,* 2001).

The quantity of anti-tuberculosis drug per ml of the pro-Pheroid™ was determined by the following calculations:

- 10 mg rifampicin per kg dosage = 0.2 mg rifampicin per 20 g mouse, which needs to be ingested in a volume of 200 µl. 0.2 mg per 200 µl = 1 mg/ml.
- 5 mg isoniazid per kg dosage = 0.1 mg rifampicin per 20 g mouse, which needs to be ingested in a volume of 200 µl. 0.1 mg per 200 µl = 0.5 mg/ml.
- 18.3 mg ethambutol per kg dosage = 0.37 mg rifampicin per 20 g mouse, which needs to be ingested in a volume of 200 µl. 0.37 mg per 200 µl = 1.83 mg/ml.
- 26.6 mg rifampicin per kg dosage = 0.53 mg rifampicin per 20 g mouse, which needs to be ingested in a volume of 200 µl. 0.53 mg per 200 µl = 2.66 mg/ml.

The percentage fatty acid fraction to raw material in the pro-Pheroid™ formulation was designed to form Pheroid™ vesicles when emulsified in a liquid and was also based on previously developed formulations used in the preceding pilot mouse study by Matthee (2007). Formulations were produced under Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP) conditions as far as possible. Although the laboratories at the Unit for Drug Research and Development, North-West University, are not accredited by any regulatory

authority such as South African National Accreditation System (SANAS) or ISO 9001 (an internationally accepted

Table 4.2: Pro-Pheroid™ formulation developments.

Pro-Pheroid™ Formulation	Actives	% Fatty acid	Physical characteristics	
Formulation 1	Rifampicin and pyrazinamide (RMP+PZA)	44.3		Very hard and brittle – not even a paste
	Isoniazid and ethambutol (INH+EMB)	40.9		Oily powder clumping together – not even a paste
Formulation 2	Rifampicin and pyrazinamide (RMP+PZA)	64.8		Smooth paste fluid enough to pass through an oral gavage needle
	Isoniazid and ethambutol (INH+EMB)	64.8		Smooth paste flowable enough to pass through an oral gavage needle

quality standard), the guidelines and principles were followed as closely as possible. Anti-oxidants i.e, butylated hydroxyanisole and butylated hydroxytoluene were added at concentrations of 0.026% and 0.131% respectively. Ascorbic palmitate at a concentration of 6.82 mg/g rifampicin was added to the formulations containing rifampicin in an attempt to decrease degradation of rifampicin. The pro-Pheroid™ formulation development process and results obtained are illustrated in Table 4.2. Due to the flowability of formulation 2, it was chosen as the experimental formulation. This decision was based on the fact that this formulation could flow through a gavage needle, which was important in the animal studies to follow.

4.3.3.2 Formulation preparation for administration.

Rifafour® e-275 (batch 803A) tablets were obtained from Medirex Pharmacy, South Africa. Each tablet contains a tuberculostatic fixed dose combination with 150 mg rifampicin, 75 mg isoniazid, 400 mg pyrazinamide and 275 mg ethambutol (Gibbon, 2008:300).

All formulation preparations were performed under dark room conditions in amber glassware to minimise degradation of photosensitive APIs.

Rifafour® e-275: One commercially available Rifafour® e-275 tablet was grinded, using a mortar and pestle, and dissolved/suspended in 150 ml distilled water by sonification for 15 min. This resulted in a formulation with API concentrations of 1 mg/ml RMP, 0.5 mg/ml INH, 2.66 mg/ml PZA and 1.83 mg/ml EMB.

Pheroid™: Pheroid™ vesicles were produced by adding $N_2O{\cdot}H_2O$ to pro-Pheroid™ test formulation batch 2. 1.05 g of the RMP+PZA pro-Pheroid™ formulation (P08021) and 6.61 g of the INH+EMB pro-Pheroid™ formulation (P08020) were weighed and added together. The mixture was emulsified to produce Pheroid™ vesicles by adding N_2O gassed H_2O up to a volume of 100 ml and sonicating for 15 min. This resulted in a formulation with API concentrations of 1 mg/ ml RMP, 5 mg/ml INH, 2.67 mg/ml PZA and 18.2 mg/ml EMB.

Free drug: Free pharmaceutical actives were dissolved/suspended in distilled water. The following were weighed and added together: 100 mg RMP (RMP/NC-020/06), 50 mg INH (IF070501), 266 mg PZA (PYR/P-228/06) and 183 mg EMB (IF070608). RMP was dissolved in 2-5 ml DMSO before hand. ddH_2O up to a volume of 100 ml was added and the mixture was sonicated for 15 min. This resulted in a formulation with API concentrations of 1 mg/ ml RMP, 0.5 mg/ml INH, 2.66 mg/ml PZA and 1.83 mg/ml EMB.

Pro-Pheroid™: An additional fatty acid fraction was added to pro-Pheroid™ test formulation batch 2. The following were weighed and added together: 1.05 g of the RMP+PZA pro-Pheroid™ formulation (P08021) and 6.61 g of the INH+EMB pro-Pheroid™ formulation (P08020). Fatty acids were added as a placebo pro-Pheroid™ (P08022) up to a volume of 100 ml and sonicate for 15 min. This resulted in a formulation with API concentrations of 1 mg/ ml RMP, 5 mg/ml INH, 2.67 mg/ml PZA and 18.2 mg/ml EMB.

The concentrations are well below the LD_{50} (mouse) for each active:
Rifampicin – Oral LD_{50} of 829.3 mg/kg (IPCS, 2007) while 10 mg/kg will be administered;
Isoniazid – Oral LD_{50} of 176 mg/kg (IPCS, 2007) while 5 and 50 mg/kg will be administered;
Ethambutol - Oral LD_{50} of 8 900 mg/kg (ScienceLab, 2008) while 18.3 and 183 mg/kg will be administered;
Pyrazinamide - Oral LD_{50} of 2 730 mg/kg (Felder & Pitre, 1983) while 26.6 mg/kg will be administered.

4.3.4 Statistical justification for number of mice per group

Results from the pilot mice study as performed by Matthee (2007) were used by Prof Faans Steyn at the NWU Statistical Consulting Service to project possible results if the study done initially on 2-3 mice per group were to be conducted on 6 or 8 mice per group respectively.
The t-test is used to judge the difference between the means of groups relative to the spread or variability of values with-in a group. Once t-values have been obtained a table of significance will indicate a corresponding p-value, which again determines whether the difference between the groups is unlikely to have been a chance finding. To test the significance, a risk level has to be determined. This risk level will determine to what degree a value obtained is statistically significant. For example, a p-value = 0.05 means that a statistically significant difference between the means will be found five out of a hundred times even if there were no difference.
Since both the pilot study and the planned study provided only one concentration-time point per individual, the t-test was conducted according to a t-test proposed by Jawien (1992).

Table 4.3: t-values & p-values for 6 mice as a reflection on the pilot study done by Matthee (2007).

	t-values	p-values
Rifampicin	5.05	$p < 0.001$
Isoniazid	2.18	$p < 0.025$
Pyrazinamide	3.65	$p < 0.001$
Ethambutol	2.42	$p < 0.025$

Table 4.4: t-values & p-values for 8 mice as a reflection on the pilot study done by Matthee (2007).

	t-values	p-values
Rifampicin	5.8	$p < 0.001$
Isoniazid	2.5	$p < 0.01$
Pyrazinamide	4.2	$p < 0.001$
Ethambutol	7.6	$p < 0.0001$

An $p \leq 0.01$ is preferred for all four actives and the decision was made to use 8 mice per time point for each treatment in order to obtain statistical meaningful results.

4.3.5 Sampling procedures for random assigning of mice to groups.

The total number of test subjects were randomly selected by means of systematic random sampling as suggested by the Web Center for Social Research Methods (2008) and assigned into groups. The systematic random sampling method is depicted as follows:

- Number the subjects 1 to N.
- Sample size will be n.
- Interval size (k) = N/n.
- Randomly select an integer between 1 to k.
- Then take every k^{th} unit.

Applied to this study: N = 352, n = 8, random starting number = 4, k=N/n = 44. Selection was started at number 4 and then every 44^{th} numbered individual thereafter in order to complete the 44 plasma collection time groups.

The first animal identified went to the Rifafour® e-275 treatment group; 0 min, 2^{nd} animal to Rifafour® e-275 treatment group; 5 min, 3^{rd} animal to Rifafour® e-275 treatment group; 10 min until time 240 min were reached. Assignation then moved over to Pheroid™ treatment group, 0 min to 240 min. This process was continued to the free drug treatment groups and after that to the pro-Pheroid™ treatment groups. When one subject has been assigned to each of the 44 groups, the second subject for each group was randomly assigned in the same fashion. The assigning pattern was repeated until each of the 44 time groups had 8 mice each.

4.3.6 Excecution of investigation

A volume of 200 µl of the prepared test formulations was administered via oral gavage with a ball tipped needle. Each mouse was firmly restrained using the double handed manual restraint method (Shimizu, 2004: 528) while the neck is extended to get the mouth, pharynx and stomach in a straight line. The needle was carefully inserted through the oesophageal orifice and injected 200 µl directly into the stomach. This method is described as the intragastric administration (Shimizu, 2004:533)

Eight mice were sacrificed for each time point 0, 5, 10, 15, 22½, 30, 45, 60, 90, 120, and 240 minutes after administration. The practical design for administration and collection is set out in Table 4.5.

Each mouse was caught, restrained and the test formulations administered. Mice were marked using a permanent marker on the base of the tail to identify order of administration.

4.3.7 Plasma collection

The mice were sacrificed to collect whole blood. The mice were identified in order of administration by the markings on the base of their tails. The correct mouse was then transferred from the cage to a chamber containing enough halothane, an inhalation anaesthetic, to ensure a painless sacrifice. According to Otto (2004:563), halothane is an efficient and non-invasive anaesthetic regularly used on mice. Blood is collected directly from the heart under thoracotomy. In short this procedure involves cutting open the thorax and withdrawing blood directly from the right heart ventricle using a syringe (Fukuta, 2004:543). Blood was then transferred to microfuge tubes with 17 IU heparin added to prevent blood coagulating (Heparin Sodium for injection: Mucosal –Fresenius, Lot 31102). All tubes were kept at 0 °C inside a refrigeration centrifuge, which was used to separate blood cells from plasma at 4000 rpm for 7 minutes.

Separated plasma was transferred to cryovails and stored in liquid nitrogen. Rifampicin undergoes rapid oxidation to 3-formyl-rifampicin and 1-amino-4-methyl-piperazine. It was, therefore, critical that the blood samples were collected, centrifuged and the plasma obtained and stored within 15 minutes. The frozen cryotubes were transported overnight on dry ice to the University of Cape Town (South Africa) where the samples were assayed blindly.

Table 4.5: Dosing and blood collection schedule for bioavailability study

Formulation	Rifafour® e-275	Pheroid™	Free drug	Pro-Pheroid™	Rifafour® e-275	Pheroid™	Free Drug	Pro-Pheroid™
Day & Time points	Mon 1 Sept	Tue 2 Sept	Wed 3 Sept	Thurs 4 Sept	Mon 8 Sept	Tue 9 Sept	Wed 10 Sept	Thurs 11 Sept
0 min	Collect 9:00	Collect 9:00	Collect 9:00	Collect 9:00				
5 min					Feed 11:45 Collect 11:50	Feed 11:45 Collect 11:50	Feed 11:45 Collect 11:50	Feed 11:45 Collect 11:50
10 min	Feed 9:30 Collect 9:40	Feed 9:30 Collect 9:40	Feed 9:30 Collect 9:40	Feed 9:30 Collect 9:40				
15 min	Feed 10:45 Collect 11:00	Feed 10:45 Collect 11:00	Feed 10:45 Collect 11:00	Feed 10:45 Collect 11:00				
22.½ min	Feed 12:00 Collect 12:22½	Feed 12:00 Collect 12:22½	Feed 12:00 Collect 12:22½	Feed 12:00 Collect 12:22½				
30min					Feed 10:00 Collect 10:30	Feed 10:00 Collect 10:30	Feed 10:00 Collect 10:30	Feed 10:00 Collect 10:30
45 min	Feed 14:00 Collect 14:45	Feed 14:00 Collect 14:45	Feed 14:00 Collect 14:45	Feed 14:00 Collect 14:45				
60 min					Feed 09:45 Collect 10:45	Feed 09:45 Collect 10:45	Feed 09:45 Collect 10:45	Feed 09:45 Collect 10:45
90 min					Feed 09:30 Collect 11:00	Feed 09:30 Collect 11:00	Feed 09:30 Collect 11:00	Feed 09:30 Collect 11:00
120 min					Feed 09:15 Collect 11:15	Feed 09:15 Collect 11:15	Feed 09:15 Collect 11:15	Feed 09:15 Collect 11:15
240 min					Feed 09:00 Collect 13:00	Feed 09:00 Collect 13:00	Feed 09:00 Collect 13:00	Feed 09:00 Collect 13:00

4.3.8 Pheroid™ vesicle formation and characterisation.

Pheroid™ vesicle formation and characterisation was determined for the test formulations administered. Pheroid™ vesicles (produced by adding $N_2O{\cdot}H_2O$ to Pro-Pheroid™) and pro-Pheroid™ (with added fatty acid fraction) were investigated using the Confocal Laser Scanning Microscope (CLSM) (Nikon D- eclipse C1, The Netherlands). The CLSM is equipped with a He/Ne laser with an excitation of 543 nm, an Argon ion laser with an excitation of 488 nm and a UV laser with and excitation of 405 nm. Detection with the Nikon D- eclipse C1 is possible with emission of 605±75nm, 450±35nm and 515±30nm respectively. A 60 X Apoplanar objective (1.4) and a small pinhole was used.

