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A preclinical evaluation of the possible enhancement of the efficacy of antituberculosis drugs by Pheroid™ technology

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Dissertation submitted in fulfillment of the requirements for the degree *Master of Science* in Pharmaceutics of the Potchefstroom campus of the North-West University

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A thorough literature review on tuberculosis highlighted two key problems that will be addressed with this study. These problems were identified as:

- Interactions that counter the stability of drugs in fixed-dose combination (FDC) products;
- Reduced *in vivo* bioavailability of rifampicin when formulated in FDC products.

The main aims and objectives of this study are to:

- Identify and correct any contributing factor(s) which may lead to the poor bioavailability of rifampicin with regards to the manufacturing process of a pro-Pheroid™ fixed-dose combination.
- Determine and compare the plasma levels of first-line anti-tuberculosis drugs after administration of the current FDC product (Rifafour-e275®) and the pro-Pheroid™ FDC product to experimental animals.
- Determine the stability of the pro-Pheroid™ FDC product and suggest corrective action should problems occur.

Other aims and objectives include:

- A better understanding of the Pheroid™ drug delivery technology and the identification of new applications for this versatile system.
- Optimization of research methodology for future *in vivo* animal experiments and stability studies.
- Understanding and practicing the art of scientific reporting.

ABSTRACT

A PRECLINICAL EVALUATION OF THE POSSIBLE ENHANCEMENT OF THE EFFICACY OF ANTI-TUBERCULOSIS DRUGS BY PHEROID™ TECHNOLOGY

With the rise of the HIV pandemic and persistent global poverty, tuberculosis (TB) was declared a global emergency, claiming thousands of lives with each passing year and putting severe pressure on the socio-economic status of affected countries. Co-infection with human immunodeficiency virus (HIV) complicates the treatment of the disease even further.

The treatment of this dreadful disease currently involves administration of a combination of rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E) for the initial 2 months, followed by rifampicin and isoniazid for 4 months. The World Health Organization (WHO) strongly encourages the use of fixed-dose combination (FDC) products. FDC products containing anti-tuberculosis drugs were introduced to the market as a means to simplify treatment and to increase patient compliance. The use of FDCs is hampered by poor bioavailability when rifampicin, isoniazid, pyrazinamide and ethambutol are formulated together. Various causes have been proposed, including raw material characteristics, changes in the crystalline forms of rifampicin, degradation in the gastro-intestinal tract and inherent variability in absorption and metabolism or a combination of any of these.

The discovery and development of a new regimen including four novel drugs usually takes up to 14 years and it is an extremely expensive process. Although a bouquet of drugs with novel mechanisms of action are ready for phase II and III clinical trials, the annual increase in new tuberculosis cases necessitates immediate action.

The search for a means to increase bioavailability of existing drugs entered a new era when the Pheroid™ drug delivery system was developed. This technology involves the entrapment of drug molecules into stable submicron and micron sized structures, referred to as Pheroids™. Pheroids™ consist of three components, namely fatty acids, sterile water and nitrous oxide gas. Research of this system promises many advantages in the oral or transdermal delivery of drug molecules.

An application of Pheroid™ technology in the treatment of tuberculosis presented itself when a Pheroid™-based combination product was tested for the possible enhancement of bioavailability of especially rifampicin in humans. However, the test formulation in the above-mentioned study had considerable stability problems. These stability problems were directly related to the instability of rifampicin when it is formulated in combination with isoniazid.

The next step in the development process entailed a few changes being made to the formulation to rule out any known drug instabilities. These changes included the use of pro-Pheroid™ technology and the separation of rifampicin and isoniazid within the formulation. A pilot study was carried out to determine and compare the levels of R, H, E and Z in the plasma of mice who received a pro-Pheroid™ formulation with those who received the current 4-drug FDC (Rifafour e-275®), dissolved in water. The aim of the pilot study was to determine if the new pro-Pheroid™ formulation would also increase the absorption of rifampicin, isoniazid, ethambutol and pyrazinamide. This study also consisted of a 3-month stability study under accelerated climatic conditions and drug content and microbial growth were determined on a monthly basis.

An increase (300%) in the absorption of rifampicin was found with the pro-Pheroid™ formulation, when plasma concentrations were compared with the current commercial product, Rifafour e-275®. The accelerated stability test was unfortunately hampered by some apparatus-based inconsistencies and formulation problems, which made it difficult to determine drug content after three months. This was unfortunate, but it was concluded that the drug content within the pro-Pheroid™ formulation did remain between 90% and 110% of the initial values. Furthermore, no microbial growth was detected in the formulations. Therefore, the pro-Pheroid™ formulations were regarded as stable.

In conclusion, the pro-Pheroid™ formulation did succeed in delivering more of the drug molecule across the intestinal epithelia of mice. Furthermore, the proposed formulation was found to be stable at various temperatures ranging from 5°C to 40°C, when protected from light and moisture. A complete bioequivalence study in mice will be based on the experimental methods used and the data obtained from this study.

The results of this study are herewith reported in the article format as described in section A.13.7.3 of the general academic rules of the North West University. The first three chapters deal with the global burden of tuberculosis, current treatment and control strategies and the Pheroid™ drug delivery system and its application in tuberculosis treatment. Chapter 4 includes a proposed article for submission to the Open Drug Delivery Journal, and Chapter 5 gives a final summary and conclusion of this study. Results for all experiments are organized into appendices 1-3.

UITTREKSEL

'N PREKLINIESE EVALUERING VAN DIE MOONTLIKE VERHOGING IN DIE EFFEKTIWITEIT VAN MIDDELS TEEN TUBERKULOSE DEUR PHEROID™-TEGNOLOGIE

Met die groei in die MIV-pandemie en volgehoue wêreldwye armoede is tuberkulose (TB) as 'n wêreldwye noodtoestand verklaar wat elke jaar duisende lewens eis en erge druk op die sosio-ekonomiese bronne van geaffekteerde lande plaas. Gelyktydige infeksie met die menslike immuniteitsgebrekvirus (MIV) kompliseer die behandeling van die siekte verder.

Die behandeling van hierdie verskriklike siekte behels tans toediening van 'n kombinasie van rifampisien (R), isoniasied (H), pirasienamied (Z) en etambutol (E) vir die eerste 2 maande gevolg deur rifampisien en isoniasied vir 4 maande. Die Wêreldgesondheidsorganisasie (WGO) beveel die gebruik van 'n kombinasie van produkte teen 'n vaste dosis (KPVD) sterk aan. KPVD wat middels teen tuberkulose bevat, is op die mark gebring om behandeling te vereenvoudig en om pasiëntmeewerkendheid te verbeter. Die gebruik van KPVD's word deur swak biobeskikbaarheid belemmer as rifampisien, isoniasied, pirasienamied en etambutol saam geformuleer word. Verskeie oorsake is hiervoor voorgestel, waaronder eienskappe van die grondstowwe, veranderings in die kristalvorms van rifampisien, ontbinding in die gastro-intestinale weg en inherente wisseling in absorpsie en metabolisme of 'n kombinasie hiervan.

Die ontdekking en ontwikkeling van 'n nuwe regimen met vier nuwe middels neem gewoonlik tot 14 jaar en is 'n uiters duur proses. Hoewel 'n versameling middels met nuwe werkingsmeganismes gereed is vir fase II- en fase III-kliniese proewe, vereis die jaarlikse toename in nuwe gevalle van tuberkulose onmiddellike optrede.

Die soeke na 'n nuwe manier om die biobeskikbaarheid van bestaande geneesmiddels te verbeter het 'n nuwe era binnegegaan toe die Pheroid™-geneesmiddelafleweringstelsel ontwikkel is. Hierdie tegnologie behels die inbou van geneesmiddelmolekules in stabiele strukture van submikron- en mikrongrootte, bekend as Pheroids™. Pheroids™ bestaan uit drie komponente, naamlik vetsure, steriele water en stikstofoksiedgas. Navorsing van hierdie stelsel hou belofte van talle voordele vir orale en transdermale aflewering van geneesmiddel-molekules in.

'n Toepassing van Pheroid™-tegnologie vir die behandeling van tuberkulose het vanself na vore gekom toe 'n kombinasieproduk op Pheroids™ gebaseer vir die moontlike verbetering van die biobeskikbaarheid van veral rifampisien in mense getoets is. Die toetsformulering van die bogenoemde studie het egter aansienlike stabiliteitsprobleme gehad. Hierdie

probleme het direk te doen gehad met die onstabiliteit van rifampisien as dit in kombinasie met isoniasied geformuleer word.

Die volgende stap in die ontwikkelingsproses was om 'n paar veranderings aan die formulering aan te bring om alle bekende onstabiliteit van die middels uit te skakel. Hierdie veranderinge was onder meer die gebruik van Pheroid™-tegnologie en die skeiding van rifampisien en isoniasied in die formulering. 'n Loodsstudie is gedoen om die vlakke te bepaal van R, H, E en Z in die plasma van muise wat 'n pro-Pheroid™-formulering ontvang het en dit te vergelyk met dié wat die huidige KPVD met 4 middels (Rifafour e-275®) opgelos in water gekry het. Die doel van die loodsstudie was om te bepaal of die nuwe pro-Pheroid™-formulering ook die absorpsie van rifampisien, isoniasied, etambutol en pirasienamied sal verbeter. Hierdie studie het ook 'n stabiliteitstudie oor 3 maande onder versnelde toestande ingesluit en die geneesmiddelinhoud en mikrobiese groei is op 'n maandelikse basis bepaal.

'n Toename (300%) in die absorpsie van rifampisien is met die pro-Pheroid™-formulering gevind toe plasmakonsentrasies met dié van die huidige kommersiële produk, Rifafour e-275®, vergelyk is. Die versnelde stabiliteitstoets is ongelukkig deur probleme in die apparaat en met die formulering belemmer wat dit moeilik gemaak het om die geneesmiddelinhoud na drie maande te bepaal. Dit was jammer, maar daar is tot die gevolgtrekking gekom dat die geneesmiddelinhoud in die pro-Pheroid™-formulering tussen 90% en 110% van die oorspronklike waardes gebly het. Verder is geen mikrobiologiese groei in die formulerings waargeneem nie. Daarom is die pro-Pheroid™-formulerings as stabiel beskou.

Ten slotte het die pro-Pheroid™-formulering daarin geslaag om meer van die geneesmiddelmolekules oor die intestinale epiteel van muise af te lewer. Verder is gevind dat die voorgestelde formulering by temperature van 5°C tot 40°C stabiel is as dit teen lig en vog beskerm word. 'n Volledige studie van die bioekwivalensie in muise sal op die eksperimentele metodes en data van hierdie studie gebaseer wees.

Die resultate van hierdie studie word hiermee in artikelformaat aangebied soos in afdeling A.13.7.3 van die algemene akademiese reëls van die Noordwes-Universiteit uiteengesit. Die eerste drie hoofstukke bespreek die wêreldwye las van tuberkulose, huidige behandeling en beheerstrategieë en die Pheroid™-geneesmiddelafleweringstelsel en die toepassing daarvan vir die behandeling van tuberkulose. Hoofstuk 4 is 'n voorgestelde artikel vir voorlegging aan die Open Drug Delivery Journal en Hoofstuk 5 gee 'n laaste opsomming en gevolgtrekking van die studie. Die resultate van al die eksperimente word in bylae 1-3 aangebied.

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CHAPTER 1

TUBERCULOSIS

Tuberculosis (TB) is a slowly progressive infection with a slumber period following exposure to *Mycobacterium tuberculosis.* It most commonly affects the lungs. Pulmonary symptoms include a productive cough, chest pain and dyspnea. Diagnosis is most commonly done by sputum culture and a smear. Treatment is with multiple antimicrobial agents.

1.1 The History of tuberculosis

Mycobacterium tuberculosis has been present in humans since 2400 BC. Fragments of the spinal column from Egyptian mummies show definite pathological signs of tubercular decay (Sarrel, 2006).

Around 460 BC tuberculosis was referred to as phthisis, the most widespread and often fatal disease of those times. Hippocrates went as far as to warn his colleagues not to visit patients in the late stages of the disease, because their inevitable deaths might blemish the reputation of the attending physician. It was not until 1679 that Sylvius identified actual tubercles as a consistent and characteristic change in the lungs and other organs of patients. He further described their progression with terms such as abscesses and cavities (Sarrel, 2006).

Benjamin Marten, an English physician, published "*A new theory of consumption" in* 1720 in which he postulated that tuberculosis might be caused by "wonderfully minute living creatures". He further stated that it would be possible to catch consumption when in close proximity of a consumptive person, constantly eating and drinking with them or inhaling the breath they emit from their lungs. Marten's publication led to a break in the chain of infection and enabled prevention of the disease. Finding a cure for the disease, however, remained a mystery (Sarrel, 2006).

Only towards the latter half of the nineteenth century did studies done by Villemin demonstrate the infectious nature of tuberculosis. This finally broke the centuries-old belief that tuberculosis was a hereditary disorder (Sarrel, 2006).

In 1882, Robert Koch excited the world when he discovered a staining technique that enabled him to see *Mycobacterium tuberculosis.* The world realized that the fight against this deadly enemy could really begin (Sarrel, 2006).