Vesicle formation was analysed on different levels:

- Pheroid™ formulation as administered. See Figure 4.1a.
- A fluid component was added to Pro-Pheroid™ formulation in order to simulate Pheroid™ formation in the stomach. 9.90 g 0.1 N HCl (Saarchem batch 1032286) was added to 0.1 g pro-Pheroid™ formulation. The solution was vortexed for 1 min. See Figure 4.1 b.
- Stomach content after 90 min and 120 min from subjects that received pro-Pheroid™ formulation. See Figure 4.2.
- Plasma collected after 90 min from subjects that received the Pro-Pheroid™ formulation. See Figure 4.3.

All samples were vortexed to ensure a homogenous preparation and 50 µl was transferred using a calibrated pipette to a microfuge tube. Nile Red (Molecular Probes, Inc. USA) was previously diluted in DMSO to 1 mg/ml. 1 µl of the diluted Nile Red was added to the sample in the microfuge tube and incubated in the dark for 15 min. After incubation a 20 µl sample was transferred with a micro pipette to a microscope slide and covered with a cover slip. An added 1 µg Acridine Orange (Molecular Probes, Inc. USA) was added to the plasma sample in order to identify DNA containing white blood cells.

Nile red is a lipid specific fluorescent probe which will after excitation at 543 nm emit light in the red spectrum which the Nikon D-eclipse C1 will detect at 605-675nm . Nile Red is almost non-fluorescent in water and other polar solvents (Hauhland, 2005:630) and will, therefore, only emit light once associated with a fatty acid. Acridine Orange is a cationic binding fluorescent probe that interacts with DNA and RNA by intercalation (Hauhland, 2005:279). Acridine Orange has green fluorescence with an emission maximum at 525 nm when bound to DNA and red fluorescence with an emission of 650 nm when bound to RNA (Hauhland, 2005:279).

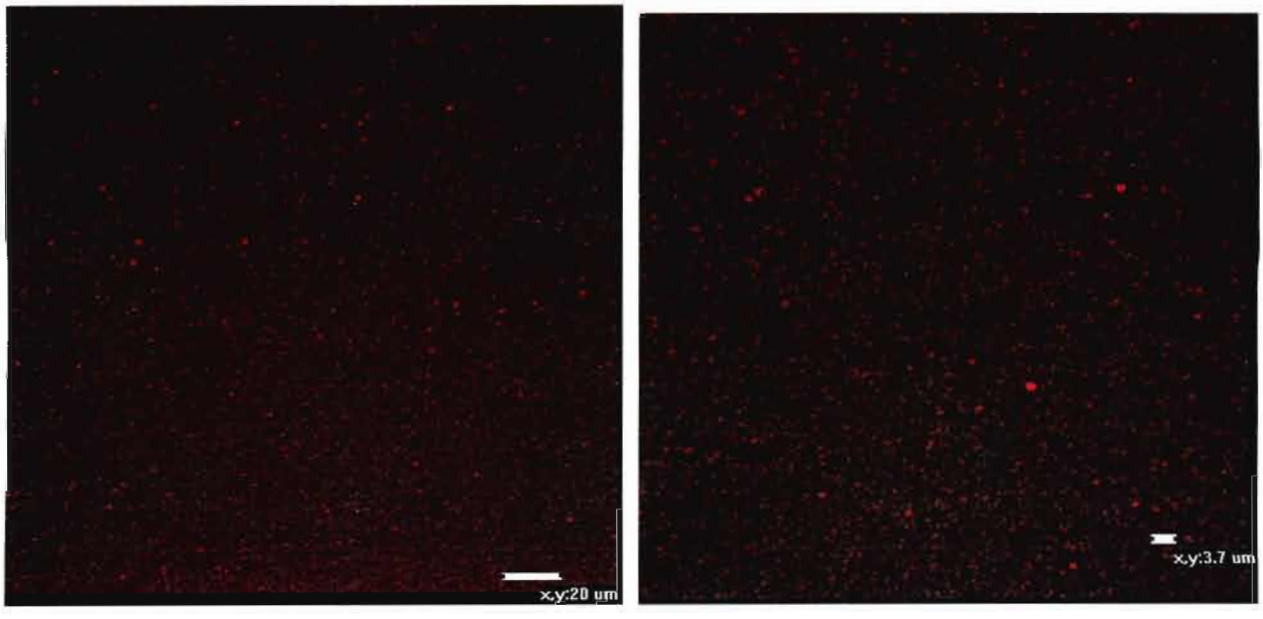
4.3.9 Plasma analysis

The plasma samples were analysed by an independent laboratory at UCT under guidance of Prof. Pete J. Smith. His laboratory developed validated analysis methods to determine quantities of the tested drugs from plasma samples. This SANAS accredited laboratory is also an accredited WHO Reference Centre for Analysis specifically for the plasma analysis of anti-TB agents.

4.4 RESULTS

4.4.1 Pheroid™ vesicle formation and characterisation

The following figures confirm Pheroid™ formation on different levels *in vitro* and *in vivo*. The fluorescent probe, Nile Red, associated with the fatty acids in all the samples and formed Pheroid™ vesicles which can, therefore, be observed in CLSM images.

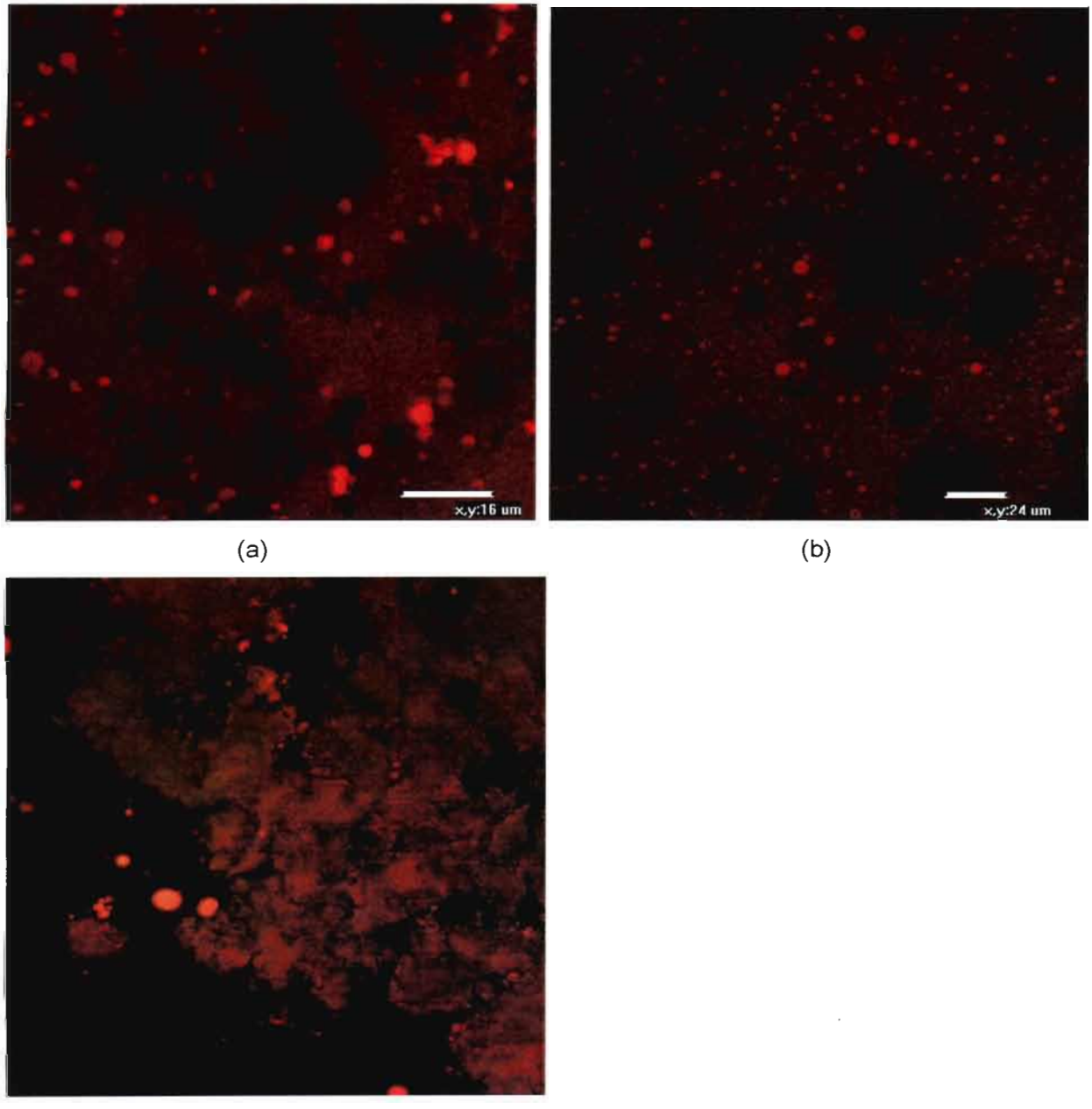


(a) (b)

Figure 4.1: CLSM micrographs of Pheroid™ vesicle formation of a) the Pheroid™ formulation as administered and b) pro-Pheroid™ formulation + 0.1 N HCl.

The Pheroid™ formulation as well as the pro-Pheroid™ formulation + 0.1 N HCl samples exhibited Pheroid™ vesicles with a diameter of ≤ 2 µm as seen in Figure 4.1. No visible

crystals were observed in either of the formulations which led to the assumption that all the API's have been dissolved.



(a) (b)

(c)

Figure 4.2: CLSM micrographs of stomach content of a mouse a) 90 min and b) 120 min after pro-Pheroid™ formulation administration and c) no Pheroid™ formulation: negative control.

The stomach content of mice 90 and 120 min after pro-Pheroid™ formulation administration shows Pheroid™s in various stages of development. Vesicles, sponges and depots from

submicron diameter size to 10 µm are present. Non lipid based content can also be observed as dark areas in the micrographs. Again no visible crystals were observed at 90 and 120 min, which leads to the assumption that all the API's have been dissolved during forming of the Pheriod™s in the stomach. Although the negative control (Figure 4.2 c) contains a few lipid globules they are far fewer that those observed in the stomachs of mice after pro-Pheroid™ indigestion. The pellet diet fed to the mice does contain lipids and will, therefore, form observable globules. It can be concluded that they are not Pheroid™s because the lipid globules do not exhibit typical spherical Pheroid™s.

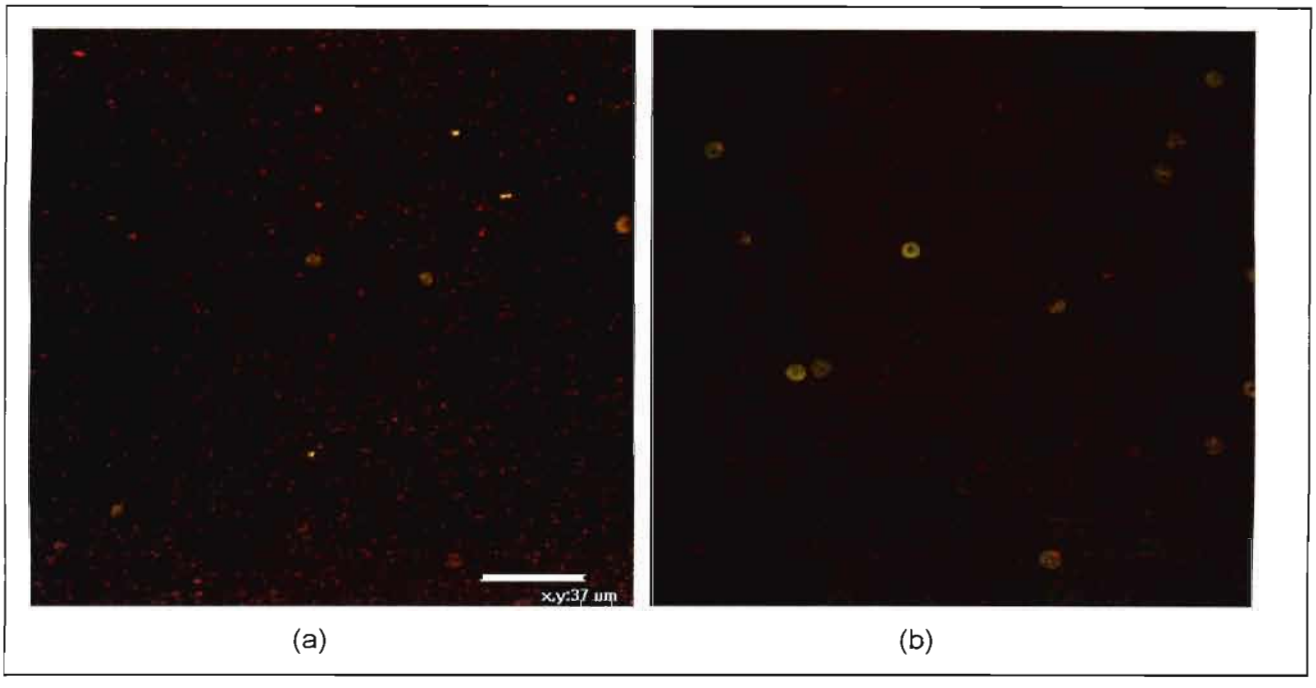


Figure 4.3: CLSM micrographs of plasma (a) 90 min after administration of pro-Pheroid™ formulation and (b) with-out administration of the pro-Pheroid™ formulation (negative control).

Pheroid™ vesicles (≤ 2 µm) can be observed in plasma of mice 90 min after administration with the pro-Pheroid™ formulation. The control showed no Pheroid™ vesicles present in the plasma. Although care was taken when separating the plasma from the hematocrit and buffy layer, the plasma did contain a few blood cells. The fluorescent marker, Acridine Orange, makes it possible to distinguish between DNA containing white blood cells and non-DNA containing red blood cells in Figure 4.3. The DNA in the white blood cells fluoresces in the green spectrum while red blood cells can be observed as black objects surrounded by a red membrane. This result indicates that Pheroid™ vesicles are not only successfully formed in the

stomach, but are transferred over the intestines and into the blood stream. Although it does not show that the API's were packaged into the vesicles before they were transported to the blood, it was assumed to be the case.

4.4.2 Plasma results

Statistical analysis was performed by Prof. F. Steyn from the Statistical Services at the North-West University. C_{max}, T_{max} and AUC calculations are based on a method for statistical comparison of bioavailability when only one concentration-time point per individual is available as described by Jawien (1992). If the area under the plasma concentration curve (AUC) and the therapeutic plasma level of an API is known, efficacy can be estimated. Since this study was restricted to a time frame of 0 to 240 min, complete curves are not available from which a simple AUC can be calculated. A second complication posed to the statistic scrutiny is that each value at each time point was derived from different individual animals. Jawien (1992) presents the following formula according to which an estimated AUC can be calculated in such a case:

$$\overline{AUC} = \overline{C_0}\frac{t_1 - t_0}{2} + \overline{C_n}\frac{t_n - t_{n-1}}{2} + \sum_{i-1}^{n-1}\overline{C_i}\frac{t_{i+1} - t_{i-1}}{2}$$

Where C_i is the unknown concentration points estimated by arithmetical mean of observations:

$$\overline{C_i} = \frac{1}{n_i}\sum_{i=1}^{n_i} C_n$$

The differences between the average AUC's were determined using the following calculation and expressed as a percentage of the difference.

$$\%Difference = \frac{\overline{AUC}formulation1 - \overline{AUC}formulation2}{\overline{AUC}formulation2} \times 100$$

The pro-Pheroid™ and Pheroid™ formulations resulted in the administration of 5 mg/ml isoniazid and 18.2 mg/ml ethambutol as set out in 4.3.3.2, whereas the Rifafour® e-275 and Free drug formulations resulted in 0.5 mg/ml isoniazid and 1.83 mg/ml ethambutol to be administered. This difference in dosage between pro-Pheroid™ and Rifafour® e-275 formulations was unplanned, but the results were adapted to accommodate this difference in initial administration quantities and enabled one to compare the results.

The terminal half life was determined by Dr. Helen McIlleron (Department of Clinical Pharmacology, University of Cape Town, South Africa) using the Stata™ 10.0 (StataCorp,

Texas USA) statistical/ data analysis program. Terminal plasma half-life is the time required to reduce the plasma concentration by 50% after reaching pseudo-equilibrium, and not the time required to eliminate half the administered dose. When the process of absorption is not a limiting factor (as the case with intra venous administration), half-life is a combined parameter controlled by plasma clearance and extent of distribution. In contrast, when the process of absorption is a factor, the terminal half-life reflects rate and extent of absorption and not the elimination process (flip-flop pharmacokinetics) (Toutain & Bousquet-Mélou, 2004).

The Stata™ 10.0 (StataCorp, Texas USA) statistical/ data analysis program calculates terminal half life by determining a least squares regression line using the points after T_{max}. The slope of the regression line represents the elimination rate constant to which the terminal half life is calculated.

The terminal half-life is especially relevant to multiple dosing regimens, because it controls the degree of drug accumulation, concentration fluctuations and the time taken to reach equilibrium (Toutain & Bousquet-Mélou, 2004). A long terminal half-life can be associated with a large volume of distribution (the amount of drug in the body) and/or indicates a small clearance of the drug from blood plasma. These parameters are very important when dose and dosage interval are determined in order to optimize efficacy and/or to minimize side-effects. The relationship between terminal half-life and dosing interval determines the amplitude of fluctuations in drug plasma concentrations during the dosing intervals and if the dosing interval is large relative to terminal half-life, there will be wide fluctuations in concentrations with possible side-effects (C_{max} too high), or lack of efficacy (C_{min} too low) (Toutain & Bousquet-Mélou, 2004).

4.4.2.1 Bioavailability

4.4.2.1.1 Ethambutol (EMB)

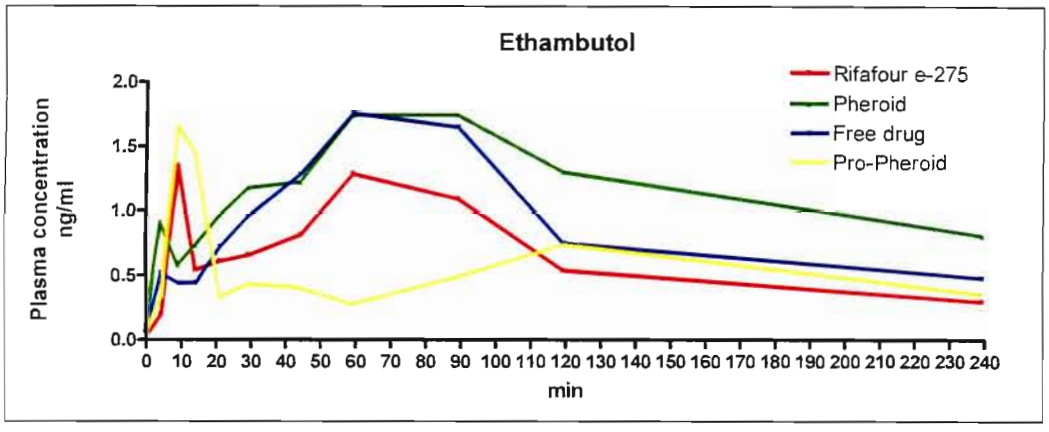
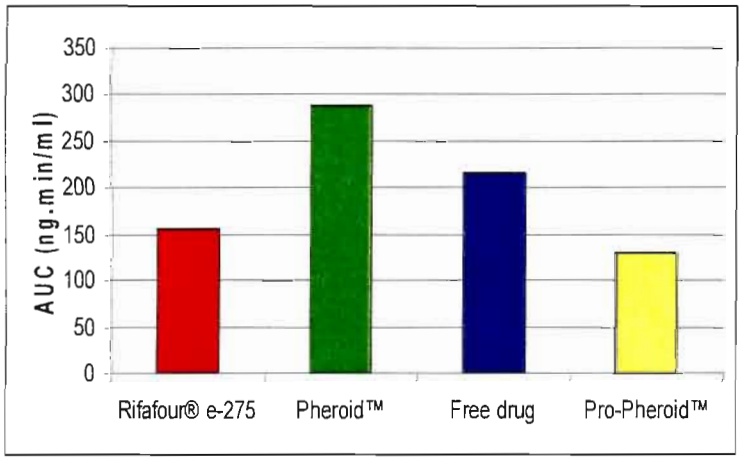


Figure 4.4: Bioavailability graph comparing plasma concentrations of EMB between Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ formulations.

The pharmacokinetic parameters of T_{max}, C_{max}, AUC and half-life for EMB were derived from Figure 4.4 and are summarised inTable 4.6 and Figure 4.5.

Table: 4.6: Summary of EMB T_{max} C_{max} and half-life in the Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ formulations.

EMB	Rifafour® e-275	Pheroid™	Free drug	Pro-Pheroid™
T_{max} (min)	10	60	60	10
C_{max} (ng/ml)	1.345125	1.73775	1.75375	1.6545
Half-life (hours)	1.55	1.08	1.71	3.99



Free drugs - Rifafour® e-275	61.7
% difference	39.8
Pheroid™ - Free drugs	71.2
% difference	32.8
Pheroid™ - Rifafour® e-275	132.8
% difference	85.7
Pro-Pheroid™ - Free drugs	-87.1
% difference	-40.2
Pro-Pheroid™ - Rifafour® e-275	-25.4
% difference	-16.4

Figure 4.5: Comparative estimated area under the curve (AUC) bar graph for EMB between Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ formulations.

The highest plasma (C_{max}) of around 1.74 ng/ml was reached for EMB in the Pheroid™ and Free drug formulations. This indicates an increase of peak plasma concentrations of 30% when compared to Rifafour® e-275. The pro-Pheroid™ formulation also showed a 22% increase when compared to Rifafour® e-275. The T_{max} was obtained 50 minutes earlier for the Rifafour® e-275 and pro-Pheroid™ formulation when compared with the Pheroid™ and Free drug formulations. The Pheroid™ and Free drug formulations had the highest C_{max} and also the longest T_{max}. Ingestion of 25mg/kg EMB leads to a blood peak level of 2-5 µg/ml with in 2 to 4 hours in humans (Chambers 2004:785; Mandell & Petri, 1996:1161). When blood peak levels are converted to match the 18.5 mg/kg used in this study, plasma levels of 1.48-3.7 µg/ml are expected. Blood plasma in the murine model did not reach the expected levels for any of the formulations tested. The therapeutic concentration of between 2.5 and 6.5 µg/ml in human blood (Moffat *et al.,* 2004: 990) is also not reached in the murine model.

In conjunction with the increased C_{max} for the Pheroid™ and Free drug formulations, the area under the curve increased by 85.7% and 39.8% respectively when compared to Rifafour® e-275. The pro-Pheroid™ formulation, however, showed a 16.4% decrease when compared to Rifafour® e-275. The Pheroid™ formulation outperformed the Free drug by 32.8%.

The half-life of EMB in the pro-Pheroid™ is more than twice that of any of the other formulations, which possibly indicates a much better volume of drug distribution and a slower clearance from plasma. Although the pro-Pheroid™ formulations seem to be less sufficient when the T_{max}, C_{max} and AUCs are compared, the elongated presence in the plasma presents the possibility to increase dosage intervals by at least double the time. The elevated terminal half life of EMB in the pro-Pheroid™ must, however, be evaluated carefully and might not reflect the true half life. The plasma levels showed a very sharp decrease after T_{max} at 10 min and increased steadily to 120 min whereafter another gradual decrease occurred. This atypical plasma concentration curve might result in an inaccurate regression line that lengthens the half life considerately. In addition, the scenario where the last points used for the extrapolation of the regression line was of such orientation that the slope of the corresponding line ran virtually parallel to the x-axis and thereby never intersected it, may have resulted in a calculation of an inflated half life. The only formulation that has the typical half life of 3-4 hours for humans as stipulated by Mandell and Petri (1996:1161) is the pro-Pheroid™ formulation at a half life of 3.99 hours.

4.4.2.1.2 Isoniazid (INH)

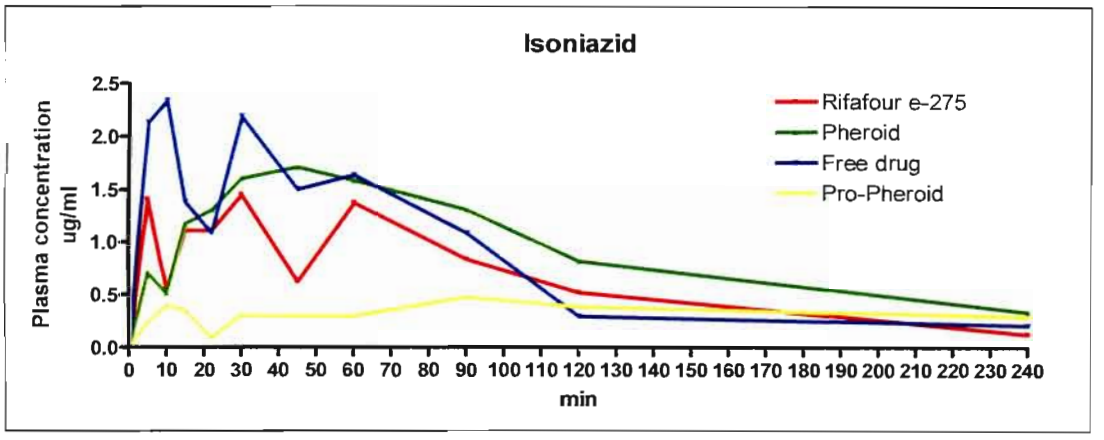
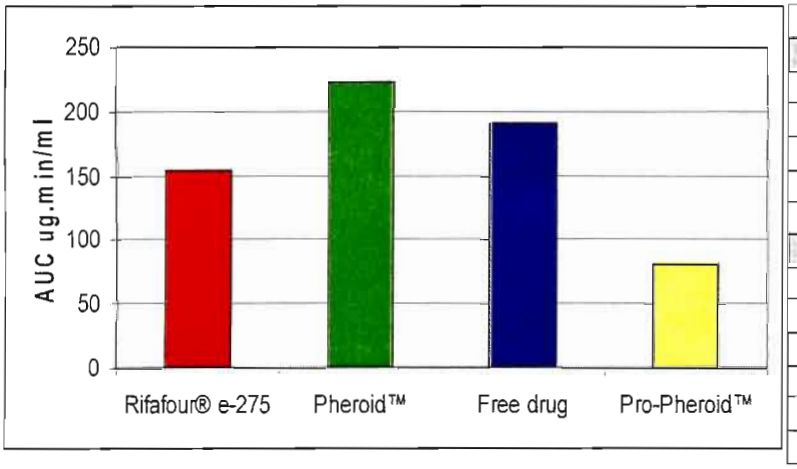


Figure 4.6: Bioavailability graph comparing plasma concentrations of INH between Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ formulations.