Still, all that could be done was to improve social and sanitary conditions, and to ensure adequate nutrition as no medicine was available yet. Sanatoria were built to isolate the sick from the general population and simultaneously control the transmission of the disease (Sarrel, 2006). Physiologists encouraged exercise as a means to increase the ability of the heart to pump efficient quantities of blood through the lungs. This was believed to inhibit growth of the tubercles. Thus, climate, exercise and diet were fundamental aspects in tuberculosis therapy.

In November 1895, Wilhelm Konrad Röntgen was the first scientist to observe and record X-rays. Since then, the progress and severity of a patient's disease could be followed and reviewed (Sarrel, 2006).

Another important development came when Calmette and Guerin used specific culture media to reduce the virulence of the bovine TB bacterium. This created the basis for the BCG vaccine that is still in use today (Sarrel, 2006).

In 1943, the first real success came when Streptomycin was administered to a critically ill tuberculosis patient and was effective almost immediately. The advanced disease was visibly arrested, the bacteria disappeared from his sputum and he made a rapid recovery. Despite side effects of the drug, the fact remained that tuberculosis could be beaten (Sarrel, 2006).

A rapid succession of anti-tuberculosis drugs appeared in the following years: p-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampicin (1963) (Sarrel, 2006).

1.2 Etiology of tuberculosis

Tuberculosis is an infectious disease that is caused by one of four closely related species comprising the *Mycobacterium* complex:

- *Mycobacterium tuberculosis;*
- *Mycobacterium bovis;*
- *Mycobacterium africanum;*
- *Mycobacterium microti.*

Humans are the main reservoir for *mycobacterium tuberculosis* and other animals serve this function for the rest of the Mycobacterium complex.

Mycobacterium tuberculosis is a bacillary-shaped microorganism and a strict aerobe. The lungs therefore are ideal to provide in the maintenance and replication need of these slow-

growth organisms (Ait-Khaled & Enarson, 2003). The bacilli are extremely resistant to cold, freezing, and drying, while being very sensitive to heat, sunlight, and ultraviolet radiation.

The chemical structure of *M. tuberculosis* comprises proteins, carbohydrates, vitamins belonging to the B complex, and minerals such as phosphorus, magnesium, and calcium. The protein component functions as a substrate that is responsible for delayed hypersensitivity reactions like the tuberculin skin reaction. The microorganism lacks a cerulean capsule, but contains a considerable amount of mainly complex lipids of which mycolic acid is the most characteristic. This lipid-rich wall is responsible for a number of its biological characteristics, such as resistance to macrophage action and drying. Although *M. tuberculosis* is unable to produce toxins, it consists of a very important and complex antigenic component that is responsible for pathogenic capacity features (Caminero, 2004).

1.3 Transmission of tuberculosis

The airborne route is responsible for more than 80% of new tuberculosis cases. When performing actions such as speaking, laughing, and especially coughing, the infected individual expels micro-droplets into the air, which contain the mycobacteria. Although the largest droplets contain more bacilli, the smaller droplets are highly infectious since they can be deposited within the alveolar spaces of the sub-pleural zone of the lower lobes. This proves to be the ideal site of deposition because of the high oxygen partial pressure needed for microbial multiplication (Frieden *et al.*, 2003).

1.4 Pathogenesis of tuberculosis

The bacilli are lodged in the alveoli in the distal airways. From there it is assimilated by macrophages. A cascade of events is initiated that starts with the slow, continuous replication of *Mycobacterium tuberculosis* (Frieden *et al.*, 2003).

The bacilli spread to the hilar lymph nodes via the lymphatic system and cell-mediated immunity normally develop after 2-8 weeks following infection. Granulomas, nodules of activated monocytes and macrophages, are formed because of a delayed type hypersensitivity reaction. The formation of granulomas subsequently leads to a decrease in the replication and number of bacilli in part by enhancing macrophage activation and creating an oxygen and nutrient deprived environment. In the case of non immune-suppressed individuals, the bacilli in the centre of the necrotic granulomas are not viable and active disease might never occur (Frieden *et al.*, 2003).

Under certain conditions such as immature or deregulated immunity, alveolar macrophages may fail in limiting the replication of the bacilli, leading to primary progressive tuberculosis. Primary progressive tuberculosis refers to a state where the bacilli were not contained and were viable from the start of infection. The immune status of the host may change in time

due to factors such as age, nutritional status and bacterial infective load (Ioachimeshu, 2004). This scenario will then lead to the caseation of granulomas subsequently spilling viable, infectious bacilli into the airways. This process refers to secondary progressive tuberculosis. Figure 1.1 gives a schematic representation of the pathogenesis of tuberculosis.

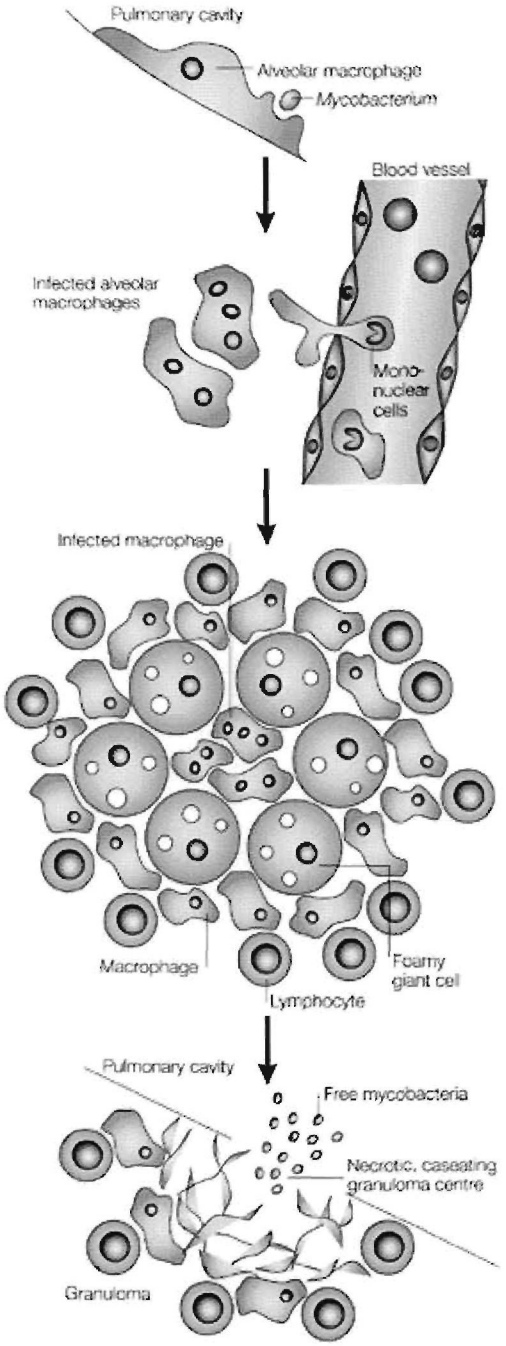

Figure 1.1: Pathogenesis of Tuberculosis (*Reprinted from* Russel, 2001).

On a cellular level the immune response against tuberculosis can be explained as follow: Infected macrophages are responsible for the release of interleukins 12 and 18, which stimulate CD4 T-cells to produce interferon γ. Interferon γ stimulates the phagocytosis of *Mycobacterium tuberculosis* in the macrophage and the release of TNF-α. According to Scott-Algood *et al.* (2003), TNF-α has an important role in the formation of granulomas. This is because ganulomas did not form in mouse models that lack TNF-α. However, the mechanism of action is not clearly understood yet. It is evident that any defect in this immune response will lead to the occurrence of the active disease (Frieden *et al.*, 2003).

1.5 Symptoms of tuberculosis

Symptoms will normally have a slow onset and will mimic other less serious respiratory diseases. A persistent cough for more than 2-4 weeks is most common and may indicate the possibility of pulmonary tuberculosis. Other commonly associated symptoms are hemoptysis and dyspnea due to lung involvement, malaise, weight loss, night sweat and chest pain. The symptoms are less pronounced in children, and any exposure to an active tuberculosis patient should raise more caution (Ioachimeschu & Tomford, 2004).

1.6 Diagnosis of tuberculosis

One of the key aspects and issues of concern in fighting the disease is the accurate and timely diagnosis of new tuberculosis cases. Timely diagnosis can have a significant impact on transmission of the disease by treating infected individuals before they could spread the disease. Possible reasons for the low case detection are inaccessibility of health facilities to patients, misdiagnosis on first visit to health care professional, fear of not being able to work and losing income as well as the stigma associated with the disease (WHO, 2005).

Given the lack of a single, sensitive and simple test, the current approach to diagnose active disease combines clinical assessment and laboratory tests in a complex algorithm (figure 1.2) (WHO, 2005).

Tuberculin skin testing is done by intradermal injection of a tuberculin purified protein derivative (PDD) into the forearm of an individual suspected of having active tuberculosis. The skin test reaction should be evaluated after 48 to 72 hours. Classification of the skin test reaction is given in table 1.1.

The result of chest radiography functions as an indication of the next step in the algorithm. A positive chest radiograph is known to show characteristic upper lobe cavitations (Ait-Khaled & Enarson, 2003).

Sputum smear examination remains a very useful diagnostic tool, since the Ziehl-Neelsen stain can identify 50-80% of culture-positive tuberculosis cases. In countries with a high prevalence of tuberculosis, a positive smear signifies tuberculosis in 95% of the cases (Ioachimeschu & Tomford, 2004). Mycobacterial culture is the standard for the definitive diagnosis of active disease. Utilization of culture smear microscopy is limited by the delay before interpretable results can be obtained and the inability of low-income settings to support the use of culture methods at primary care level (WHO, 2005). Sputum from patients is normally obtained for culture, but *M. tuberculosis* can also be recovered from gastric aspirates. Bronchoscopy or induced sputum is reserved mainly for patients who are unable to provide good-quality sputum.

Table 1.1: Classification and interpretation of the tuberculin skin test (CDC, 2007).

An induration of 5 or more millimeters is considered positive in:	An induration of 10 or more millimeters is considered positive in:	An induration of 15 or more millimeters is considered positive in:
- HIV-infected persons - A recent contact of a person with TB disease - Persons with fibrotic changes on chest radiograph consistent with prior TB - Patients with organ transplants - Persons who are immunosuppressed for other reasons (e.g., taking the equivalent of >15 mg/day of prednisone for 1 month or longer, taking TNF-α antagonists)	- Recent immigrants (< 5 years) from high-prevalence countries - Injection drug users - Residents and employees of high-risk congregate settings - Mycobacteriology laboratory personnel - Persons with clinical conditions that place them at high risk - Children < 5 years of age - Infants, children, and adolescents exposed to adults in high-risk categories	Any person, including persons with no known risk factors for TB. However, targeted skin testing programs should only be conducted among high-risk groups.

Since roughly 50% of all cases of active tuberculosis currently go undetected, there is a compelling need to pursue research aimed at improving diagnostic methods of tuberculosis.

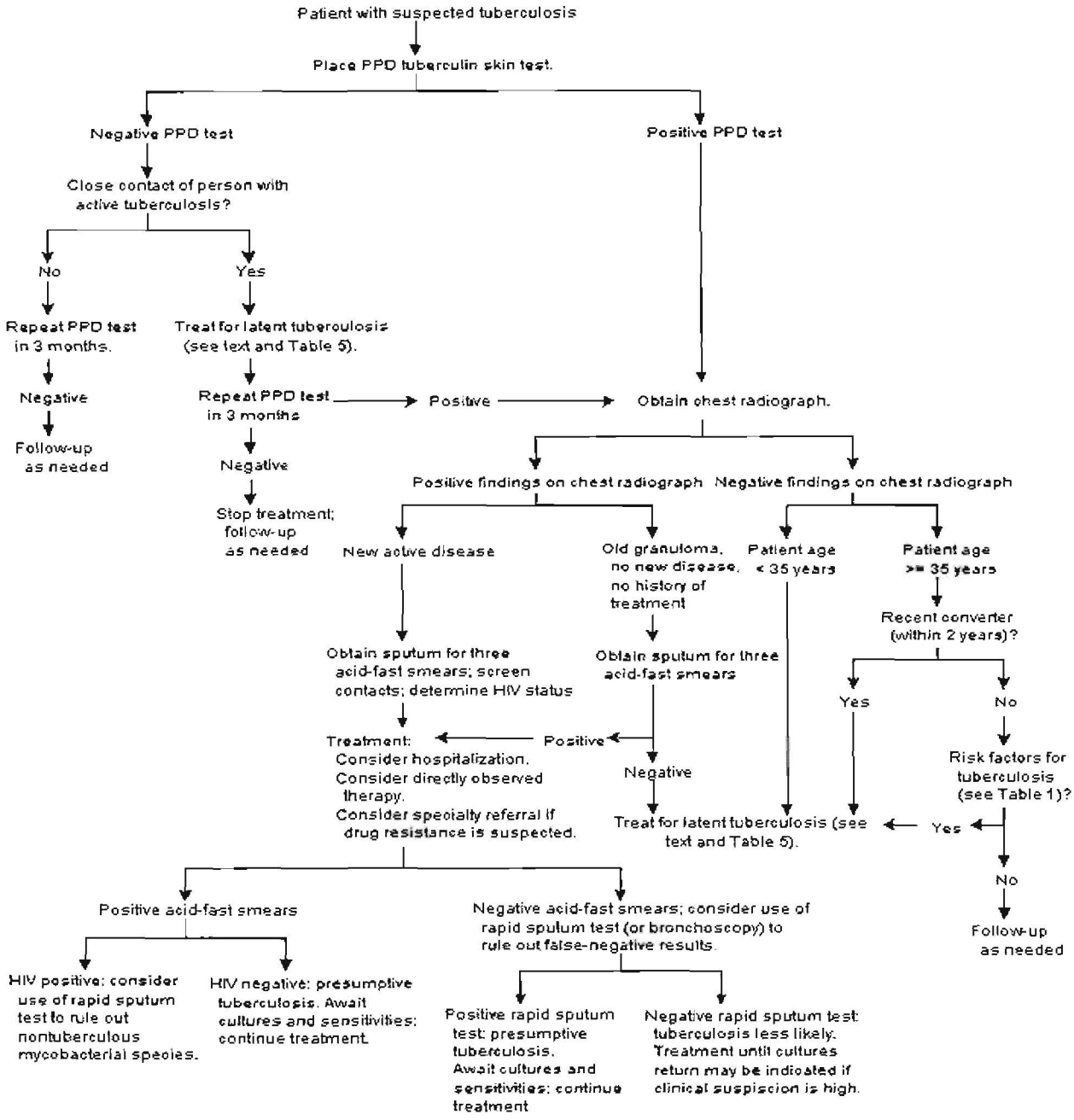

Figure 1.2: Algorithm for the evaluation of patients with suspected tuberculosis. (PPD = purified protein derivative; HIV = human immunodeficiency virus) (*Reprinted from* Jerant *et al.*, 2000).

1.7 Treatment of tuberculosis

The treatment of tuberculosis is essential in turning a global emergency around into a long forgotten disease. Modern treatment strategies are based on a standardized short-course chemotherapy regimen under direct observation, at least during the initial phase of treatment,

and proper case management to ensure that an individual is completely treated and cured. The essential services needed to control tuberculosis were developed and packaged as the directly observed treatment strategy (DOTS) from the early 1990's. Countries that applied DOTS successfully witnessed a significant decrease in transmission, mortality and drug resistance. Properly applied chemotherapy is effective in curing infectious individuals and interrupting the chain of transmission. A more detailed description of the treatment of tuberculosis is given in chapter 2.

1.8 Tuberculosis: a Global emergency

Tuberculosis remains a grave burden to public health. According to recent estimates, 9 million new cases of tuberculosis disease are reported annually (figure 1.3). Furthermore, more than 2 million deaths are reported each year (Dukes Hamilton *et al.*, 2007). Because of various factors, such as, HIV co-infection and persistent poverty, the global incidence rate of tuberculosis is now growing at approximately 1% per annum. The growth in global incidence is unevenly distributed with a notable explosion of new infections in sub-Saharan Africa (figure 1.4) (WHO, 2007).

In 1993, the World Health Organization (WHO) took an unprecedented step and declared tuberculosis to be a global emergency (Grange & Zumla, 2002). Despite the existence of cheap and effective treatment regimes, the incidence of tuberculosis is increasing worldwide due to the rise of the HIV-pandemic, persistent global poverty and the emergence of multi-drug resistant tuberculosis strains.

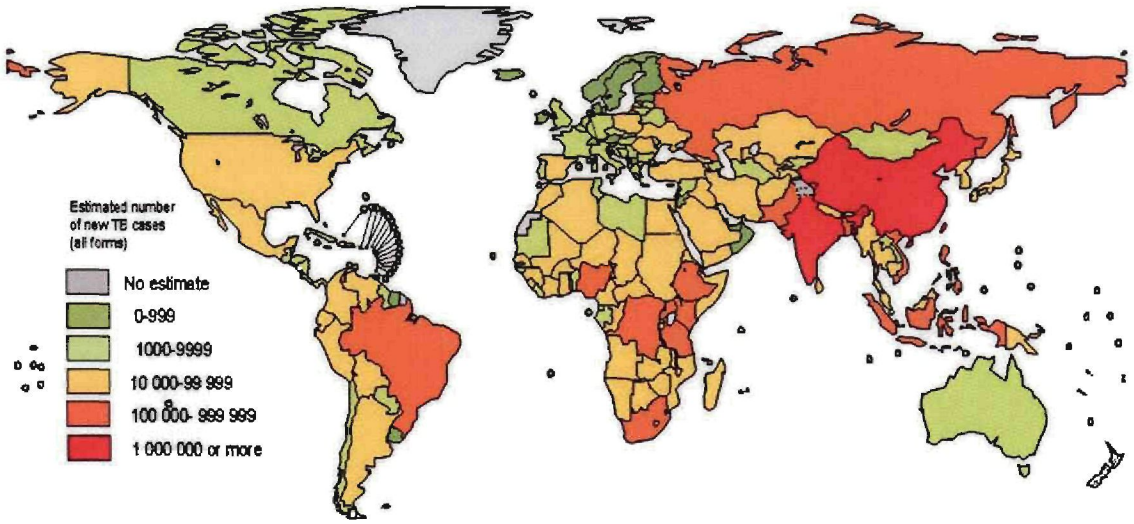

Figure 1.3: Estimated number of new tuberculosis cases (WHO, 2007).

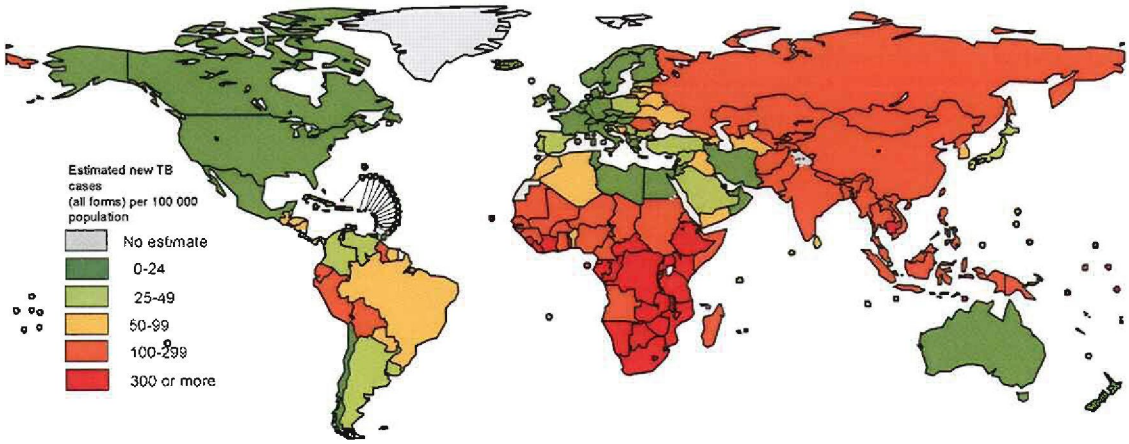

Figure 1.4: Estimated tuberculosis incidence rate in 2004 (WHO, 2007).

1.8.1 The effect of poverty on tuberculosis control

The connection between poverty and tuberculosis is well established. Even in the developed world the highest rate of infection occurs in the poorest sections of the community (Davies, 1999). Tuberculosis incidence is higher in the impoverished communities due to overcrowded living or working conditions, poor nutrition, and co-infections such as HIV.

In addition, individuals suffering from tuberculosis are less able to generate income for themselves and their dependents. This situation further limits their access to effective treatment (WHO, 2005).

1.8.2 Tuberculosis and HIV

According to the WHO approximately 11,5 million HIV-infected individuals worldwide were co-infected with *M. tuberculosis* by the end of 2000. Of these individuals, 70% were in sub-Saharan Africa, 20% in South-East Asia, and 4% in Latin America and the Caribbean (WHO, 2005).

HIV is fuelling the tuberculosis epidemic by increasing susceptibility to infection with *M. tuberculosis* and increasing the risk of progression of latent tuberculosis into the active progressive disease. This is due to increased immune-suppression brought on by HIV. Ninety percent (90%) of all individuals have the tubercle bacilli under control in a dormant state throughout his/her lifetime, because of an effective immune system. 5 % develop the progressive primary disease, while another 5 % develop the disease in late stages of life. This situation changes in the case of individuals co-infected with HIV, of whom 50% to 60%

will develop active tuberculosis in the course of their lifetime. *The impact of tuberculosis on HIV can be described as follows*: in an individual infected with HIV, the presence of other infections, including tuberculosis, may allow the virus to multiply more quickly. This leads to more rapid progression of HIV disease (WHO, 2005).

The principles of tuberculosis control are the same when there are many co-infected patients, but health service providers may suffer under the large and rising numbers of tuberculosis cases.

The consequences include:

- Misdiagnosis of tuberculosis cases
- Inadequate supervision of anti-tuberculosis chemotherapy
- Low cure rates
- High morbidity and mortality rates during treatment
- High rate of tuberculosis recurrence
- Increased transmission of drug-resistant strains among HIV-infected patients in congregate settings (WHO, 2005).

Treatment of HIV-patients with anti-retroviral drugs is complicated when co-infected with TB due to rifampicin's stimulation of the cytochrome P450 liver enzyme system. This system is necessary for the metabolism of non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI). This can lead to decreased blood levels of PI's and NNRTI's and consequently ineffective treatment of HIV (Gibbon, 2003).

1.8.3 Multi-drug Resistant Tuberculosis (MDR-TB)

Multi-drug resistant tuberculosis is defined as being resistant to at least rifampicin or isoniazid, the two first-line drugs in the treatment of tuberculosis. The rationale behind this definition lies in the fact that isoniazid is the most powerful mycobactericidal drug available and it ensures early sputum conversion, thereby decreasing transmission of the disease. Rifampicin is crucial for the prevention of relapses due to its mycobactericidal and sterilizing properties (Sharma & Mohan, 2004; Mitchison, 2000). Resistance to isoniazid involves mutations at either the *kat G* or *inh A* genes. Resistance to rifampicin occurs due to point mutations in the *rpo* gene in in the beta subunit of DNA-dependent RNA polymerase (Ormerod, 2005). MDR-TB holds one of the most important threats to the control of the

epidemic. At the end of 2005, about 19 000 laboratory confirmed MDR-TB cases were reported by 104 countries (WHO, 2007). According to published literature MDR-TB is a problem that was present as early as the discovery of the first drugs and worsened because of poor tuberculosis control and negligence of the disease.

Incomplete and inadequate treatment with a single drug is the most common means of acquiring drug resistance. This could have occurred because of ignorance, the use of rifampicin for other diseases or economic constraints. Another reason that is given for the emergence of drug resistance is poor patient compliance with the changeover from fully supervised sanatorium treatment to unsupervised domiciliary treatment (Sharma & Mohan, 2004). In a study conducted in South India, it was observed that only 43% of the patients receiving short-course treatment and 35% of those receiving standard chemotherapy completed 80% or more of their treatment regime (Datta *et al.*, 1993).

1.8.3.1 Treatment of multi-drug resistant tuberculosis

Treatment of MDR-TB is frequently unsuccessful, requiring the use of more toxic, expensive drugs, and/or surgery. Treatment regimes employing second-line reserve drugs as suggested by the American Thoracic Society, Centers for Disease Control and Prevention and the Infectious Diseases Society of America (ATS/CDC/IDSA) should be used in patients when MDR-TB is suspected. Where resistance to isoniazid and rifampicin (with or without resistance to streptomycin) is present during the initial phase, a combination of ethionamide, fluoroquinolone and another bacteriostatic drug such as ethambutol, pyrazinamide or an aminoglycoside are used for 3 months until sputum conversion. Ethionamide, fluoroquinolone and another bacteriostatic drug should be used for a further 18 months. Where there is resistance to isoniazid, rifampicin and ethambutol (with or without resistance to streptomycin) a combination of ethionamide, fluoroquinolone and another bacteriostatic drug such as cycloserine or para-amino salicylic acid (PAS), pyrazinamide and an aminoglycoside are suggested for 3 months. For the continuation phase a combination of ethionamide, ofloxacin and another bacteriostatic drug such as cycloserine or PAS should be used for a further 18 months (Ormerod, 2005).

Second-line drugs are difficult to obtain in small towns or rural areas making reliable supply of drugs a problem. These drugs also have a wide price range variation between different pharmaceutical brands. As tuberculosis and poverty are closely interwoven, even the cheapest brands may be too expensive for a patient. These factors make it very difficult to treat and control MDR-TB and emphasis should rather be placed on avoiding the emergence of drug resistance.

1.8.4 Extensively drug-resistant tuberculosis (XDR-TB)

XDR-TB is defined as a disease caused by a strain of *M. tuberculosis* that is resistant to isoniazid and rifampicin, plus any of the fluoroquinolones and at least one second-line injectable drug such as amikacin, capreomycin or streptomycin. The loss of the fluoroquinolones and the injectable drugs limit the treatment options to the more toxic and least potent second-line drugs. The emergence of XDR-TB in recent years threatens to exhaust all available drugs for the treatment of tuberculosis, returning the disease to the preantibiotic era. XDR-TB is documented throughout the world, with the highest incidence rates in Eastern Europe and Asia. Recent outbreaks of XDR-TB in KwaZulu Natal (RSA) were the result of a strain of highly resistant tuberculosis that was introduced into a particular vulnerable population. Patients with TB and HIV/AIDS showed excessive mortality despite the use of standard TB therapy (Gandhi *et al.*, 2006). XDR-TB is the motivation behind the research for a new drug regimen with four novel drugs.