The pharmacokinetic parameters of T_{max}, C_{max}, AUC and half-life for INH were derived from Figure 4.6 and are summarised in Table 4.7 and Figure 4.7.

Table: 4.7: Summary of T_{max} and C_{max} of INH in Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ formulations.

INH	Rifafour® e-275	Pheroid™	Free drug	Pro-Pheroid™
T_{max} (min)	30	45	10	90
C_{max} (µg/ml)	1.4525	1.70975	2.33875	0.476875
Half-life (hours)	1.02	1.13	0.99	2.51



Free drugs - Rifafour® e-275	37.2
% difference	24.3
Pheroid™ - Free drugs	32.6
% difference	17.1
Pheroid™ - Rifafour® e-275	69.7
% difference	45.5
Pro-Pheroid™ - Free drugs	-109.8
% difference	-57.7
Pro-Pheroid™ - Rifafour® e-275	-72.6
% difference	-47.4

Figure 4.7: Comparative estimated area under the curve (AUC) bar graph for INH for Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ test formulations.

A very high plasma (C_{max}) of 2.34 µg/ml was reached for INH by the Free drug formulation, followed by Pheroid™ at 1.7 µg/ml and Rifafour® e-275 at 1.4 µg/ml. This indicates an increase of peak plasma concentrations of 66% by the Free drug formulation when compared to Rifafour® e-275. The Pheroid™ formulation also showed a 21% increase when compared to Rifafour® e-275. The Pheroid™ based formulation had a T_{max} very close to that of Rifafour® e-275, but the Pro-Pheroid™ formulation had considerable delayed T_{max} when compared to the other formulations.

Although the Free drug formulation had a much higher C_{max} for INH than any of the other formulations, the Pheroid™ formulation showed the highest AUC. The area under the curve increased by 45.5% for the Pheroid™ formulation and 24.3% for the Free drug formulation when compared to Rifafour® e-275. The pro-Pheroid™ formulation however showed a 47.4% decrease when compared to Rifafour® e-275. The Pheroid™ formulation outperformed the Free drug by 17.1%. Ingestion of 5mg/kg INH by humans lead to a blood peak level of 3-5 µg/ml with-in 1 to 2 hours (Chambers 2004:783; Mandell & Petri, 1996:1157). Murine blood plasma did not reach the expected levels by any of the formulations tested. The therapeutic concentration of between 3 and 10 µg/ml in human blood (Moffat *et al.*, 2004: 1143) is also not reached in the murine model.

Isoniazid's half-life in the pro-Pheroid™ formulation is more than 2.5 times higher than that of any of the other formulations, which indicates a much better volume of drug distribution and a slower clearance from plasma. Although the pro-Pheroid™ formulations seem to be less suitable when the T_{max}, C_{max} and AUCs are compared, it has a lengthened presence in the plasma which opens the possibility to increase dosage intervals by at least double the time. The elevated terminal half life of INH in the pro-Pheroid™ must also be carefully evaluated and might not reflect the true half life for the same reasons discussed for EMB. Expected half lives are at an average of 1 hour and 3 hours for fast and slow human acetylators respectively. The half life of all the formulations in the murine model corresponds with what is expected in humans (Katsung 2004:783).

4.4.2.1.3 Pyrazinamide (PZA)

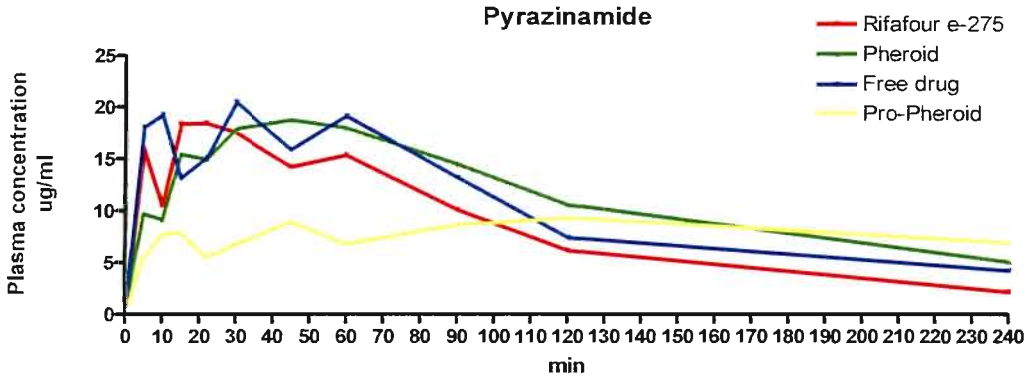
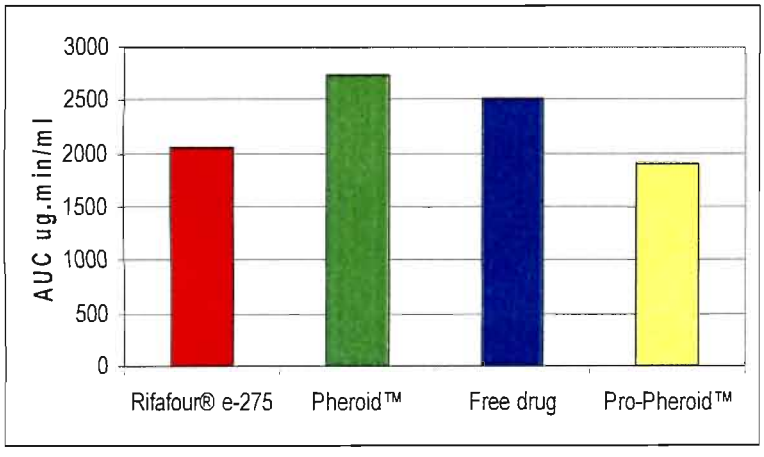


Figure 4.8: Bioavailability graph comparing plasma concentrations of PZA between Rifafour® e-275, Pheroid™, Free drugs and pro-Pheroid™ formulations.

The pharmacokinetic parameters of T_{max}, C_{max}, AUC and half-life for PZA were derived from figure 4.8 and are summarised in table 4.8 and figure 4.9.

Table: 4.8: Summary of T_{max} and C_{max} of PZA in Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ formulations.

PZA	Rifafour® e-275	Pheroid™	Free drug	Pro-Pheroid™
T_{max} (min)	22	45	10	120
C_{max} (µg/ml)	18.4125	18.76875	19.22625	9.37375
Half-life (hours)	1.07	1.31	1.51	4.13



Free drugs - Rifafour® e-275	448.7
% difference	21.9
Pheroid™ - Free drugs	231.3
% difference	9.3
Pheroid™ - Rifafour® e-275	680.0
% difference	33.2
Pro-Pheroid™ - Free drugs	-597.3
% difference	-23.9
Pro-Pheroid™ - Rifafour® e-275	-148.6
% difference	-7.2

Figure 4.9: Comparative estimated area under the curve (AUC) bar graph for PZA between Rifafour® e-275, Pheroid™, Free drugs and Pro-Pheroid™ test formulations.

C_{max} for all the formulations excluding the pro-Pheroid™ formulation were more or less equal and in the vicinity of 19 µg/ml. This indicated an increase of peak plasma concentrations of 51% when compared to the Pro-Pheroid™ formulation. Both the Pheroid™ based formulations had a delayed T_{max} of between 23 and 100 minutes when compared to both the Free drug and Rifafour® e-275 formulations. Ingestion of 25mg/kg PZA leads to a blood serum level of 30-50 µg/ml with in 1 to 2 hours in the human system (Chambers, 2004:786). Murine blood plasma did not reach the expected levels by any of the formulations tested.

The area under the curve increased 33.2% for the Pheroid™ formulation and 21.9% for the Free drug formulation when compared to Rifafour® e-275. The pro-Pheroid™ formulation only showed a 7.2% decrease when compared to Rifafour® e-275. The Pheroid™ outperformed the Free drug by 9.3%.

The pro-Pheroid™ formulation increased PZA's half-life between 2.5 and 4 times when compared to the other formulations, which may indicate much better volume of drug distribution and a slower clearance from plasma. Although the pro-Pheroid™ formulations seem to be less satisfactory when the T_{max}, C_{max} and AUC's are compared, it has an elongated presence in the plasma which opens the possibility to increase dosage intervals by at least double the time. Again, the elevated terminal half life must be carefully evaluated since it might not reflect the true half life for the same reasons discussed for EMB. Although a half life of between 8 and 11 hours is expected in humans (Chambers, 2004:786), none of the formulations reached the expected half life in the murine model.

4.4.2.1.4 Rifampicin (RMP)

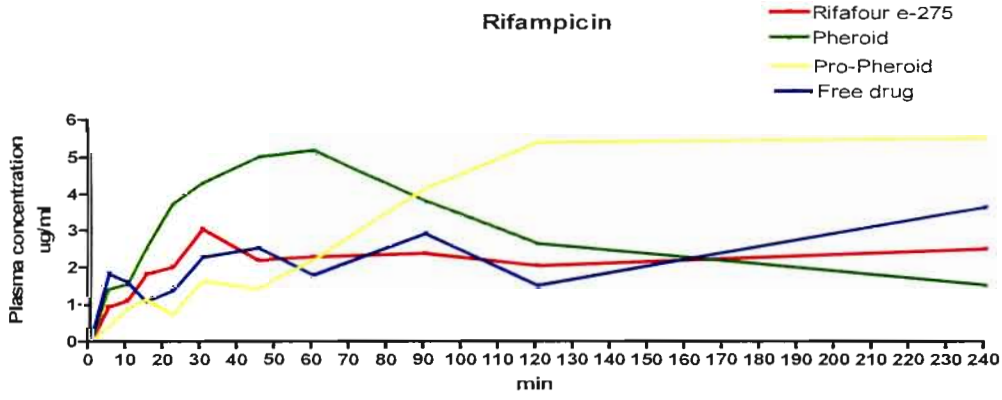
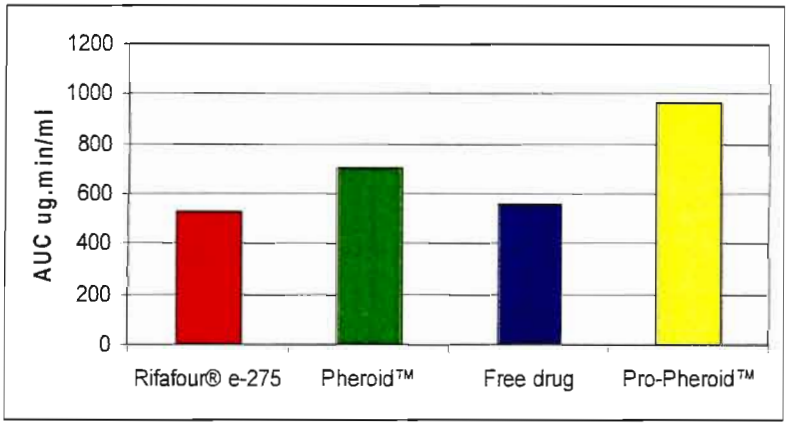


Figure 4.10: Bioavailability graph comparing plasma concentrations of RMP for Rifafour® e-275, Pheroid™, Free drugs and pro-Pheroid™ formulations.

The pharmacokinetic parameters of T_{max}, C_{max}, AUC and half-life for RMP were derived from figure 4.10 and are summarised in table 4.9 and figure 4.11.

Table: 4.9: Summary of T_{max} and C_{max} of RMP in Rifafour® e-275, Pheroid™, Free drugs and pro-Pheroid™ formulations.

RMP	Rifafour® e-275	Pheroid™	Free drug	pro-Pheroid™
T_{max} (min)	30	60	240	240
C_{max} (µg/ml)	3.0325	5.1675	3.62875	5.50125
Half-life (hours)	-	1.56	-	-



Free drugs - Rifafour® e-275	28.9
% difference	5.5
Pheroid™ - Free drugs	145.9
% difference	26.4
Pheroid™ - Rifafour® e-275	174.8
% difference	33.3
Pro-Pheroid™ - Free drugs	412.4
% difference	74.5
Pro-Pheroid™ - Rifafour® e-275	441. 3
% difference	84.1

Figure 4.11: Comparative Area Under the Curve (AUC) bar graph for RMP between Rifafour® e-275, Pheroid™, Free drugs and pro-Pheroid™ test formulations.

Higher plasma concentrations (C_{max}) of 5.12 µg/ml and 5.5 µg/ml were reached for RMP by the Pheroid™ and pro-Pheroid™ formulations respectively. This indicates an increase of peak plasma concentrations of between 30% and 45% when compared to both the Free drug and Rifafour® e-275. Both the Pheroid™ and Rifafour® e-275 test formulations had a T_{max} of between 180 and 210 minutes faster when compared to both the Free drug and pro-Pheroid™ formulations. Ingestion of 10mg/kg RMP leads to a average blood plasma level of 7 µg/ml with in 2-4 hours in the human system (Mandell & Petri, 1996:1160). Murine blood plasma did not reach the expected levels by any of the formulations tested.