1.9 Global efforts in the control of tuberculosis

It became evident that drastic measures should be taken to control tuberculosis. As a global movement to stop the spread of the disease, the Stop TB Partnership came to life. This campaign provides the basis for international organizations, countries, donors, governmental and non-governmental organizations, patient organizations, and individuals to contribute to a collective goal to stop TB. In order to make the most of this partnership, the Global Plan to Stop TB was developed for employment during the period of 2006-2015 (WHO, 2006).

The development of the Global Plan relied strongly on the eight working groups from the Stop TB Partnership – DOTS expansion, DOTS plus for MDR-TB, TB-HIV, new TB diagnostics, new TB drugs, new TB vaccines, advocacy, communication and social mobilization. *The working groups have also contributed to the two key dimensions of the Plan:*

- Regional scenarios which include projections of the expected impact and costs involved towards achieving the Partnership's targets for 2015 in each region, and
- The strategic plans of the working groups and the Secretariat (WHO, 2006).

An overview of the "Stop TB" strategy can be given by briefly discussing the six key components.

1. **Pursuing high quality DOTS coverage.** In order to ensure that services of the highest quality are made readily available to all those who need them, DOTS coverage to even the remotest areas are required. In 2004, 183

countries were implementing DOTS in at least part of the country. These included all 22 of the high burden countries.

2. **Addressing challenges (TB-HIV and MDR-TB).** This requires much greater action than DOTS implementation and is essential in order to achieve the targets for 2015.

3. **Contributing to the strengthening of current health systems.** National Tuberculosis Control Programmes must be able to contribute to overall strategies regarding financing, planning, management, information and supply systems, and innovative service delivery improvement.

4. **The use of all care providers.** The treatment of a TB patient relies on the care from a wide array of service providers, thereby necessitating the engagement of all types of service providers.

5. **The empowerment of TB patients and communities.** Previous community TB care projects have shown some important tasks that people can perform in tuberculosis control. A good example of this is the supervisor in the DOTS strategy.

6. **Enabling and promoting research.** The current tools are sufficient in controlling tuberculosis, but without new diagnostics, drugs and vaccines, elimination will not be possible.

By successful implementation of this strategy, it is hoped that:

- Equal access for all patients to quality TB diagnosis and treatment will be possible;
- During the next ten years, about 50 million people will be treated, including about 800 000 patients with MDR-TB and about 3 million HIV-TB co-infected patients will be enrolled on antiretroviral therapy;
- Approximately 14 million lives will be saved from 2006-2015;
- A new TB drug (the first in 40 years) will be introduced in 2010, with a new 1-2 months TB regimen shortly after 2015;
- By 2010, diagnostic tests will allow rapid, sensitive and inexpensive detection of active tuberculosis at the point of care;

- By 2015, a new, safe, effective and affordable vaccine will be available.

All of these should reverse the rise in the incidence of TB by 2015, and halve the prevalence and death rates in all regions except Africa and Eastern Europe (WHO, 2006).

1.10 Conclusion

This chapter gives an overview of tuberculosis as a disease posing a definite threat worldwide, as it is a leading cause of death. This is even more true when tuberculosis is combined with HIV-infection. Tuberculosis also has a huge socio-economic impact on a country by targeting the most productive age groups.

It is suggested that poor patient compliance exists because of the side effects of the medicines available. Patients tend to stop their treatment as soon as they feel better. Poor patient compliance led to the emergence of drug resistant strains that are extremely difficult to treat, as it requires more toxic and more expensive drugs.

Fixed-dose combination products were discovered to be successful in the treatment of tuberculosis and multi-drug resistant tuberculosis, and relieved poor patient compliance. Problems still exist with the duration of treatment. The world is in desperate need of a shorter treatment regimen for tuberculosis with fewer side effects.

This review has revealed the most important aspects of the disease, as well as the extent of the disease as a global health problem. In the next chapter, the first-line drugs used in the treatment of tuberculosis will be discussed, leading to a better understanding of recent trends in treatment regimens as well as possible shortcomings.

2 Introduction

Treating tuberculosis with antimicrobial agents is troublesome for several reasons. The mycobacterial cell wall is nearly impermeable due to the mycolic acid component, which consequently increases resistance to the drug. Antimicrobial therapy must continue for months to years due to the slow growth rate of *M.tuberculosis.* Combination therapy must be used to prevent the emergence of drug-resistant strains. The ability of the active pharmaceutical ingredient to enter human cells poses a further challenge as *M. tuberculosis* is an intracellular pathogen (Ingraham & Ingraham, 1995).

Fixed dose combination (FDC) products containing anti-tuberculosis drugs were introduced to the market as a means to simplify treatment and increase patient compliance (Blomberg *et al.*, 2001). Numerous cases of poor bioavailability have been reported for rifampicin in FDC products. Various reasons for the poor bioavailability include raw material characteristics, changes in the crystalline forms of rifampicin, degradation in the gastro-intestinal tract and inherent variability in absorption and metabolism (Panchagnula & Agrawal, 2004). The physical and chemical properties of the current first-line drugs against *M. tuberculosis* were investigated in this study. This chapter gives an overview of the characteristics that were important for the study. Special reference was made to the instability of these active compounds within formulations as well as the *in vivo* interaction existing between isoniazid and rifampicin.

2.1 The history of tuberculosis chemotherapy

Treatment of tuberculosis with chemotherapeutic agents had its beginning with the discovery of streptomycin in 1944. Streptomycin was used as monotherapy until clinical studies revealed that resistance to this agent increased readily. This problem was solved by combination therapy with other agents. The basis for initial chemotherapy consisted of isoniazid, streptomycin and para-aminosalicylic acid (PAS). The duration of therapy was 12-18 months or more. More problems were encountered because of the difficulty in ensuring patient adherence to such a lengthy course of therapy with painful injections and many side-effects. Later on, ethambutol began to replace PAS due to increased efficacy and the lack of distressing gastro-intestinal irritation associated with PAS. An interesting fact is that current first-line drugs such as pyrazinamide and rifampicin were considered more toxic than isoniazid combined with streptomycin and PAS (Holvey, 1972).

By 1992, PAS was completely removed from first-line treatment regimes. The modern chemotherapeutic era began with the introduction of rifampicin into the first-line treatment of tuberculosis. Treatment duration was shortened from 18 months to 6 months. Therapy was no longer aimed at just curing the patients and preventing relapse, but also to render patients non-infectious as quickly as possible (Onyebujoh *et al.*, 2005). Anti-TB agents was therefore carefully selected to instantly kill active metabolizing bacilli, to kill near-dormant bacilli and to destroy slow-replicating bacilli found in acidic and anoxic closed lesions. This profile was best fitted by a regimen of isoniazid, rifampicin, pyrazinamide with either streptomycin or ethambutol for the first two months (Table 2.1), followed by rifampicin and isoniazid for an additional four months. Patients infected with HIV received more intensive treatment for a period of 9 months. This regime consisted of 2 months with isoniazid and rifampicin followed by a further 7 months with isoniazid and rifampicin. Ethambutol was added to the regime if bacterial resistance to isoniazid was suspected (Mandel & Sande, 1992).

Fixed-dose combination (FDC) products became superior over monotherapy in an attempt to simplify treatment and facilitate patient compliance. The administration of drugs under direct observation, became a concept around which the World Health Organization built its Directly Observed Therapy Short Course (DOTS) strategy. DOTS has been shown to achieve high cure rates when it is applied rigidly (Frieden & Munsiff, 2005). However, the possibility of DOTS failing does exist due to the use of inferior medicines. Poor quality relates to the rifampicin bioavailability in FDC tablets.

In South Africa, a 4-drug FDC (Rifafour e-200®) had been placed on the essential drug list in 1998 as the only option for intensive phase treatment of tuberculosis (table 2.2). By this time, the use of streptomycin was reserved for resistant strains of *M. Tuberculosis* (Mitchison, 2000).

Current tuberculosis chemotherapy in South Africa includes a two months intensive phase with Rifafour e-275® and 4 months with a 2-drug FDC containing isoniazid and rifampicin. Although 2-drug FDCs (containing isoniazid and rifampicin) are not generally available, it is used in South Africa during the continuation phase (see table 2.3). The main difference between Rifafour e-200® and Rifafour e-275® includes an increase in the rifampicin concentration. Dosages of anti-tuberculosis medications are based on body weight. Thus, dosages should be continuously revised as a patient's weight changes with time. Adults are divided into four weight categories. The dosages of tuberculosis medications corresponding to these weights, while minimizing toxicity, have been carefully calculated based on multiple clinical trials. Therapeutic dosages currently used are given in table 2.4.

Table 2.1: Tuberculosis treatment regimen (early 1990's) (Mandel & Sande, 1992).

Initial Intensive Phase					**Continuation Phase**
Daily during month 1 and 2					**3 times a week for 4 months**
Pretreatment weight	R, H	Z	S or E		R, H
	H = 100 mg, R = 150 mg (combined tablet)	Z = 500 mg (tablet)	S (injection)	E = 400 mg (tablet)	H = 100 mg, R = 150 mg (combined tablet) + H = 300 mg (tablet)
Less than 33 kg	2	2	500 mg	2	2 HR + 1 H
33 kg - 50 kg	3	3	750 mg	2	3 HR + 1 H
51 kg or more	4	4	1000 mg	3	4 HR + 1 H

Abbreviations:

HR = isoniazid + rifampicin
E = ethambutol
Z = pyrazinamide
H =isoniazid
R = rifampicin
S = streptomycin

Table 2.2: Treatment of tuberculosis (For adults in 1998) (SADOH, 1998).

Initial Intensive Phase		**Continuation Phase**	
Daily during month 1 and 2		**5 times a week for 4 months**	
Pretreatment weight	R, H, Z & E	R, H	R, H
	H = 60 mg, R = 120 mg, Z = 300 mg, E = 200 mg (combined tablet)	R = 150 mg, H = 100 mg (combination tablet	H = 150 mg, R = 300 mg (combination tablet)
≤50 kg	4	3	
≥50 kg	5		2

Abbreviations:

HR = isoniazid + rifampicin
E = ethambutol
Z = pyrazinamide
H =isoniazid
R = rifampicin

Table 2.3: Current tuberculosis treatment regimen in South Africa (SADOH, 2006).

Initial Intensive Phase		**Continuation Phase**			
Daily during month 1 and 2		**5 times a week for 4 months**		**3 times a week for 4 months**	
Pretreatment weight	R, H, Z & E	R, H	R, H	R, H	R, H
	RHZE (150, 75, 400, 275) (combination tablet)	RH (150, 75) (combination tablet)	RH (300, 150) (combination tablet)	RH (150, 150) (combination tablet)	RH (300, 150) (combination tablet)
30 - 37 kg	2	2		2	
38 - 54 kg	3	3		3	
55 - 70 kg	4		2		3
≥71 kg	5		2		3

Abbreviations:

HR = isoniazid + rifampicin
E = ethambutol
Z = pyrazinamide
H =isoniazid
R = rifampicin

Table 2.4 Current therapeutic dosage ranges of first-line anti-tuberculosis agents (SADOH, 2006).

Drugs	**Dosage**					
	Daily (mg/kg)		**2 times/week (mg/kg)**		**3 times/week (mg/kg)**	
	children	**Adults**	**children**	**adults**	**children**	**Adults**
INH	10-20 max 300 mg	5 max 300 mg	20-40 max 900 mg	15 max 900 mg	20-40 max 900 mg	15 max 900 mg
RIF	10-20 max 600 mg	10 max 750 mg	10-20 max 600 mg	10 max 750 mg	10-20 max 600 mg	10 max 750 mg
PZA	15-30 max 2 g	15-30 max 2 g	50-70 max 4 g	50-70 max 4 g	50-70 max 3 g	50-70 max 3 g
EMB	15-25 max 2.5 g	15-25 max 2.5 g	50 max 2.5 g	50 max 2.5 g	25-30 max 2.5 g	25-30 max 2.5 g

2.2 The DOTS-strategy

With the realization that compliance is critical to successful chemotherapy, DOTS emerged as a means of ensuring compliance through directly observing the ingestion of every dose by someone other than the patient. DOTS comprises that the health worker and the patient select a supervisor who will safeguard the supply of medicine, the patient's treatment chart and will observe the patient as s(he) takes the medication, and record this in the appropriate manner. The supervisor need not be a health worker, but can for example be a family member or even the local shopkeeper. The important criterion is accessibility of the supervisor to the patient. Health care workers are responsible for regular visits to the supervisor to provide the necessary support and to check the patients' treatment charts (Fanning, 1999).

The DOTS-strategy consists of five components: political commitment to support the treatment of TB, the passive detection of active tuberculosis by the use of sputum microscopy, direct observation of the short-course therapy, ensuring a regular supply of medicines, and the reporting of treatment outcomes and program performance (Fanning, 1999).

By the end of 2004, 83% of the world's population lived in DOTS-covered countries. These programs succeeded in notifying 4.4 million new and relapsed TB cases. In total, 21.5 million patients, and 10.7 million AFB smear-positive patients were treated in DOTS programs over the 10-year period from 1995-2004 (Sharma & Liu, 2006).

2.3 DOTS-plus strategy

Based upon DOTS, DOTS-plus is a comprehensive management strategy currently under development and includes the five tenets of the DOTS strategy. DOTS-plus is especially concerned with the use of second-line drugs in areas with high prevalence of MDR-TB. These drugs should be stored and dispensed at specialized health centers with the appropriate facilities and well-trained staff. DOTS-plus can therefore be seen as a supplement to the standard DOTS strategy (The stop TB partnership, 2006).