Although Rifafour® e-275 had a T_{max} between 180 and 210 minutes faster than the Free drug and Pheroid™ formulations, the area under the curve of the last two increased by 5.5% and 33.3% respectively. The pro-Pheroid™ formulation also showed a significant increase of 84.1%

when compared to Rifafour® e-275. Both the Pheroid™ and the pro-Pheroid™ formulations outperformed the Free drug by 26.4% and 74.5% respectively.

The half-life for the Rifafour® e-275, pro-Pheroid™ and Free drug formulations could not be calculated because no decrease in blood plasma levels was observed by the time that the study was terminated at four hours. This resulted in a progression line rather than a regression line needed to calculate terminal half life. Although a half life of between 1.5 and 5 hours is expected in humans (Mandell & Petri, 1996:1160), none of the formulations reached the expected half life in the murine model.

4.4.2.2 Significance (t) tests and probability (p)

The null hypothesis (H_0) tested states that no difference in mean plasma levels for the four formulas administered results in equal bioavailability (Rifafour® e-275 = Pheroid™ = Free drugs = pro-Pheroid™). To test if the hypothesis is true in the light of this set of bioavailability data, the *significance* of differences is determined by the probability (p) that the average values obtained are a true reflection of the differences within a population. The t-test is used to determine the difference between test formulations and takes into account the mean, standard error from the mean and estimated population mean for each test formulation.

A significance test (t-test) as described by Jawien (1992) is performed to determine an effective difference between the test formulations statistically.

Table 4.10: T-test values from challenging Rifafour® e-275 vs. Pheroid™ vs. Free drugs vs. pro-Pheroid™ for the four APIs.

	ETH	INH	PZA	RMP
Rifafour® e-275 vs. Pheroid™	4.22	4.24	3.52	1.94
Rifafour® e-275 vs. Free drugs	3.00	2.53	2.85	0.29
Rifafour® e-275 vs. Pro-Pheroid™	1.67	7.10	1.01	4.74
Pheroid™ vs. Free drugs	2.11	1.80	1.17	1.35
Pheroid™ vs. Pro-Pheroid™	5.17	9.65	4.37	2.64
Free drugs vs. Pro-Pheroid™	4.47	8.60	3.90	3.73

Probability values for a two tailed t-test with in a normally distributed population with degrees of freedom (number of subjects/mice) at 70 were determined using a statistics calculator online at the tutor and homework help website (Roth, 2009).

Table 4.11: Probability when challenging Rifafour® e-275 vs. Pheroid™ vs. Free drugs vs. Pro-Pheroid™ for the four APIs.				
	EMB	INH	PZA	RMP
Rifafour® e-275 vs. Pheroid™	0.0001	0.0001	0.0008	0.0564
Rifafour® e-275 vs. Free drugs	0.0037	0.0137	0.0057	0.7727
Rifafour® e-275 vs. Pro-Pheroid™	0.0994	0	0.316	0
Pheroid™ vs. Free drugs	0.0384	0.0762	0.246	0.1814
Pheroid™ vs. Pro-Pheroid™	0	0	0	0.0102
Free drugs vs. Pro-Pheroid™	0	0	0.0002	0.0004

The hypothesis will be tested against a confidence level of 99% ($p \leq 0.01$) that the average bioavailability values obtained are a true reflection of the differences within a population when challenging Rifafour® e-275 vs. Pheroid™ vs. Free drugs vs. pro-Pheroid™ for the APIs, ethambutol, isoniazid, pyrazinamide and rifampicin.

Table 4.12: Statistically significant difference at a probability of $p \leq 0.01$.				
	ETH	INH	PZA	RMP
Rifafour® e-275 vs. Pheroid™	y	y	y	n
Rifafour® e-275 vs. Free drugs	y	n	y	n
Rifafour® e-275 vs. Pro-Pheroid™	n	y	n	y
Pheroid™ vs. Free drugs	n	n	n	n (y)
Pheroid™ vs. Pro-Pheroid™	y	y	y	n
Free drugs vs. Pro-Pheroid™	y	y	y	y

y= yes, significantly different

n= no, not significantly different

Although a probability of $p \leq 0.01$ is preferred a $p \leq 0.05$ is also statistically acceptable since $p \leq 0.05$ have 95% confidence that the data are still a reflection of the population. For RMP, the probability that the difference between the response to Pheroid™-entrapped and Free drug is very close to $p \leq 0.01$ and can, therefore, still be considered significant for $p \leq 0.01$. Although the probability is statistically not significant for INH with the probability at $p \leq 0.01$ when challenged in Rifafour® e-275 vs. Free drugs, it is statistically significant at $p \leq 0.05$. The same scenario applies when looking at the probability of EMB when challenged in Pheroid™ vs. Free drugs.

4.5 CONCLUSION

4.5.1 Pheroid™ vesicle formation and characterisation

Pheroid™ vesicles of a diameter ≤ 2 µm successfully formed when either $N_2O{\cdot}H_2O$ or 0.1 N HCl were added as liquid phases. Pheroid™ vesicles (≤ 2 µm) are observable in plasma of mice 90 min after administration with the pro-Pheroid™ formulation. This indicates that Pheroid™ vesicles have formed when the Pro-Pheroid came into contact with the stomach fluids. The vesicles were then successfully absorbed/moved across the stomach and intestine lining and into the blood steam. Any significantly elevated plasma levels above the Free drug as positive control has to the result of the active transport of Pheroid™ vesicles accross the stomach and intestine linings as well as capillary walls. Due to the absence of crystals in all the images the assumption can be made that all crystals were dissolved completely or were unobservably small.

The degradation of RMP in the presence of INH (Singh *et al.,* 2001) *in situ* is accelerated by EMB (Singh *et al.* 2006). This poses fix dose dosage form development challenges. The ideal future formulation for human trials will be the separation of RMP from INH and EMB. The formulations developed in this study were thus two separate formulas, one containing RMP and PZA while the other contains INH and EMB.

4.5.2 Plasma results

The null hypothesis - H_0 that plasma levels for the four formulas administered results in equal bioavailability (Rifafour® e-275 = Pheroid™ = Free drugs = pro-Pheroid™) is rejected.
Bioavailability was enhanced by ≈40% for EMB, ≈24% for INH, ≈22% for PZA and ≈5.5% for RMP in the Free drug formulation when compared with Rifafour® e-275. Bioavailability was enhanced at ≈ 32.8% for EMB, ≈ 17.1% for INH, ≈ 9.3% for PZA and ≈ 26.4% for RMP in the Pheroid™ formulation when compared with the Free drugs. Bioavailability was enhanced at ≈ 85.7% for EMB, ≈ 45.5% for INH, ≈ 33.2% for PZA and ≈ 33.3% for RMP in the Pheroid™ formulation when compared with Rifafour® e-275, resulting in the highest difference when all the formulations are compared.

Bioavailability decreased between ≈ 7% and ≈ 58% for EMB, INH, and PZA when Free drugs and Rifafour® e-275 are compared with the pro-Pheroid™ formulation. Rifampicin on the other hand showed its highest increase of ≈75% to ≈ 84% in the pro-Pheroid™ formulation. If the assumption is made that RMP half-life would increase as was the case for the other drugs, it

could mean that a much lower dose of all four drugs be administered at longer intervals. This could contribute significantly to the attempt to lower side effects associated with RMP.

Recommendations for future formulations will be to lower the RMP concentration by about 60% when a pro-Pheroid™ dosage form is developed. The observed half-life of INH, EMB and PZA indicated that there is a strong possibility for longer dosage intervals when the pro-Pheroid™ formulation is re-investigated. The API concentration for EMB can be lowered by ± 30%, that of INH ± 17% and that of PZA ± 9% when a Pheroid™ dosage form is developed.

Although the mice were administered with the same dosage per body weight as with what would have been the case in humans, the blood plasma levels in the murine model did not reach the expected human plasma levels for all four APIs. With the exception of INH and the EMB in the pro-Pheroid™ formulation, the plasma half lives also do not correspond with what is expected in humans.

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CHAPTER 5

EFFICACY OF THE PHEROID™ ENTRAPPED ANTI-TUBERCULOSIS DRUGS, RIFAMPICIN, ISONIAZID, ETHAMBUTOL AND PYRAZINAMIDE IN THE MURINE MODEL

5.1 INTRODUCTION

Proving *in vivo* efficacy of pharmaceutical active ingredients (APIs) is an imperative step in drug development. Efficacy of any API or combination of APIs can be greatly influenced by the dosage form since formulation plays a role in stability and absorbtion rate of the API. Preceding *in vivo* mouse and human studies on anti-TB APIs in a Pheroid™ based dosage form (as described in Chapter 3) indicate elevated bioavailability, but a need exists for efficacy data in a mammal model. The mouse model is prefered over other mammalian models due to practical reasons discussed in Chapter 2. A study with the title: "Evaluation of Pheroid™ as delivery system for first-line TB drugs in a murine model of Tuberculosis" has, therefore, been contracted to the South African Medicine Research Council (SAMRC). This study has been approved and monitored by the MRC ethics committee (ECRA approval number: MRC ECRA 13/07).

5.2 AIM

The main aim of this study was to evaluate the efficacies of combination therapy with Pheroid™ entrapped essential TB drugs rifampicin, pyrazinamide, ethambutol and isoniazid in a murine model of tuberculosis. Results will give an indication about the level of efficacy of anti-tuberculosis drugs entrapped in the Pheroid™ delivery system over a 12 week treatment period and a 3 month post treatment period. The results will test a null hypothesis stating that a Free drug formulation is equally effective as a Pheroid™ formulation (see 5.3.3.3 below for definitions of each formulation).

5.3 METHODS

5.3.1 Subjects

Inbred, specified pathogen free female BALB/C mice, 4 – 5 weeks of age with an average mass of 16-18 g were purchased from South African Vaccine Producers (Sandringham, South Africa). Females are preferred over males due to their more placid nature and less fighting is experienced than with males. Twenty mice were housed per cage at the Laboratory Animal

Unit of the TB epidemiology and intervention research unit of the SAMRC in a Techniplast Sealsave Individually Ventilator Cage Rack Isolator System equipped with input and exhaust fan HEPA filters under the following environmental conditions: temperature of 21 ± 2°C, relative humidity of 52% with 12 hours light and 12 hours dark, and a ventilation rate of 23 supplied air changes and 47 extracted changes per hour. Subjects were allowed to acclimatise for 1 week prior to infection. The animals were fed a standard pellet diet (EPOL, South Africa) and deionised water was supplied *ad libitum*. All water, food and dust free wood shavings and bedding were sterilised. Mice were ear punched using a modified ear punch numbering system of Nagy *et al.* (2003). Figure 5.1. illustrates the modified ear punch numbering system that was used to randomly assign animals to three experimental groups.

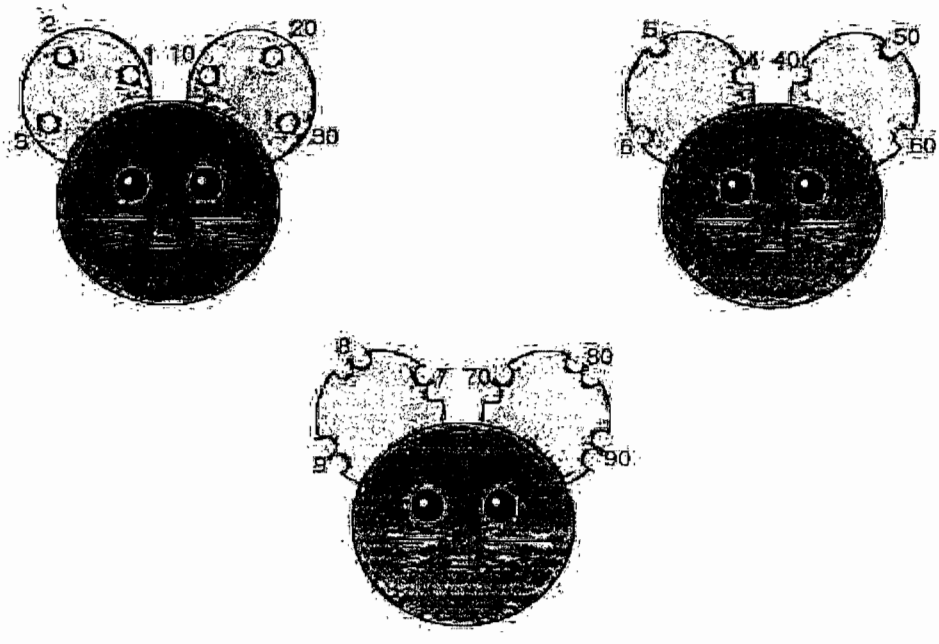


Figure 5.1: Diagram illustrating the ear punch numbering system (Nagy *et al.*, 2003:86)

5.3.2 Test formulations

5.3.2.1 Anti-tuberculosis drugs

Rifampicin (RMP/NC-020/06) and pyrazinamide (PYR/P-228/06) powders were obtained from Lanaria Chemicals Limited (Thailand). Isoniazid (IF070501) and ethambutol (IF070608) powders were obtained from Iffect Chemphar (HK) Company Limited (China) through BD fine Chemicals (Pty) Limited (South Africa).

All pharmaceutical active compounds were used as received. Particle sizes of powders were determined by the method of laser diffraction using a Malvern Mastersizer Micro 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The methods used are the same as described in section 4.3.2. Samples of each raw material were measured in quadruple and the average particle sizes set out in Table 4.1. The Mastersizer analysis result reports are shown in Annexure A.

5.3.3.2 Pheroid™ technology based formulation development

A Pheroid™ based formulation was developed and manufactured by the Unit for Drug Research and Development, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. The pro-Pheroid™ concept was used to develop this formulation. Pro-Pheroid™ entails the oil phase, containing the fatty acid fraction as well as the N_2O fraction of Pheroid™ with the exclusion of the fluid in which the vesicles are emulsified. Pheroid™ vesicles will form once pro-Pheroid™ comes into contact with fluids. The future dosage forms developed for human trials will probably be pro-Pheroid™ and, therefore, it is the logical choice for this study.

Formulations were based on previously developed formulations used in the preceding human phase 1 trial (see Chapter 3). The test formulations were produced under the same GLP/GMP conditions as discussed in 4.3.3.1. The fatty acid ratios of the test formulations were based on the Pyriftol C formulation used in the phase 1 human trial. Anti-oxidants butylated hydroxyanisole and butylated hydroxytoluene were added at concentrations 0.01% and 0.1% respectively. Ascorbic palmitate at a concentration of 6.82 mg/g rifampicin was added to formulas containing rifampicin in an attempt to decrease degradation (Le Guellec *et al.,* 1997). Although Le Guellec *et al.* (1997) prolonged rifampicin stability by adding ascorbic acid, the Pheroid™ formulation contained ascorbic palmitate due to better fat soluble characteristics than ascorbic acid. Poly-ethylene Glycol 400 was added to increase fluidity of the formulations. Due to the *in situ* decomposition of rifampicin in the presence of isoniazid (Singh *et al.,* 2001), which is accelerated by ethambutol (Singh *et al.,* 2006), rifampicin was separated from the formulation containing isoniazid, ethambutol and pyrazinamide. The formulations developed in this study were thus two separate formulations, one containing rifampicin and the other containing isoniazid, ethambutol and pyrazinamide. The percentage of actives was adjusted each fortnight per actual average weight of the subjects. After preparation the formulations administered resulted in dosages of 10 mg/kg rifampicin, 25 mg/kg isoniazid, 150 mg/kg pyrazinamide and 100 mg/kg ethambutol.

5.3.3.3 Formulation preparation for administration

All preparations were performed in the dark and in amber glassware to minimise degradation of APIs. Dosage treatments were prepared freshly every day.

A: Pheroid™ based treatments: Shake the bottles with the Pheroid™ formulations well. Weigh 25 g of both the Pheroid™ formulations into a 50 ml volumetric flasks; with the Pheroid™ + rifampicin in the AMBER flask. Add $N_20{\cdot}H_20$ to the volume lines of the flasks to obtain final volumes of 50 ml each. Close lid, shake well and sonicate for 15 min.

B: Free Drug treatments: Weigh the correct amount of INH, EMB and PZA together into a 100 ml volumetric flask and add ddH_20 to the volume line of the flask. Weigh off the correct amount rifampicin into a 100 ml amber volumetric flask and add 5% DMSO/H_20 to the volume line of the flask. Close lids, shake well and sonicate both flasks for 15 min.

The concentrations are well below the LD_{50} of the mouse for each active and ruled out the possibility of mortalities due to toxicity.

5.3.3 Bacterial strain and infection of mice

Mycobacterium tuberculosis H37Rv (ATCC 27294) were cultivated according to standard microbiological practices on 7H10 agar plates supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment for 3 weeks at 37°C, harvested, suspended in 15% glycerol/Dubos broth and kept frozen in 1 ml aliquots at -80°C. A random collection of vials were selected for preparations of serial dilutions, plated out on 7H11/10% OADC agar plates and incubated at 37°C for three weeks to determine theinitial infection dose.

A frozen random aliquot of *M.Tb* H37Rv (1 ml) was thawed at 37°C, declumped by passing successively through 25G, 26G, 29G, and 30G needles and suspended in 0.01% Tween 80 / saline to an estimated bacterial concentration of 5×10^7 CFU/ml.

Mice were carefully heated in a heating box for 5 minutes (not exceeding 40°C) to promote vasodilatation. Each mouse was placed in the crutch on top of a special lid, the tail tautened through the crutch and 0.1 ml of the *M.Tb* H37Rv suspension was slowly injected into one of the two lateral tail veins. The inoculum was determined to be 10^6 colony forming units (CFUs) per dose by serial dilution in triplicate on 7H11 agar plates supplemented with 10 % Middlebrook OADC enrichment, and selective anti-microbials; polymyxin B (200 units/ml), amphotericin B (10 µg/ml), carbenicillin disodium salt (100 µg/ml) and trimethoprim lactate (20 µg/ml)(Mitchison *et al.,* 1972). Plates were incubated at 37°C in the dark for 3 weeks prior to counting viable CFU by means of an automated colony counter (Q-Count, Spiral Biotech, Massachusetts,

USA). Mice were subjected to health surveillance weekly during which body mass was measured and each mouse inspected for signs of morbidity or injuries.

5.3.4 Implementation of investigation

Treatment started 2 weeks after infection. A volume of 200 µl of the prepared test treatments were administered once daily five times a week for twelve weeks via oral gavage with a 24G ball tipped feeding needle (Popper & Sons, New York, USA). Each mouse was firmly restrained using the double handed manual restraint method (Shimizu, 2004: 528) while the neck is extended to get the mouth, pharynx and stomach in a straight line. The needle was carefully inserted through the oesophageal orifice and 200 µl directly inject into the stomach. This method is described as the intragastric administration procedure (Shimizu, 2004:533).

The rifampicin containing treatments were administered at least one hour after the isoniazid, ethambutol and pyrazinamide treatments to minimise drug interactions (Dhillon *et al.*, 2004).

Four untreated TB infected mice were sacrificed on day 1 to determine baseline infection and six each at week 1, 2 & 4 after infection to monitor infection kinetics. Six more untreated TB infected mice were allocated for weeks 6 and 8, but showed a 20% loss in body mass and had to be terminated. Six mice per group for the Free drug and Pheroid™ treatment groups were sacrificed at weeks 2, 4, 6, 8, 10 and 12 to determine the effect of treatment. To determine relapse, five mice for each of the treatment groups were sacrificed at the end of *months 1, 2* and 3 of the *post* treatment observation period.

5.3.5 Assessment of treatment

5.3.5.1 Necropsy and organ harvesting

The mice were identified by the punched ear markings. Mice were then transferred from the cage to a chamber containing 100% CO_2 and euthanised by means of inhalation and death ensured by cervical dislocation. CO_2 inhalation is readily available, inexpensive and safe (Otto, 2004:564). Lungs and spleens were aseptically removed by an incision made through the thoracic cavity, and stored in a -80°C freezer if immediate cultivation was not possible. Based on the method described by Orme and Roberts (1999), the lungs were cut loose at the bronchial tree close to the heart and the spleen was carefully separated from the pancreas and removed.

5.3.5.2 Bacterial cultivation

Lungs were thawed (if frozen previously), weighed, placed in 2 ml of 0.05% Tween 80 / phosphate buffered saline solution (PBS, pH = 7.4) and homogenised by means of a motorised homogeniser with dispersing tool (Heidolph DIAX 900, Germany). Ten-fold serial dilutions of homogenates were plated in triplicate on 7H10 agar plates supplemented with 10% Middlebrook OADC enrichment, polymyxin B (200 units/ml), cyclohexamide (10 µg/ml), carbenicillin (50 µg/ml) and trimethoprim (20 µg/ml). Plates were then incubated at 37°C in the dark for 3 weeks prior to counting viable CFU by means of an automated colony counter (Q-Count). The plates of which the colonies were not distinctive due to over-growing were not included in the data, but those that resulted in singles colonies were counted and relayed back to the CFUs per ml of lung homogenate.

Dhillon *et al.* (2004) indicated that about only about 1.4% of the spleen and 3.7% of the lung *M. Tb* bacilli population in chronic infected mice will grow on agar plates. In addition to bacterial enumeration on agar plates, 0.1 ml of the ten fold dilutions of organ homogenates were inoculated in duplicate into *Mycobacteria* Growth Indicator Tubes (MGIT) supplemented with 10 % MGIT OADC and the same selective anti-microbials at identical concentrations as were used for agar plates. All inoculated MGIT tubes were scanned into and incubated in the BACTEC MGIT 960 instrument (Becton, Dickinson & Company, New Jersey, USA) at 37°C for 6 weeks (Shin *et al.,* 2007). Bacterial growth in MGIT tubes causes fluorescence which is automatically detected by the instrument and flagged as positive and these results are reported as time to detection (TTD). If no growth occurs after 42 days, a tube is flagged as negative. Presence of the *Mycobacterium tuberculosis complex* in a positive MGIT tube was confirmed by an immuno-chromatographic assay (SD Bioline TB Ag MPB64, Kat Medical Laboratory, Roodepoort, South Africa) (Abe *et al.,* 1999).

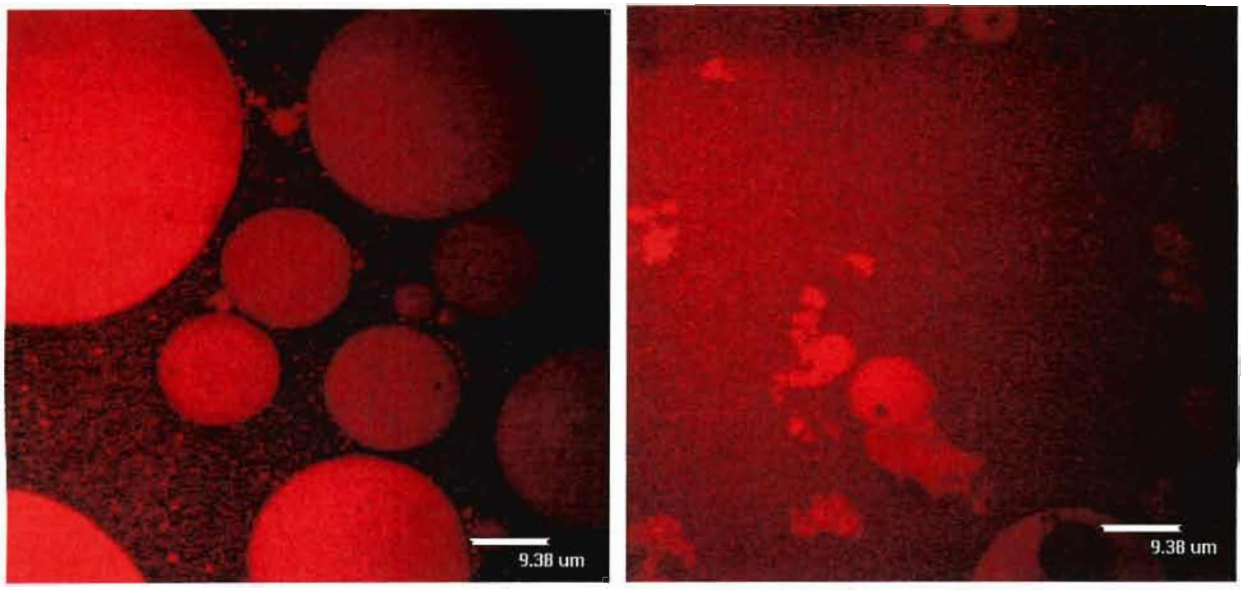
5.3.6 Pheroid™ vesicle formation and characterisation

Pheroid™ vesicle formation and characterisation were determined for prepared test formulations i.e. Pheroid™ vesicles (produced by adding $N_2O{\cdot}H_2O$ to pro-Pheroid™) using the Confocal Laser Scanning Microscope (CLSM) (Nikon D-eclipse C1, The Netherlands). The lasers used were a He/Ne laser with an excitation at 543 nm and an Argon ion laser with an excitation at 457 nm. A 60 X Apoplanar objective (1.4) and a small pinhole was used. Light microscope images were obtained using a Nikon PCM 2000 with digital camera DMX 1200 (The Netherlands) with 60 X Apoplanar objective (1.4).

The samples were vortexed to ensure a homogenous sample and 50 µl transferred using a calibrated pipette to a microfuge tube. 1 µl (10nM) Nile Red (Molecular Probes, Inc. USA) diluted in DMSO was added and the sample was incubated in the dark for 15 min. After incubation, a 20 µl sample was transferred with a pipette to a microscope slide and covered with a cover slip. Nile red is a lipid specific fluorescent probe which will after excitation at 540 nm emit light in the red spectrum which the Nikon D-eclipse C1 will detect at 605-675nm . Nile Red is almost non-fluorescent in water and other polar solvents (Hauhland, 2005).