2.4 The challenges of global drug research and development

The goal of tuberculosis research is the discovery and implementation of a simpler, safer and/or shorter multidrug regimen. Beyond the development of new compounds, several challenges need to be addressed. The primary challenge is to create a foolproof system for the identification of the best possible combination regimen worthwhile of clinical testing. The second major challenge will be to identify new potential drug targets for persisting microorganisms (WHO, 2005).

2.5 Fixed-dose combination products

The justification for the recommendation of FDC tablets to replace single-drug tablets was set out by the WHO as follows:

- FDCs prevent monotherapy and therefore reduce the risk for the emergence of drug-resistant tuberculosis.
- FDCs simplify treatment, minimize prescription errors and increase patient and doctor compliance.
- FDCs minimize out-of-stock situations; reduce strain on shipping and distribution.
- FDCs reduce the risk of misuse of rifampicin for other health conditions (WHO, 1999).

Concern has been expressed that FDC tablets might be seen as an opportunity to undermine the DOTS strategy by encouraging self-supervision. If FDC tablets are given unsupervised, patients can interrupt treatment repeatedly, and this scenario may lead to the emergence of drug resistance. The WHO therefore emphasizes the need for supervision in the treatment of tuberculosis (WHO, 1999).

The major challenge in using FDC tablets is to ensure that FDC tablets of sufficient quality are used. The two major problems associated with quality of FDCs are loss of bioavailability of rifampicin upon administration and instability of drugs within the formulation environment (Singh *et al.*, 2001).

2.6 Role of individual drugs in current treatment regimens

As mentioned before, the treatment of tuberculosis is divided into two phases. The initial or bactericidal phase usually lasts for 2 months but officially ends when a patient's sputum is smear and culture negative. The second phase (4 months) is known as the continuation or sterilizing phase. These two phases should be kept apart since each of them entails a different approach. Four populations of tubercle bacilli have been described based on their location or metabolic activity, namely extracellular (in the cavity walls), intracellular (macrophage), semi-dormant (caseum) and dormant. Metabolic activity slows down in order from extracellular (highest) to dormant (lowest). Isoniazid rapidly destroys extracellular bacilli, while pyrazinamide is highly effective against the intracellular bacilli. Thus, it justifies these drugs' inclusion during the initial phase. Ethambutol is included in the initial phase because it is bacteriostatic to the slow-growing mycobacteria. Patients are usually relieved of all symptoms once the initial phase ends, but semi-dormant bacilli are still present. Rifampicin is the key drug here since it possesses high activity against all the tubercle populations,

excluding dormant bacilli. Not one of the current first-line drugs show activity against dormant bacilli. The dormant bacilli are responsible for relapses in immunocompetent patients when they periodically become active. If one were able to kill all populations right from the start of treatment, tuberculosis could be cured within two months (Mitchison, 2000).

2.7 Shortcomings of existing first-line drugs

Rifampicin induces cytochrome P450 enzymes and has a significant impact on the metabolism of antiretroviral agents. This makes it especially troublesome to treat tuberculosis patients co-infected with HIV. Ethambutol is associated with red/green visual disturbances, and this drug adds little to the potency of the current regimen. Isoniazid may lead to a decrease in the efficacy of other first-line drugs in the regimen, is widely associated with a high prevalence of drug resistance and does not contribute to treatment shortening. Pyrazinamide exhibits activity only during the first two months of treatment (WHO, 2005).

2.8 Molecular structures of active pharmaceutical ingredients (APIs)

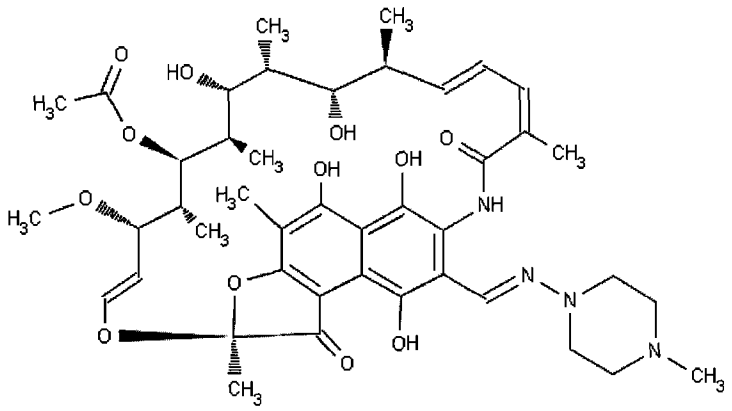

Figure 2.1: Rifampicin

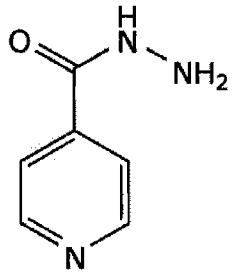

Figure 2.2: Isoniazid

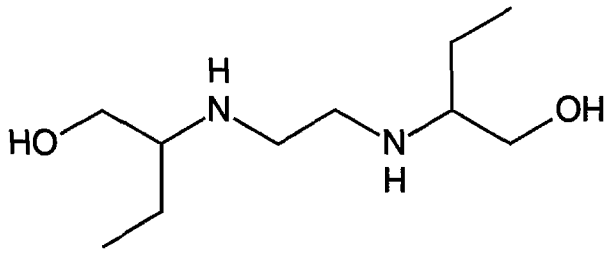

Figure 2.3: Ethambutol

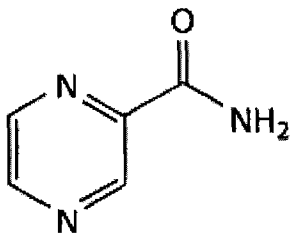

Figure 2.4: Pyrazinamide

A compound can only be optically active if it is chiral and all chiral compounds are inevitably optically active. According to Gallo & Radaelli (1976), rifampicin and ethambutol do have optical rotation and therefore are intrinsically chiral compounds. Conversely, isoniazid and pyrazinamide are non-chiral compounds.

2.9 Physical and chemical properties of active pharmaceutical ingredients (APIs)

2.9.1 Physical appearance

A thorough description of each active drug compound was obtained from the literature and used in this study to determine any physical changes that may occur during stability testing.

2.9.1.1 Rifampicin

Rifampicin is a red-orange to reddish-brown or brick red, odourless crystalline powder (Reynolds, 1989).

2.9.1.2 Isoniazid

Isoniazid is a white or colourless, odourless crystalline powder that tastes slightly sweet at first, which is followed by a bitter taste (Reynolds, 1989).

2.9.1.3 Ethambutol

Ethambutol is a white, thermo stable, crystalline powder that is odourless and bitter tasting (Reynolds, 1989).

2.9.1.4 Pyrazinamide

Pyrazinamide is a white, odourless crystalline powder (Reynolds, 1989).

2.9.2 Solubility

Solubility refers to the amount of solute that can be dissolved in a particular solvent. This value is of importance when formulating a pharmaceutical product using the Pheroid™ drug delivery system that has both an oil and water component. It can also be used to predict the intestinal absorption of a drug by making use of the Biopharmaceutics Classification System (BCS). The BCS provides a classification of drug substances into one of four classes based on its aqueous solubility and intestinal permeability. According to Yu *et al.* (2002), a drug is considered highly soluble when the highest strength is soluble in ≤ 250 ml (equivalent to a glass of water) of aqueous media over the pH range of 1.0-7.5. A drug is further considered highly permeable when the extent of intestinal absorption is determined to be greater than 90%.

The BCS consists of the following classes (Yu *et al.*, 2002):

- Class I – High permeability, high solubility.

  The compounds in this class show greater absorption rates than excretion rates.

- Class II – High permeability, low solubility

  These compounds' bioavailability is limited by their solvation rate.

- Class III – Low permeability, high solubility

  These compounds' absorption is limited by its permeability, but it does go into solution very fast.

- Class IV – Low permeability, low solubility

  These compounds show little bioavailability. High variability can be expected as they are usually not well absorbed over the intestinal mucosa.

2.9.2.1 Rifampicin

Rifampicin is a BCS class II compound and it is regarded as a slightly soluble molecule with high intestinal permeability (Pachagnula & Agrawal, 2004). Rifampicin is slightly soluble in water, freely soluble in chloroform and soluble in methanol.

The solubility of rifampicin increases at low (acidic) pH, but aqueous solutions are relatively unstable in the same pH range (Reynolds, 1989).

2.9.2.2 Isoniazid

Isoniazid is a BCS class I compound and it is regarded as a highly soluble molecule with high intestinal permeability (Pachagnula & Agrawal, 2004). Isoniazid is freely soluble in water, soluble in alcohol and slightly soluble in chloroform (Reynolds, 1989).

2.9.2.3 Ethambutol

Ethambutol is a BCS class I compound and it is regarded as a highly soluble molecule with high intestinal permeability (Pachagnula & Agrawal, 2004). Ethambutol is freely soluble in water and alcohol, slightly soluble in chloroform and practically insoluble in ether (Reynolds, 1989).

2.9.2.4 Pyrazinamide

Pyrazinamide is a BCS class I compound and it is regarded as a highly soluble molecule with high intestinal permeability (Pachagnula & Agrawal, 2004). Pyrazinamide is soluble in water, slightly soluble in alcohol and very slightly soluble in ether (Reynolds, 1989).

The physicochemical properties of the four API's are summarized in table 2.5.

Table 2.5: Summary of physicochemical properties of APIs

Description	Drug			
	Rifampicin	Isoniazid	Pyrazinamide	Ethambutol
Molecular formula	$C_{43}H_{58}N_4O_2$[1]	$C_6H_7N_3O$[2]	$C_5H_5N_3O$[3]	$C_{10}H_{24}O_2N_2$
Molecular weight	822.94 g/mol[1]	137.139 g/mol[2]	123.113 g/mol[3]	204.31 g/mol[4]
Physical appearrance	Rifampicin is a red-orange to brick red, odourless crystalline powder.[1]	Isoniazid is a white or colourless crystalline powder that is virtually odourless. When tasted, it at first has a slightly sweet and then a bitter taste.[2]	Pyrazinamide is a white crystalline powder.[3]	Ethambutol is a white, thermostable, crystalline powder which is odourless and bitter tasting.[4]
H2O Solubility	Slightly soluble[1]	Freely soluble[2]	Soluble[3]	Freely soluble[4]
Log P/Hydrophobicity	3.719 [1]	-0.64	-1.884	-0.14
Melting point	183°C[1]	171.4°C[2]	192°C[3]	88°C[4]
Pharmacology	Rifampicin is bactericidal with a broad spectrum of activity against most gram-positive and gram-negative organisms. Use is restricted to treatment of mycobacterial infections together with a few other indications because of the rapid emergence of resistant bacilli.[1,6]	Isoniazid is bactericidal against all rapidly-dividing mycobacteria and bacteriostatic if the mycobacterium is slow-growing. It is a highly specific agent, ineffective against other microorganisms.[2,6]	Pyrazinamide is active only against mycobacterium tuberculosis. This drug is only active in a slightly acidic pH.[3,6]	Ethambutol is specifically effective against actively growing mycobacterium bacilli. Inhibits RNA synthesis and decreases bacilli replication.[4,6]

Table 2.5...continued: Summary of physicochemical properties of APIs

Description	Drug			
	Rifampicin	Isoniazid	Pyrazinamide	Ethambutol
Mechanism of action	Rifampicin inhibits DNA-dependant RNA polymerase, leading to a suppression of RNA synthesis and cell death.[1,6]	Isoniazid is in fact a prodrug which is activated by bacterial catalase enzymes. Once activated, it inhibits the synthesis of mycolic acids, an essential component of the bacterial cell wall.[2,6]	It is written that pyrazinoic acid, pyrazinamide's active moiety,disrupts membrane energetics and inhibits membrane transport function at acid pH.[3,6]	Acts by inhibiting arabinosyl transferases involved in cell wall biosynthesis.[4,6]
Absorption	Rifampicin is well absorbed from the gastrointestinal tract. Variable absorption has been reported between individuals.[1,5,6]	Readily absorbed from the gastrointestinal tract, but undergo significant first pass metabolism. Food impair the bioavailibilty of isoniazid.[2,6]	Rapidly and well absorbed from the gastrointestinal tract.[3,6]	Up to 80% of the administered dose are absorbed from the gastrointestinal tract.[4,6]
Protein Binding	20 -30%[1]	Very low (less than 10%)[2]	~10% bound to plasma proteins[3]	89%[4]
Half Life	3-4 hours[1]	Fast acetylators: 0.5 to 1.6 hours Slow acetylators: 2 to 5 hours[2]	9-10 hours in normal conditions[3,6]	3 to 4 hours in patients with normal renal function and up to 8 hours in patients with impaired renal function[4,6]
Biotransformation	Primarily hepatic, rapidly deacetylated.[1,6]	Primarily hepatic. Isoniazid is acetylated by N-acetyltransferase to *N-acetylisoniazid. It is further* biotransformed to isonicotinic acid and monoacetylhydrazine; the latter is associated with hepatotoxicity via the formation of a reactive intermediate metabolite when N-hydroxylated by the cytochrome P450 oxidase system.[2,6]	Hepatic[3,6]	Mainly hepatic. Initial oxidation of the alcohol to an aldehydic intermediate, followed by conversion to a dicarboxylic acid.[4]

Table 2.5...continued: Summary of physicochemical properties of APIs

Description	Drug			
	Rifampicin	Isoniazid	Pyrazinamide	Ethambutol
Toxicity	LD50 = 1570 mg/kg (rats). Chronic exposure may cause nausea and vomiting and unconsciousness.[1]	LD50 = 100 mg/kg (human). Adverse reactions include rash, abnormal liver function tests, hepatitis, peripheral neuropathy and mild central nervous system effects.[2]	Side effects include liver injury, arthralgias, anorexia, nausea and vomiting, dysuria, malaise and fever, adverse effects on the blood clotting mechanism or vascular integrity as well as pruritis and skin rashes.[3]	The most prevalent toxic effect is optic neuropathy, especially red/green colour-blindness. This is completely reversible. Other toxic effects that have been observed are pruritis, joint pain, gastrointestinal upset, abdominal pain, malaise, headache, dizziness and possible hallucinations.[4,6]

References:

1. DRUGBANK. 2007. Rifampicin. [Web:] http://redpoll.pharmacy.ualberta.ca/drugbank/cgi-bin/getCard.cgi?CARD=EXPT02777.txt [Date of access: 30 October 2007].