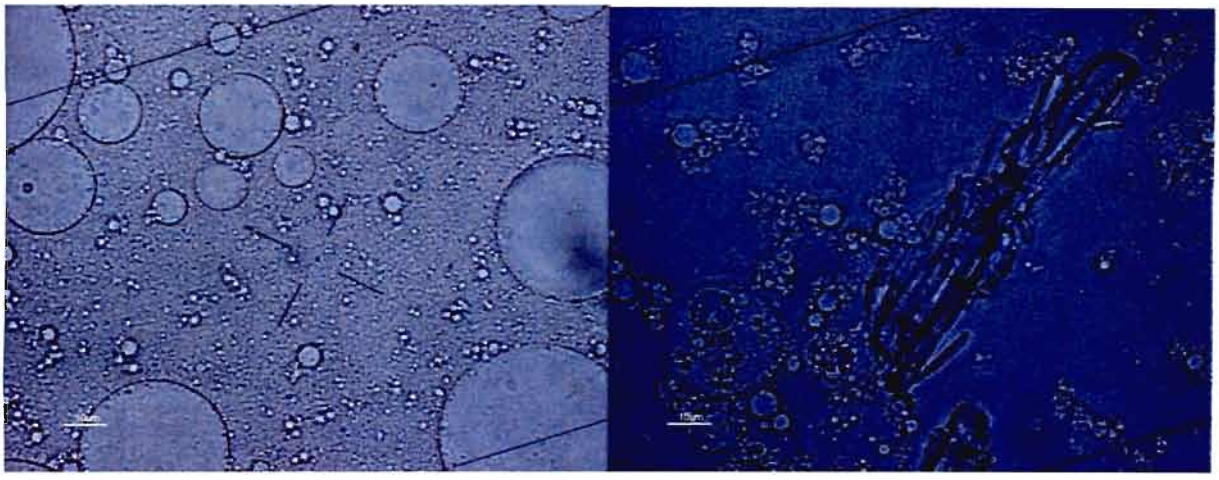
5.4 RESULTS

5.4.1 Pheroid™ vesicle formation and characterisation



(a) (b)

Figure 5.2: CLSM micrographs of Pheroid™ vesicle formation of a) formulation containing rifampicin and b) formulation containing ethambutol, pyrazinamide and isoniazid.



(a) (b)

Figure 5.3: Light micrographs of Pheroid™ vesicle formation of a) formulation containing rifampicin and b) formulation containing ethambutol, pyrazinamide and isoniazid.

The fluorescent probe, Nile Red, bound to the fatty acids in all the samples and can, therefore, be observed as CLSM micrographs. The Pheroid™ vesicle formulation containing rifampicin as well as the formulation containing ethambutol, pyrazinamide and isoniazid exhibit vesicles ≤ 2 µm, sponges and depot's. Very large (≥ 20 µm) sponges present in both formulations imply that not all the potential vesicles have formed at this stage. A longer sonication time might assist in forming the final vesicles. Light microscopy indicates the presence of crystals between 1- 40 µm in both the test formulations. The formulation containing rifampicin had 4-5 crystals per *field* while the other isoniazid, pyrazinamide and ethambutol containing formulation had only one crystal per two fields. Undissolved crystals have obviously not been packed into vesicles and this might result in less absorption and a lower efficacy.

5.4.2 Bacterial cultivation

The average CFUs per ml homogenate and TTD of bacterial growth for each treatment were determined and plotted as line graphs (Figure 5.4 and Figure 5.5).

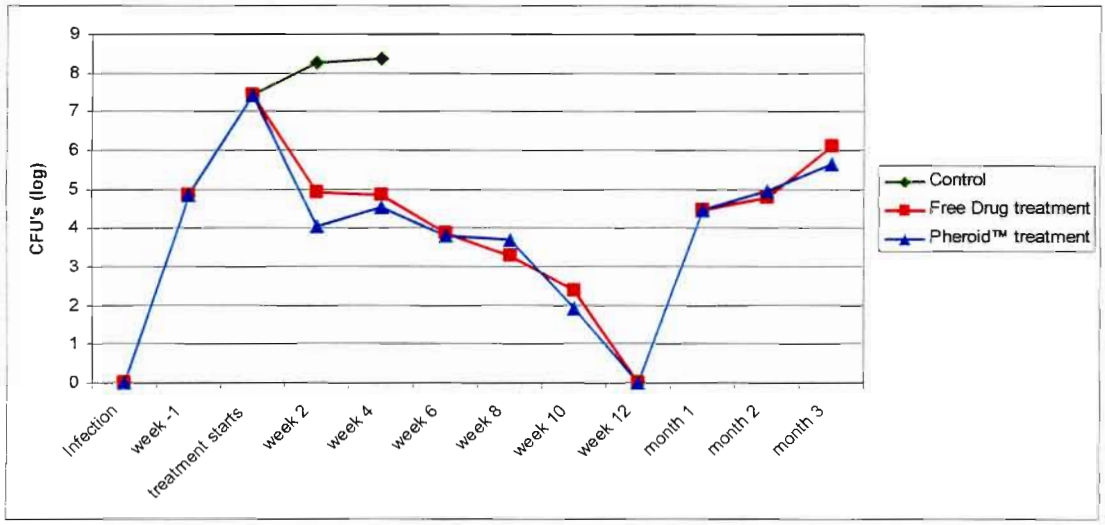


Figure 5.4: The effect of Pheroid™ and Free drug anti-TB treatments against *Mycobacterium tuberculosis* in the mouse model expressed as colony forming units.

Both the Free drug treatment and the Pheroid™ based treatment reduced CFU in the lungs from around 70 000 per ml at initial to zero in week 12. A relapse unfortunately occurred for both treatments during the post-treatment observation period. The treatments were not equally effective for each observation time point and their efficacies will be investigated below.

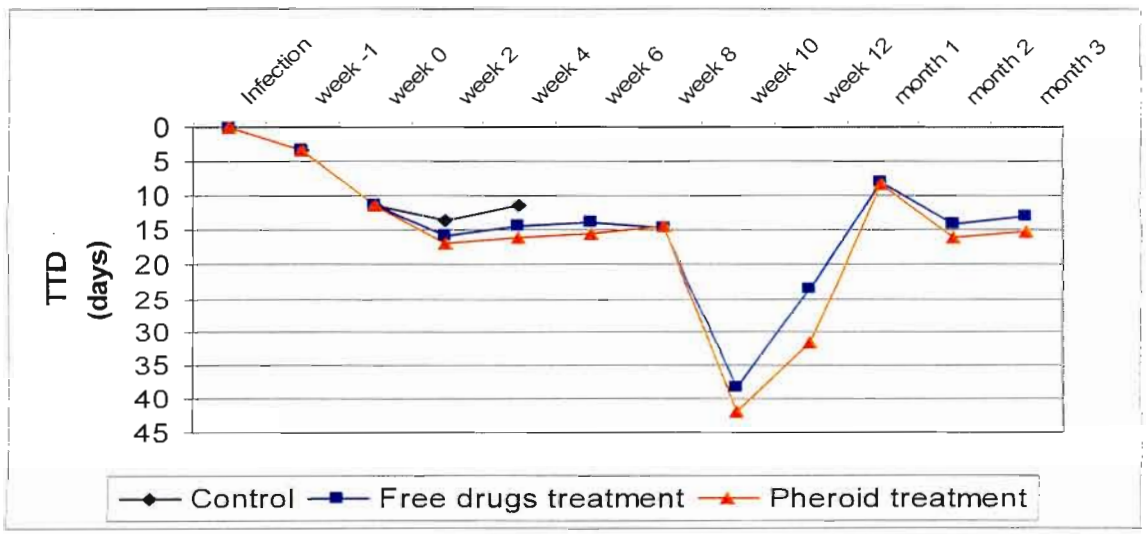


Figure 5.5: MGIT results illustrating the effect of Pheroid™ and Free drug treatments of anti-TB drugs against *Mycobacterium tuberculosis* in the mouse model.

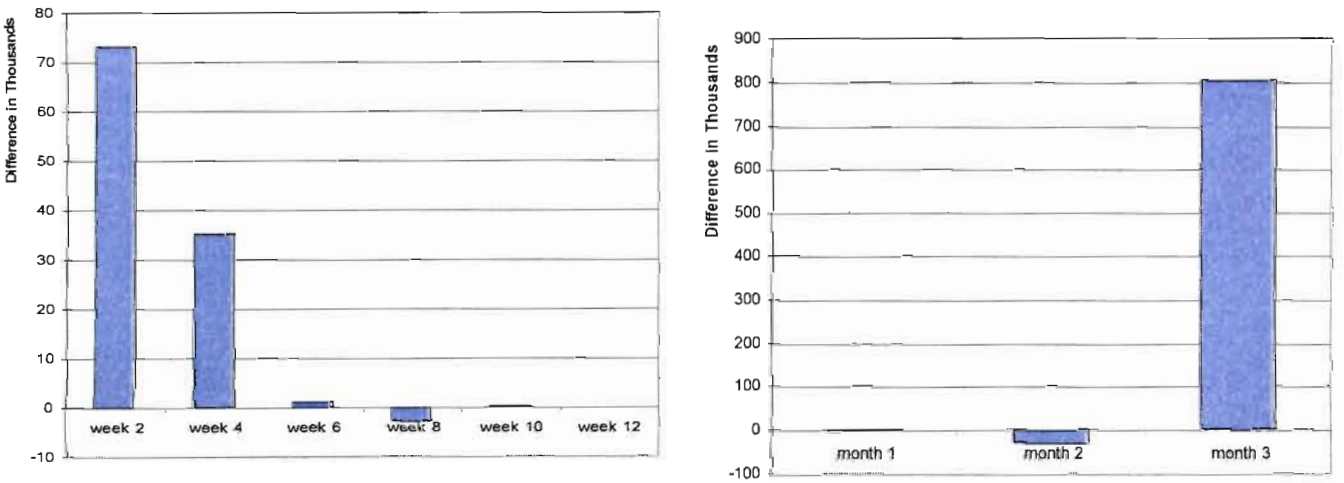
Note that the numerical values of Figure 5.5 are inverted on the y-axis.

Both treatments show a large increase in TTD between 8 and 10 weeks. The Pheroid™ based treatment showed no bacterial growth after 42 days of samples obtained at week 10 and can be relayed as zero bacteria present in the lungs. Unfortunately both the treatments showed bacterial growth again at week 12 and well into the post-treatment period. The Pheroid™ treatments did, however, take longer to indicate bacterial growth than the Free drug treatments and the significance will be discussed below.

To visually illustrate the extent of difference (Δ) between the Pheroid™ and the Free drug treatments, a graph can be drawn by deducting the average CFU counts resulting from the Pheroid™ formulation from the Free drug formulation with the following equation:

$$\Delta(CFU) = \bar{x}(Freedrug) - \bar{x}(Pheroid)$$

Figure 5.6a illustrates this difference during treatment and Figure 5.6 b the difference post treatment.



a b

Figure 5.6: Graphs illustrating the difference between Pheroid™ and Free drug of anti-TB treatments against *Mycobacterium tuberculosis* in the mouse model when expressed as CFUs during a) treatment and b) post treatment.

Weeks 2 and 4 had the largest difference in CFUs between the Pheroid™ and Free drug treatments. From week 6 to week 10 the two treatments showed virtually no difference with zero difference at week 12. Week 8 is the only time point where the Free drug treatment outperformed the Pheroid™ treatment. Figure 5.6 shows that the greatest difference in bactericidal effects between the Pheroid™ and Free drug treatments occurs early on during treatment and the Pheroid™ treatment proves to be superior.

Month 3 shows a great difference in the CFU count after the first two months showed virtually no difference. Figure 5.7 shows that the Pheroid™ have a better prolonged post treatment effect than the Free drugs.

The difference (Δ) of TTD between the two treatments can also be illustrated in a graph by deducting the TTD for the Pheroid™ from the Free drug formulation with the following equation:

$$\Delta(TTD) = \bar{x}(Freedrug) - \bar{x}(Pheroid)$$

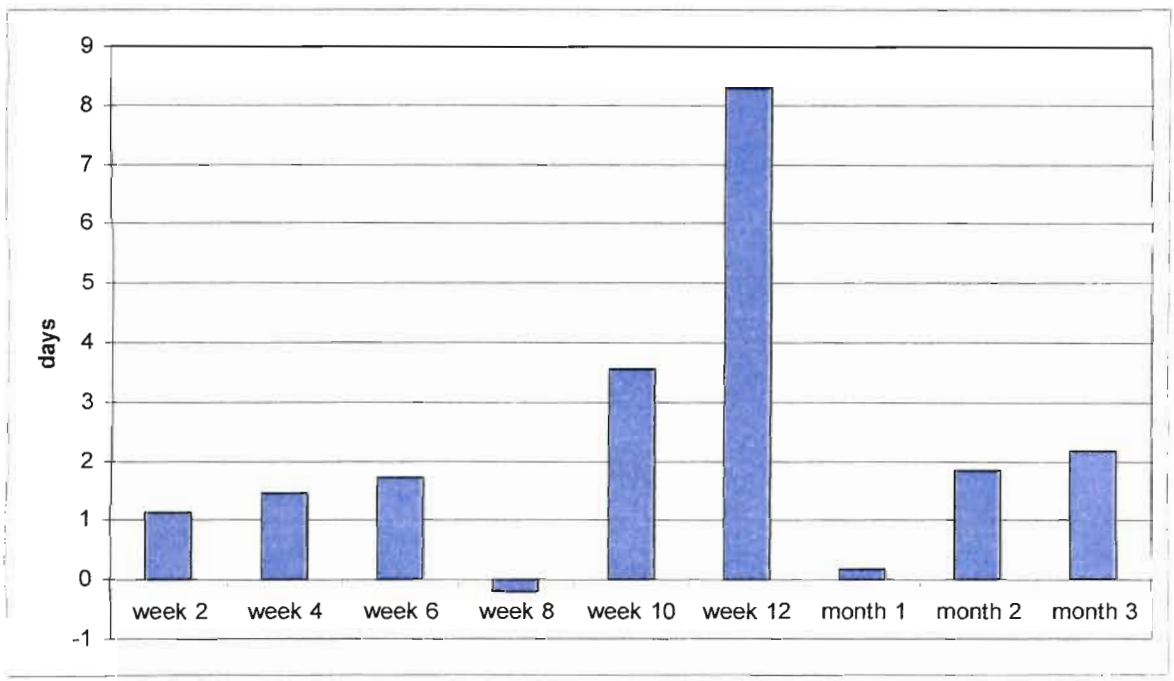


Figure 5.7: A graph illustrating the difference in days between Pheroid™ and Free drug of anti-TB treatments against *Mycobacterium tuberculosis* in the mouse model when expressed as TTD.

When the MGIT results are compared, the Pheroid™ treatment took longer to have a positive *M.tb* growth for the whole observation time with the exception on week 8. Week 12 showed the

largest difference in TTD, with the Pheroid™ treatment outperforming the Free drug treatment by more than 8 days. Since the MGIT method gives a negative detection if no bacterial growth was detected after 42 days, the difference between the two treatments could be even larger at week 12.

The null hypothesis (H_0) that was tested states that there is no difference in efficacy between the Free drug and Pheroid™ formulations. Thus: Free drugs = Pheroid™. Prof Faans Steyn at the NWU Statistical Consulting Service analysed the data with a non-parametric one way statistical test known as the Wilcoxon Rank Sums test using Statsoft version 8 statistical software. The Wilcoxon rank-sum test is a non-parametric alternative to the two-sample t-test of a normal distribution. The non-parametric calculations of the Wilcoxon test takes into account data that shifts the normal distribution due to data with a high standard deviation form the mean.

Table 5.1: Probability values and significance ($p \leq 0.05$) from infected mice challenged with Free drugs vs. Pheroid™ formulations using the Wilcoxon rank-sums test.

	CFU	Significant	TTD	Significant
Week 2 treatment	0.0305	yes	0.2007	no
Week 4 treatment	0.1208	no	0.0305	yes
Week 6 treatment	0.3203	no	0.2007	no
Week 8 treatment	0.8143	no	0.9376	no
Week 10 treatment	0.2463	no	0.0509	yes
Week 12 treatment	1.000	no	0.1203	no
Month 1 post treatment	0.5864	no	1.000	no
Month 2 post treatment	0.5864	no	0.2550	no
Month 3 post treatment	0.1564	no	0.0535	no (yes)

Yes=significantly different

No=not significantly different

When the CFU average counts are compared, there is only a significant difference between the two treatments at week 2. The TTD data, however, indicate a significant difference at week 4 and 10. At the 3 month post treatment time point the difference in probability between the response to Pheroid™-entrapped and Free drug is very close to $p \leq 0.05$ and can, therefore, still be considered significant for $p \leq 0.05$. The difference when comparing the two methods could be due to factors such as inaccurate sampling methods or clumping of the bacteria, the homogenizing may not be producing a good homogeneous mixture.

When the time points that are statistically significant for both methods are compared to the time points with the greatest differences, the results seems to be contradictive. For example; when looking at the CFU results, only week 2 turned out to be statistically significant, while the Δ(CFU) graphs give the impression that week 4 and month 3 should also be statistically significant. The same can be said for week 12 when looking at the TTD results and graphs. A possible reason could be that the standard deviation from the mean at those controversial time points are very large. However, the Wilcoxon rank sum tests took that variation into account and determined that although the difference in mean is large, the points are not statistically different.

Another matter of concern is the difference in which time points are statistically significant between the CFU and TTD data. The correlation between the data can be determined using the Pearson's Correlation Coefficients. The Pearson Correlation determines the degree to which data points are linearly related by determining the covariance between two points (Hodges *et al.*, 1975). Table 5.2 gives the Pearson Correlation cofficient between the two data sets as well as the significance thereof determined by Prof Faans Steyn at the NWU Statistical Consulting Service using Statsoft version 8, statistical software. A probability of $p \leq 0.05$ is tested.

Table 5.2: Correlation and significance of differences between the CFU and TTD data for each time point.

	Correlation	Probability	Significant
Week 2 treatment	-0.73857	0.0061	yes
Week 4 treatment	-0.50627	0.0931	no
Week 6 treatment	-0.22229	0.4874	no
Week 8 treatment	-0.72897	0.0072	yes
Week 10 treatment	0.25412	0.4254	no
Week 12 treatment	None		
Month 1 post treatment	0.39276	0.2066	no
Month 2 post treatment	-0.52772	0.0778	no
Month 3 post treatment	-0.71217	0.0094	yes

Yes=significantly different

No=not significantly different

The correlation for week 12 could not be calculated because the CFU counts were 0. A negative correlation can be seen for all the time points except weeks 10 and 12 as well as month 1 post treatment. A negative correlation means that the as the CFU counts decrease,

the TTD increase and that happens to a significant extent on week 2 and 8 and 3 months post treatment.

5.5 CONCLUSION

5.5.1 Pheroid™ vesicle formation and characterisation

Both the light microscope and the CLSM images indicate that Pheroid™ vesicles ≤ 2 µm successfully formed with the addition of the $N_2O{\cdot}H_2O$ liquid phase. As previously stated, the sponges and depots could be homogenised to form smaller vesicles by a longer/higher frequency sonication process. Visible crystals in the vesicles indicate < 100% entrapment of the actives in Pheroid™ and might play a role in efficacy. Future formulations must, therefore, incorporate a thorough grinding process to minimise the particle size of the API, in order to help with entrapment of the drugs when a liquid phase is added. The formulation containing rifampicin had more and larger crystals that the other formula. The incorporation of commercially obtainable micronised rifampicin might aid in increasing rifampicin entrapment into Pheroid™ vesicles.

5.5.2 Bacterial cultivation

The null hypothesis tested states that a formulation containing the anti-TB drugs rifampicin, ethambutol, pyrazinamide and isoniazid in their free form has equal efficacy than a Pheroid™ based formulation containing the same anti-tuberculostatics. This hypothesis is rejected at week 2, 4 and 10 of treatment depending on the analysis method as well as for month 3 post treatment, depending on the analysis method. According to the Mycobacteria Growth Indicator Tubes (MGIT) analysis method, the Pheroid™ treatment is 10% more effective at week 4 and 9% at week 10. The Pheroid™ based treatment is also 16.7% more effective at month 3 post treatment. According to the agar plate method, the Pheroid™ treatment 7% is more effective *than the* Free drug treatment at week 2 of treatment. The Free drug treatment did not outperform the Pheroid™ based treatment for the whole duration of the study.

Unfortunately the significance of correlation between data sets cannot explain the differences in significance between the test methods, i.e. CFU and TTD data. The reason is probably due to the method of data generation. The CFU agar plates were incubated for 3 weeks prior to counting viable CFU and the time of the first colony to grow not noted, which is what the MGIT tubes are ultimately determining. A general trend as to when the Pheroid™ treatment works better than the Free drugs can, however, be concluded to be from the onset of treatment to about week 10 (with the exception of week 6) and again at 3 months post treatment. The

significant difference in re-occurance of the *M.tb* bacterium to a lesser degree after treatment with a Pheroid™ formulation supports the argument that the APIs stay more effective for longer *in vivo*. This indicates that with a new anti-tuberculosis treatment regime the APIs can either be lowered, treatment time shortened or treatment intervals increased.

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CHAPTER 6

SUMMARY AND FUTURE PROSPECTS

6.1 SUMMARY

This study focused on the bioavailability and efficacy of anti-tuberculosis drugs with the aim of formulating of a new commercial dosage form. This new dosage form/treatment regime must have reduced quantities of anti-tuberculosis drugs in order to improve patience compliance. During this study attention was also given to formulating with Pheroid™ and gave attention to the incompatibilities between anti-TB agents of especially RMP and INH.

Bioavailability in the mouse was statistically enhanced by ≈ 85.7% for EMB, ≈ 45.5% for INH and ≈ 33.2% for PZA with the Pheroid™ formulation when compared with the formulation currently prescribed (Rifafour® e-275). Although not significantly different did RMP show a ≈ 33.3% increase with the Pheroid™ formulation when compared with Rifafour® e-275. The pro-Pheroid™ formulation revealed opposite behaviour with a significant increase of ≈75% to ≈ 84% when compared to Rifafour® e-275. Bioavailability decreased between ≈ 7% and ≈ 58% for EMB, INH, and PZA when Rifafour® e-275 is compared with the pro-Pheroid™ formulation. These results nevertheless provide valuable information as to which APIs can be decreased and the corresponding reduction quantity.

Efficacy of RMP, INH, PZA and EMB entrapped in Pheroid™ was compared to Rifafour® e-275 in the mouse model using the MGIT method and the colony counting method. Although the two methods gave slightly different results, could a trend be observed. The mice treated with the Pheroid™ formulations showed a significant improvement from onset of treatment to about week 10 (with the exception of week 6) and again at 3 months post treatment. The significant difference in re-occurrence of the *M.tb* bacterium to a lesser degree after treatment with a Pheroid™ formulation supports the argument that the APIs stay more effective for longer *in vivo.* This indicates that with a new anti-tuberculosis dosage form/treatment regime can the APIs quantities either be decreased, treatment time shortened or treatment intervals increased.

During the course of the bioavailability and efficacy experiments, constant attention was given to formulation challenges. Issues around formulating with pro-Pheroid™ and separating RMP and INH was addressed. A dosage form separating PZA and RMP from INH and EMB was

formulated and used in the bioavailability experiments while dosage forms separating RMP from PZA, INH and EMB were deemed the best for the efficacy experiments. Knowledge gained in formulating these dosage forms, together with the bioavailability and efficacy outcomes could be combined into a final effective dosage form.

Results from this study also paved the way forward for further development of a reduced anti-tuberculosis dosage form and registration with regulatory bodies. The bioavailability and efficacy data accumulated in this study will be able to support an application for a Phase II clinical trial.

6.2 FUTURE PROSPECTS

The next steps towards registering a commercially available TB treatment regime will be to:

1. Formulate a reduced dosage form keeping commercialisation practicalities in mind.
2. Challenge the stability of the new dosage form.
3. PK/PD animal studies on the new dosage form.
4. Efficacy animal studies on the new dosage form.
4. Application towards a Phase I human trial testing the new dosage form.

6.3 RECOMENDATIONS FOR FUTURE STUDIES

The bioavailability study used eight mice per time point multiplied by the amount of times plasma had to be analysed to produce results on all four the APIs. The ideal is to have only the six or eight subjects donating blood over the whole period investigated in order to minimize sacrifices for blood collections. Fewer mice will decrease individual mouse variations which will give statistically more solid results. Fewer mice will also be ethically more acceptable. Some method development in regards to plasma collection and analysis is there for needed. Once a new plasma analysis method using less plasma is met can alternative plasma collection methods such as orbital bleeding be investigated.

This is the first experiments on the mouse model regarding the Pheroid™ drug delivery system and anti-tuberculosis drugs. Much knowledge around the model has been gained, but a need still exists to optimize methods. One area that needs to be optimized is Pheroid™ formulations and dosages suitable to animal models. One concern from this study is whether a quantity of 200 µl of the pro-Pheroid™ might be toxic to the mouse system. It is recommend that the quantity of pro-Pheroid administered to the mouse must be adjusted proportionately to mimic the quantity a human will consume.

Plasma concentrations and half life of the anti-TB drugs in mice are not comparable with what is expected in humans when the same dosage per kg in ingested. One factor could be explained by the differences between human and mouse cytochrome P450 metabolism. Although the mouse is practically the better choice for experiments, reevaluation of the model is necessary if differences from human biosystems are too large. Models to consider are guinea-pig and rabbit.

This study only assessed bioavailability, but a full pharmacokinetic and pharmacodinamic study will be recommended. True half life of a drug is optimally investigated via the intra venous route and not derived from post-equilibrium data of a bioavailability study of an oral dosage form. An extension of this study must include plasma analysis after intra venous administration of the anti-TB drugs EMB, INH, PZA and RMP. Since a half life for rifampicin in all but the Pheroid™ formulation could not have been determined, a study stretching over a longer period (eight hours instead of four, perhaps) will produce enough data for to determine half-life. This study also assumed that half-life is an indication of the volume of distribution of the drugs through-out the body. More accurate and meaning full investigations into the volume of distribution, clearance of the drug and, therefore, the elimination constant for each drug will give a more holistic pharmacokinetic picture. Since dosing rate can be estimated as a product of drug clearance and the desired plasma concentration, will drug clearance data be one step closer to accurate dosing intervals. True volume of distribution information can determine a drug loading dose which might be needed to achieve target plasma concentrations rapidly i.e. the loading dose. Calculating terminal half life from a least square regression line derived from the last few points on a bioavailability curve might not produce accurate volume of distribution information, especially for slow release dosage forms. This method assumes that the drug elimination phase has been reached at T_{max} and that the slope of the regression line, therefore, indicates clearance at a constant rate without the accommodating a second increase in absorption.

Future recommendations of efficacy studies include refining the CFU method. The possibility exists that the initial lung size measured in weight might influence the CFU/ml. Lungs were placed in 2 ml PBS regardless of the size. A larger lung might host more CFUs and the bacteria will, therefore, be more concentrated in the 2 ml PBS in which the organs were homogenised. A more accurate reading will be generated if the volume in which the lungs are homogenised is calculated according to the weight of the lung. Alternatively the volume can be kept at 2 ml, but the weight of organ to be homogenised can be adjusted to be equal for all subjects. This will produce more comparable results. If the CFU and MGIT methods are

performed together as in the case with this study, the time the first colonies appear on the agar plates will assist in comparing the two methods.