2. DRUGBANK. 2007. Isoniazid. [Web:] http://redpoll.pharmacy.ualberta.ca/drugbank/cgi-bin/getCard.cgi?CARD=APRD01055.txt [Date of access: 30 October 2007]

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2.10 Uses of APIs

2.10.1 Indications

2.10.1.1 Rifampicin

Rifampicin is primarily indicated for the treatment of pulmonary and extra pulmonary tuberculosis and leprosy. Rifampicin is further indicated for tuberculosis prophylaxis if isoniazid is contraindicated in a patient. It is useful for elimination of *Neisseria Meningococci* in carriers and for gram positive and negative bacteria. It has some anti-chlamydial activity and *in vitro* activity against the poxvirus and adenovirus when given in high dosages (Chambers, 2001).

2.10.1.2 Isoniazid

Isoniazid is an antimycobacterial agent that is bactericidal for both extracellular- and intracellular organisms. It is a first-line drug in the treatment of tuberculosis when the disease is caused by isoniazid-sensitive strains (Chambers, 2001). Isoniazid may also be used for tuberculosis prophylaxis.

2.10.1.3 Ethambutol

Ethambutol is indicated for tuberculosis in conjunction with at least one other antituberculosis drug. Higher dosages can be used in the treatment of tuberculous meningitis (Chambers, 2001).

2.10.1.4 Pyrazinamide

Pyrazinamide is an important drug when used in combination with isoniazid and rifampicin. It is mainly used as a sterilizing agent active against residual intracellular organisms that might cause relapse. Pyrazinamide may also be used for prevention of active tuberculosis in close contacts when given in combination with either ciprofloxacin or ofloxacin (Chambers, 2001).

2.10.2 Current therapeutic dosages

The currently used dosages are summarized in table 2.4 and expanded below.

2.10.2.1 Rifampicin

For the treatment of tuberculosis, a dosage of 8-12 mg/kg ought to be given as a single daily dose (maximum 750 mg). To be taken orally in combination with other antimycobacterial agents OR 8-12 mg/kg when 3 times weekly (maximum 900mg) (Blomberg *et al.*, 2001).

2.10.2.2 Isoniazid

Tuberculosis – A usual adult dose of isoniazid is a single daily dose of 4-6 mg/kg up to a maximum of 300 mg daily (Blomberg *et al.*, 2001).

2.10.2.3 Ethambutol

Tuberculosis – 15-20 mg/kg in the initial phase as a single daily dose; or 25 to 35 mg/kg three times per week (Blomberg *et al.*, 2001).

2.10.2.4 Pyrazinamide

Tuberculosis – 20 to 30 mg/kg/day as a single dose with a maximum of 2 grams per day; or 30 to 40 mg/kg 3 times a week (Blomberg *et al.*, 2001).

2.11 Kinetics of APIs

2.11.1 Absorption and distribution

2.11.1.1 Rifampicin

Rifampicin is readily absorbed from the gastrointestinal tract. Peak plasma levels of about 7-9 µm/ml are reached after 1.5 to 4 hours after an oral dose of about 600 mg. Food may reduce or delay absorption (Reynolds, 1989; Chambers, 2001). In a study done by McIlleron *et al.* (2006), wide variations in the plasma concentrations of rifampicin occurred in infected patients. C_{max} values ranged from 1.3 – 14.9 µg/ml. About 69% of patients had a C_{max} below 8 – 24 µg/ml (McIlleron *et al.*, 2006).

Rifampicin is lipid soluble and is widely distributed in body tissues and fluids. Only about 20 - 30% of rifampicin in circulation is bound to plasma proteins (Reynolds, 1989; Chambers, 2001).

2.11.1.2 Isoniazid

Isoniazid is readily and almost completely (90-95%) absorbed from the gastrointestinal tract. In healthy individuals, peak plasma concentrations of 3-5 µg/ml are reached within 1 to 2 hours after a dose of 300 mg (5 mg/kg in children). According to McIlleron *et al.* (2006) the peak plasma levels of isoniazid was not significantly affected by the physical state of the patient. The high variability could be due to different acetylation rates between patients. Only a 10% fraction of isoniazid is bound to plasma proteins. The unbound fraction (90%) diffuses into all body tissues and fluids. The concomitant administration of isoniazid with food is known to impair absorption of the drug (Reynolds, 1989; Chambers, 2001).

2.11.1.3 Ethambutol

Following administration of a single oral dose of 25 mg/kg to healthy individuals, ethambutol hydrochloride attains peak plasma level of 2 to 5 µg/ml within 4 hours. McIlleron *et al.* (2006) found that only 2% of the infected study population had peak plasma levels of below 2 µg/ml. Thus, the physical state of individuals does not have a signifant impact on the absorption of ethambutol. However, female patients and patients co-infected with HIV had lower plasma levels of ethambutol than male patients and patients infected with tuberculosis only (McIlleron *et al.*, 2006). About 80% of the oral dose is absorbed from the gastrointestinal tract, and the rest appears in the faeces unchanged (Reynolds, 1989; Chambers, 2001).

Protein binding is 89% and absorption is not significantly impaired by food (Reynolds, 1989).

Ethambutol diffuses into red blood cells and into the cerebrospinal fluid when the meninges are inflamed. The concentration in the red blood cells is approximately twice the plasma concentration (Reynolds, 1989).

2.11.1.4 Pyrazinamide

Pyrazinamide is rapidly absorbed from the gastrointestinal tract following administration. Peak serum levels occur after 2 hours. Pyrazinamide displayed less variability in peak plasma levels in infected individuals than the other first-line drugs. Only one patient in the McIlleron study had a C_{max} of less than 20 µg/ml. This is the lower limit of the reference range for plasma concentration of pyrazinamide (McIlleron *et al.*, 2006). Pyrazinamide is widely distributed in the tissues. It penetrates inflamed meninges to reach peak levels in the cerebrospinal fluid (Chambers, 2001).

2.11.2 Metabolism and elimination

2.11.2.1 Rifampicin

Almost the complete oral dose (85%) of rifampicin is rapidly deacetylated in the liver by the microsomal enzymes to its main and active metabolite – desacetylrifampicin. Except for this metabolite, rifampicin will undergo enterohepatic recirculation. Desacetylrifampicin is eliminated through bile and urine. The amount excreted in the urine is concentration-dependent and up to 30% of a dose of 900 mg may be excreted in the urine (Reynolds, 1989).

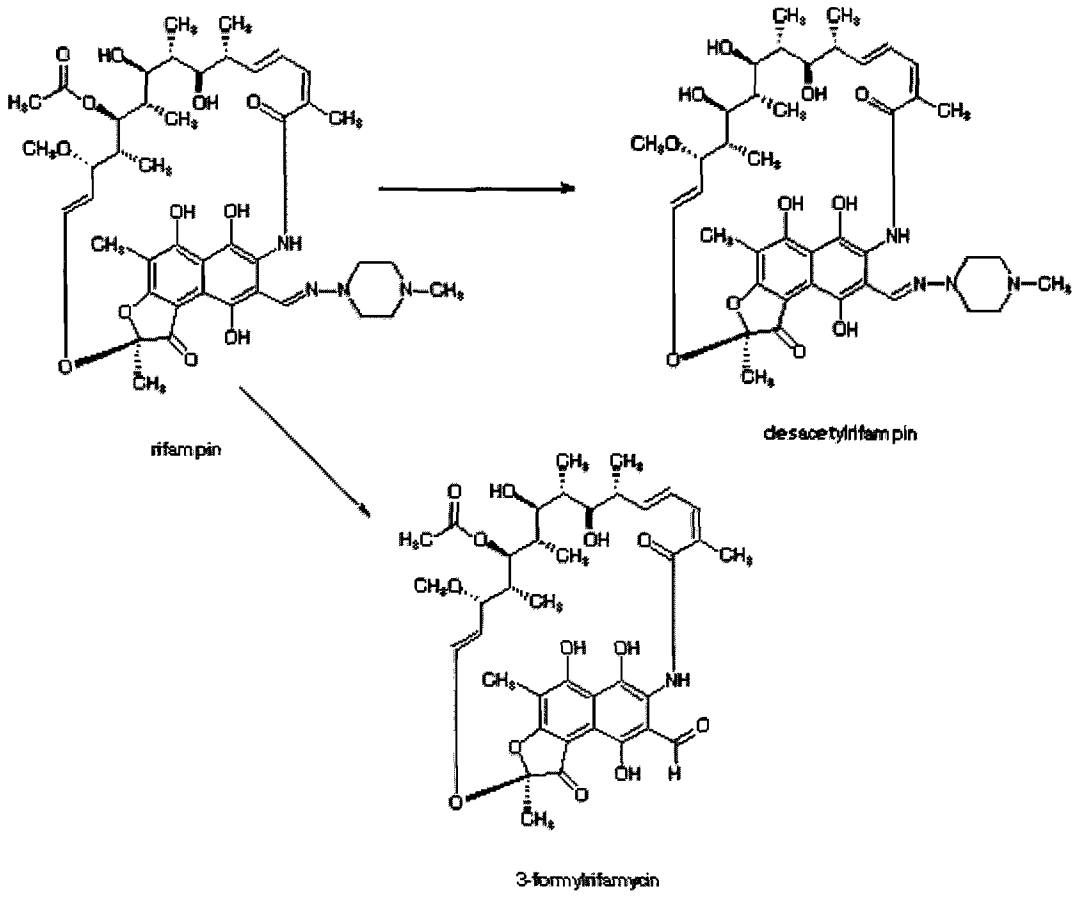

Figure 2.5: Proposed mechanism for the decomposition of rifampicin upon administration.

2.11.2.2 Isoniazid

The major route of isoniazid metabolism is acetylation by N-acetyl transferase which produces acetylisoniazid. Acetylisoniazid can be further hydrolyzed to isonicotinic acid and acetylhydrazine, both of which are excreted in the urine. Acetylhydrazine is further metabolized to diacetylhydrazine that are converted to the reactive metabolite. This metabolite is thought to be responsible for isoniazid-induced hepatotoxicity (Reynolds, 1989). Figure 2.6 is a schematic example of this process:

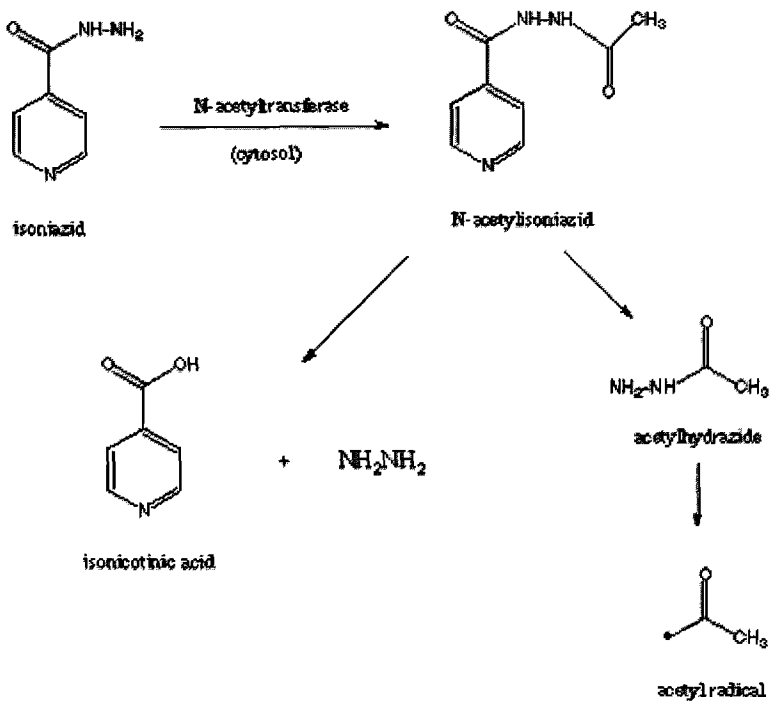

Figure 2.6: Decomposition of isoniazid upon administration.

Humans can be divided into three categories based on their rate of acetylation namely slow, normal and fast acetylators. As the main decomposition reaction is acetylation, slow acetylators have higher serum levels of isoniazid at a given dose. Conversely, fast acetylators have lower plasma levels at a given dose. Six hours after ingestion of 4 mg/kg isoniazid, plasma concentrations are 0.8 µg/ml in slow acetylators and only 0.2 µg/ml in fast acetylators. The rate of acetylation does not appear to alter efficacy when the drug is administered daily or 2 to 3 times per week as in the case of the current regimes (Reynolds, 1989).

In adults with normal renal function, 50 to 70% of the entire dose of isoniazid is excreted in the urine within 24 hours as intact drug and as metabolites. This excretion rate is also determined by the acetylator phenotype (Reynolds, 1989).

2.11.2.3 Ethambutol

The main path of metabolism is an initial oxidation reaction of the alcohol to an aldehyde intermediate, followed by a conversion to a dicarboxylic acid. The enzyme responsible for the metabolism of ethambutol is alcohol dehydrogenase (Reynolds, 1989).

After 24 hours, approximately 50% of the initial dose is excreted unchanged in the urine, while an additional 8 to 15% appears in the form of metabolites. From 20% of the initial dose is excreted in the faeces as unchanged drug (Reynolds, 1989).

2.11.2.4 Pyrazinamide

Pyrazinamide is hydrolyzed to pyrazinoic acid, a major active metabolite, in the liver. Pyrazinoic acid is further hydroxylated to the main excretory metabolite. Pyrazinamide as well as its metabolites are excreted mainly via glomular filtration in the urine (Reynolds, 1989).

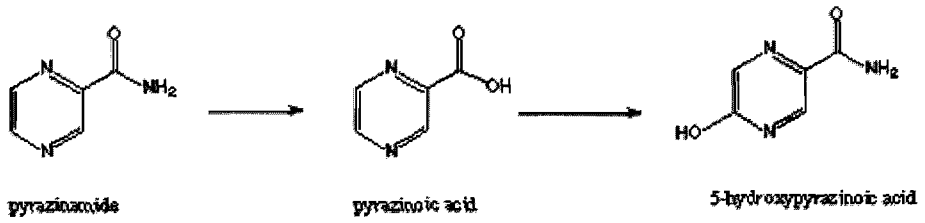

Figure 2.7: Decomposition of pyrazinamide upon administration.

2.12 Stability

2.12.1 Rifampicin

Rifampicin is very stable as a powder when stored in sealed containers at room temperature. Rifampicin in the solid state can withstand temperatures up to 70 °C. The stability of rifampicin in aqueous solution has been widely investigated and the conditions and transformation products are summarized in table 2.6. Rifampicin is subjected to desacetylation on alkaline treatment, yielding 25-desacetylrifampicin without substantial loss of antibacterial activity. In the presence of atmospheric oxygen, mildly alkaline solutions at room temperature transform it into rifampin quinone. This oxidation reaction can be prevented by adding sodium ascorbate or ascorbic acid. Under the same conditions, but at 60-70 °C, rifampicin yields three main transformation products, which were identified as 25-desacetyl-rifampicin, 25-desacetyl-23-acetyl-rifampicin and 25-desacetyl-21-acetyl-rifampicin (Gallo & Radaelli, 1976).

In acidic aqueous solutions, the main decomposition product of rifampicin is 3-formylrifamycin SV (Gallo & Radaelli, 1976).

2.12.2 Isoniazid

The stability of isoniazid has been studied in solution and in various pharmaceutical preparations. The reactions of the hydrazine group with naturally occurring ketones and aldehydes such as sugars or ketoacids to form hydrazones are of most interest. Isoniazid also forms complexes with metal ions. It was further reported that isoniazid is unstable in human or rabbit plasma, but stable for weeks in buffered aqueous solutions at pH values below 8 (Reynolds, 1989).

Alkaline hydrolysis under aerobic conditions yields isonicotinic acid, isonicotinamide and 1,2 diisonicotinoyl hydrazine. Under anaerobic conditions the principle products were isonicotinic acid and 1,2 diisonicotinoyl hydrazine (Reynolds, 1989).

At pH 3.1 and under anaerobic conditions isoniazid was found to form isonicotinic acid. At lower pH values the effect of the buffer type can be seen. Losses of isoniazid could be seen when the drug was blended with various antacid preparations (Reynolds, 1989).

Reports were given that isoniazid underwent slow oxidation in aqueous solution, but in the presence of sucrose, the isoniazid reacted with the aldohexoses formed on inversion. The sucrose reaction can be inhibited with 0.3% sodium citrate. The use of sorbitol as a replacement for sucrose is advised (Reynolds, 1989).

2.12.3 Ethambutol

Ethambutol is hygroscopic and may influence the stability of other drugs in FDC products (Singh *et al.*, 2001). However, there is no literature available that report on instability of ethambutol. Ethambutol is considered as a stable compound.

2.12.4 Pyrazinamide

Pyrazinamide is considered a stable molecule (Reynolds, 1989).

A summary of the decomposition products at a variety of conditions is given in table 2.6 (Gallo & Radaelli, 1976).

2.13 Drug decomposition in the stomach (*in situ*)

A series of studies brought forward results indicating that while some formulations had acceptable rifampicin bioavailability, others did not. In both cases, the problem has been ascribed to the rapid decomposition of rifampicin in the presence of isoniazid *in situ* in stomach acidic conditions (Singh *et al.*, 2001). A reaction pathway explaining the mechanism for this increased decomposition of rifampicin in the presence of isoniazid was postulated (Fig. 2.8).

Table 2.6: Decomposition products of first-line anti-tuberculosis drugs.

Drug	Conditions	Decomposition products
Rifampicin	pH 2-3, 20-22 °C	3-Formyl rifamycin SV
	pH 8.2, 20-22 °C	Rifampicin quinine
	pH 8.2, 60-70 °C	25-desacetyl rifampicin 25-desacetyl-21-acetyl rifampicin 25-desacetyl-23-acetyl rifampicin
	NaOH 5% in ethanol-water (1:1), 20-22 °C	25-desacetyl rifampicin
Isoniazid	pH 3.1, anaerobic	Isonicotinic acid
	Alkaline, aerobic	Isonicotinic acid Isonicotinamide 1,2-diisonicoyl hydrazine Isonicotinamide
	Alkaline, anaerobic	Isonicotinic acid Isonicotinamide 1,2-diisonicoyl hydrazine
	Alkaline, anaerobic with EDTA	Isonicotinic acid
Pyrazinamide	Wet or dry atmosphere at solid state	Stable
	Natural day light	Stable
	Autoclaving of intravenous infusions	Stable

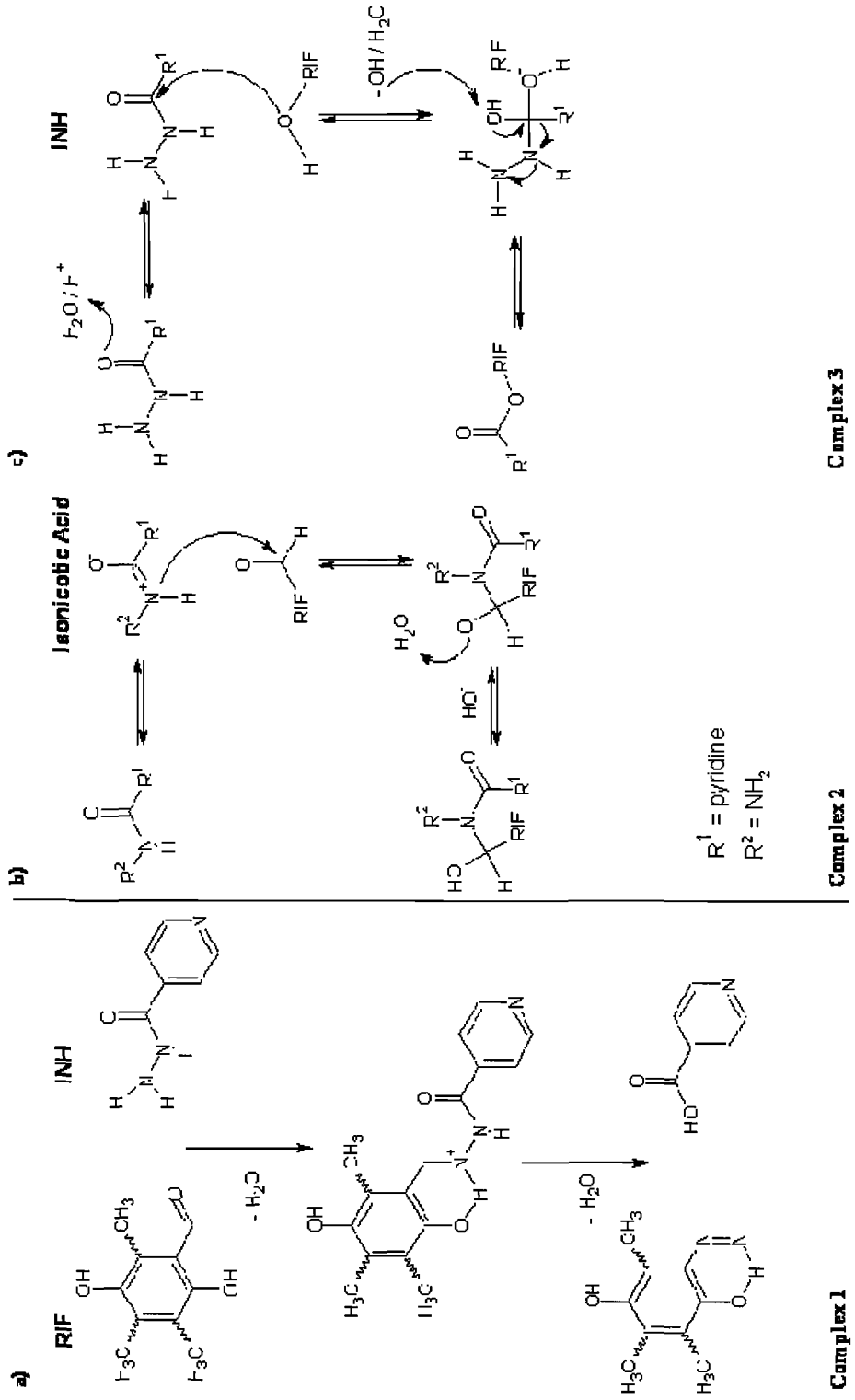

Figure 2.8: Mechanism of action for the decomposition of rifampicin in the presence of isoniazid (Du Toit *et al*, 2006).

Once 3-formylrifamycin is formed under acidic conditions, it interacts with isoniazid to form isonicotyl hydrazone, through a fast second-order reaction. Isonicotyl hydrazone will regenerate isoniazid and 3-formylrifamycin by first-order reaction due to the instability of the hydrazone at acidic pH. An overall enhancement of the degradation of rifampicin is observed

because the second-order reaction is faster than the other reactions (rifampicin to 3-formylrifamycin and the hydrazone to 3-formylrifamycin and isoniazid) (Singh *et al.*; 2001).

2.14 Conclusion

In the past five years, a robust portfolio of new drugs has been identified. However, these drugs are still in the discovery stage. Current timelines for phase I, II and III clinical testing predict that a new regimen containing four novel drugs will require 12 years to complete. The current first-line agents namely rifampicin, isoniazid, pyrazinamide and ethambutol have a list of shortcomings, but these drugs remain the principle weapons in the battle against *M. tuberculosis*. All of these drugs readily penetrate cell membranes and are highly selective by attacking biochemical targets whilst leaving human cells relatively unharmed. Fixed dose combinations are the option of choice in the treatment of tubeculosis. In South Africa a 4-drug FDC containing the four first-line drugs is the only available option during the initial intensive treatment phase. Because poor quality of FDC products relates to the bioavailability of rifampicin, it was important to identify and investigate all the factors contributing to poor bioavailability. The bioavailability is affected by various factors including absorption, intestinal permeability and metabolism of a drug.

API-related problems within the scope of this study were identified as:

- Solubility of rifampicin in water is higher at low pH values; however it undergoes rapid decomposition at low pH values (Reynolds, 1989);
- Hygroscopicity of ethambutol;
- Amounts of active ingredients needed to obtain therapeutic effect;
- Availability of API's of acceptable quality;
- Instability of rifampicin in aqueous solution;
- Assay method for quantitative determination of drug content in pharmaceutical products.

Poor quality of rifampicin-containing FDC products frequently relates to the bioavailability of rifampicin. Much can be done to improve the bioavailability of current drugs.

The Pheroid™ drug delivery system was investigated as a possible solution for most of the API-related challenges.

CHAPTER 3

THE ROLE OF THE PHEROID™ DRUG DELIVERY SYSTEM IN THE TREATMENT OF TUBERCULOSIS

3 Introduction

The treatment of tuberculosis is complicated by the need for multi-drug regimens that need to be administered over extensive periods in time. The single, most common reason for failure of the current regimens is said to be poor patient compliance. Various progressive efforts have been made to minimize toxicity and improve patient compliance. The majority of such efforts involve the development of various carrier-based drug delivery systems to either be more site-specific or aim at reducing the dosing frequency. Recent trends in controlled drug delivery include the micro-encapsulation of drugs in biodegradable polymers. Some liposome or micro sphere formulations proved more efficient in the sustained delivery of anti-tuberculosis drugs. However, these formulations need to be administered either intravenously or subcutaneously, which is often unacceptable due to pain and discomfort caused by the administration. Hence, there is a definite need for oral drug delivery systems that are convenient for patients.

Pheroid™ technology was founded by Piet Meyer while searching a cure for his own psoriasis. The novelty of this technology can be underlined by various patents that describe the use of Pheroid™ technology as delivery system to enhance absorption and efficacy of dermatological and oral medicines in various pharmacological groups (Grobler, 2004).

A few of the key advantages of the Pheroid™ are discussed in this chapter, which include increased delivery of active pharmaceutical compounds, decreased time to onset of action, reduction of MEC (minimal effective concentration) and increased therapeutic efficacy amongst others.

From previous studies discussed later in this chapter, the application of Pheroids™ in the pharmaceutical industry is potentially limitless and its effect on worldwide epidemics such as tuberculosis, malaria and AIDS could be staggering.

3.1 Structural characteristics of Pheroids™

Although the Pheroid™ delivery system is often confused with other lipid-based delivery systems, it can be described as a colloidal system that contains stable, lipid-based submicron- and micron-sized structures, referred to as Pheroids™ (Grobler *et al.*, 2007). The

Pheroids™ are uniformly dispersed in a dispersion medium and can be manipulated in terms of morphology, structure, size and function (Grobler, 2004).

Three types of Pheroids™ that are formulated include:

- Lipid-bilayer vesicles in both the nano- and micrometer size range (80-300 nanometer);
- Micro-sponges (0.5-5 micrometer) ; and
- Depots or reservoirs that contain pro-pheroids™ (size is determined by the amount of pro-pheroid™ contained in the reservoir).

Each type of Pheroid™ has a specific composition and the size and shape of the vesicles are reproducible (Grobler, 2004).

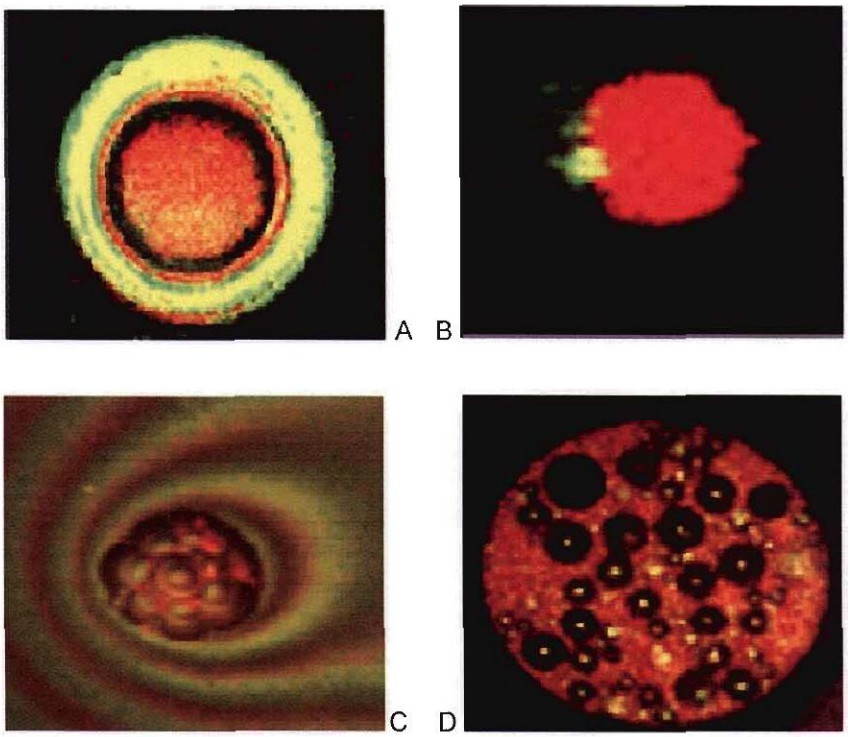

Figure 3.1: Different types of Pheroid™ formulations (Grobler, 2004)

Pheroids™ can entrap drugs with various solubilities by changing one or more of the following:

- The specific fatty acids used in the manufacturing,
- The ratios of the fatty acids used,
- The saturation state of the used fatty acids,

- The modification state of the fatty acids,
- Manufacturing procedure (to a lesser extent) (Grobler *et al.*, 2007).

Lipid-soluble drugs are often better entrapped into vesicles (figure 3.1 A) as it readily diffuses through the solid bilayer membrane composed of fatty acids. Drugs with high water-solubility tend to diffuse more easily through the sponge-like membrane packing of microsponges (Figure 3.1 B). Reservoirs may be used to entrap insoluble compounds because of its large loading capacity to surface area ratios (Figure 3.1 C). Pro-pheroids™ is used in formulations containing drugs that are unstable in aqueous solutions (Figure 3.1 D) (Grobler *et al.*, 2007).

3.2 Fatty acids as the cornerstone of the Pheroid™ drug delivery system

Lipids with the most biomedical importance are commonly known as fats. Fats are classified as esters of 3 fatty acid molecules and 1 glycerol molecule. It serves as insulation around certain organs while lipoproteins are important cellular constituents, occurring both in cell walls and in the mitochondria. Lipids are also a main source of energy for the human body. Fatty acids form important components of all known lipids. A fatty acid is defined as a carboxylic acid with a long (4 to 28 carbons) unbranched aliphatic chain, which is either saturated or unsaturated. Saturated fatty acids do not contain any double bonds or other functional chains along the chain, allowing all carbons to contain as much hydrogen atoms as possible. Unsaturated fatty acids have a similar form as saturated fatty acids, except that unsaturated fatty acids contain a double bond in the carbon chain.

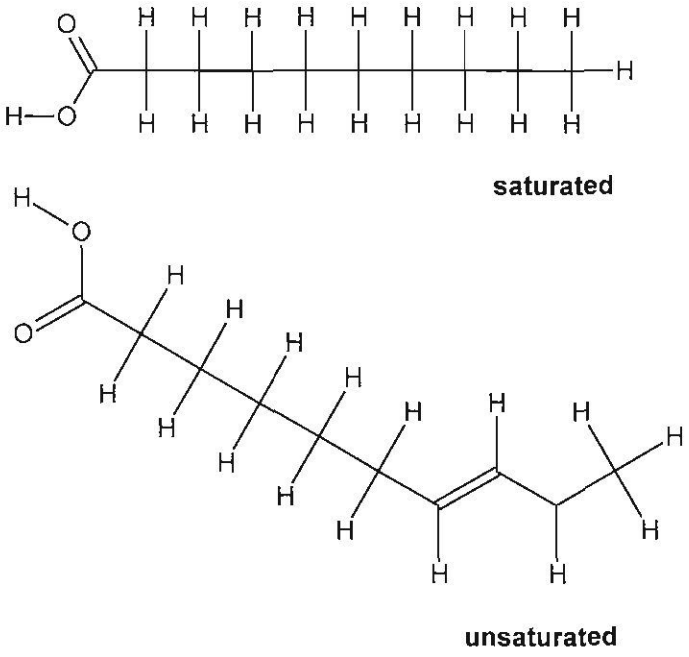

Figure 3.2: Molecular structure of saturated and unsaturated fatty acids

The two carbon atoms bound to either side of the double bond can occur in a *cis* or *trans* configuration. The *cis* configuration refers to the adjacent carbon atoms that are on the same side of the double bond. Conversely, when the adjacent carbon atoms are bound to opposite sides of the double bond, it is known as a *trans* configuration. The rigidity of double bonds freezes the conformation of fatty acids, while the *cis* isomer causes the chain to bend slightly (Mayes, 1993).

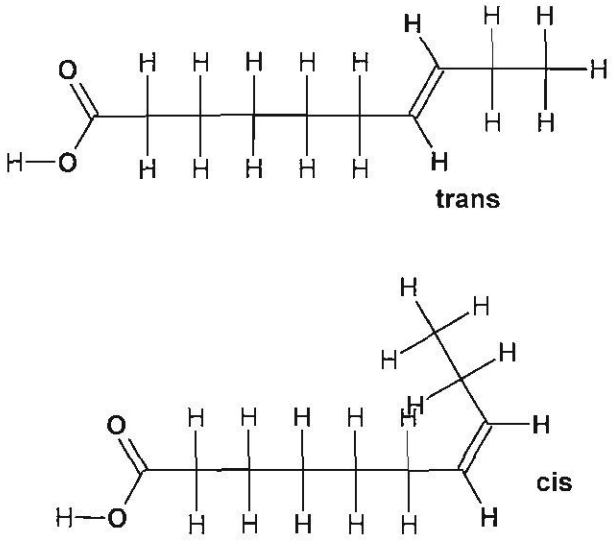

Figure 3.3: Different structural configurations of fatty acids

3.3 The composition of Pheroids™

Pheroids™ consist of three phases:

- An aqueous phase;
- An oil phase;
- Nitrous oxide.

The aqueous phase is generally made up of sterile water during the manufacturing process, but it may also consist of a buffer solution. In the case of pro-Pheroids™, the aqueous component of body fluids is necessary for the formation of Pheroids™ (Grobler *et al.*, 2007).

The oil phase is composed mainly of ethylated and pegylated polyunsaturated fatty acids, including omega-3 and -6 fatty acids but excluding arachidonic acid (Grobler *et al.*, 2007).

The human body synthesizes most of the necessary fatty acids, with the exception of two. These two, linoleic acid and alpha-linolenic acid, are referred to as essential fatty acids that must be ingested through food (Mayes, 1993). The lack of essential fatty acids in the modern western diet provides an advantage for Pheroid™ drug delivery as it also includes these fatty

acids in its composition. More double bonds along the chain with adjacent carbons in the *cis* configuration, as in the case of linoleic acid, will have a more pronounced bend with less flexibility (Mayes, 1993). This may be part of the reason for the stable structure of Pheroids™, when considered that the fatty acids used in Pheroids™ are compatible with the orientation of the fatty acids in humans due to its cis-formation.

The Pheroid™ consists of one component that distinguishes it from all other lipid-based delivery systems, namely nitrous oxide (N_2O) (found distributed throughout the continuous phase and in association with the dispersed phase).The addition of nitrous oxide has been shown to have at least three functions:

a.) Contributing to the miscibility of the fatty acids in the dispersal medium,

b.) An essential contributor in the self-assembly process of the Pheroid™,

c.) To improve stability of the formed Pheroid™ (Grobler *et al.*, 2007).

3.4 The Pheroid™ system versus other lipid-based drug delivery systems

Liposomes, emulsions and microemulsions, polymeric microspheres and macromolecular microspheres are all colloidal systems most often used in dosage forms. The design of the Pheroid™ incorporates one or more features of each of these systems. Some features common to the Pheroids™ and the other colloidal systems are highlighted. Pheroids™ generally contain a lipid-bilayer as is the case with liposomes, but it contains no phospholipids or cholesterol. Pheroids™ share the characteristic of self-assembly with low-energy emulsions and microemulsions and no lyophylization or hydrations of the lipid components is necessary. Pheroids™ differ from emulsions by consisting of not only two liquid phases, but also an additional dispersed gas phase which is associated with the fatty acid dispersed phase. A feature taken from polymeric microspheres include the specific ratio of pegylated to ethylated fatty acids used in the assembly of Pheroids™, whilst the formulation of natural depots are representative of macromolecular microspheres (Grobler *et al.*, 2007).

3.5 Advantages of the Pheroid™ in the treatment of tuberculosis

The Pheroid™ system has shown its superiority to most other drug delivery systems due to the following characteristics:

3.5.1 Decrease in T_{max}

Research indicated repeatedly that the Pheroid™ delivery system rapidly moves across most physiological barriers and delivers the active (Grobler, 2004). Figures 3.4 – 3.7 illustrates that the time needed to obtain maximum plasma concentration (T_{max}) was shortened for all the

actives in the Pheroid™-based formulation. The clinical significance of this phenomenon could be a shorter treatment regimen.

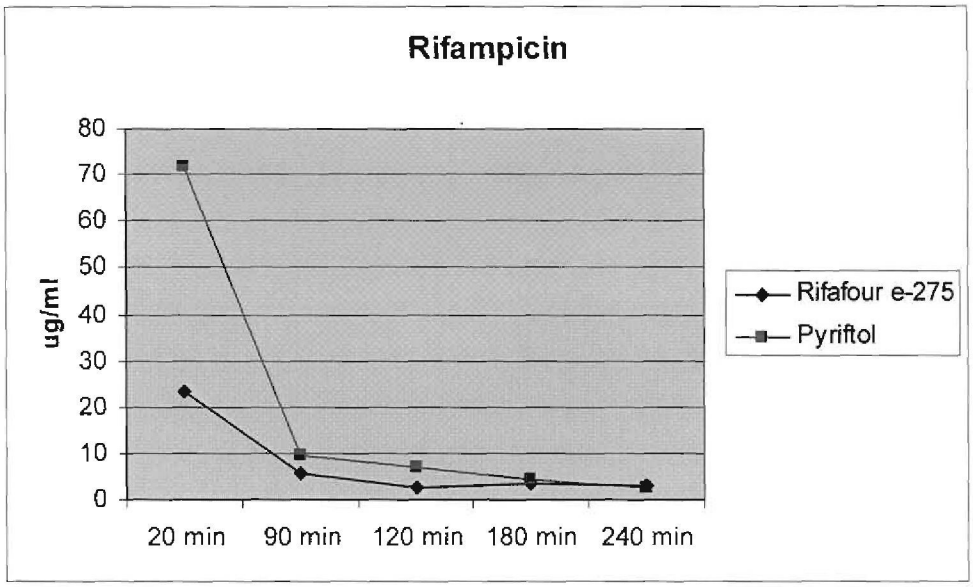

Figure 3.4: Comparative graph of plasma concentrations of rifampicin between the test formulation (Pyriftol) and Rifafour e-275®. *Reprinted with permission of L.M Nieuwoudt.*

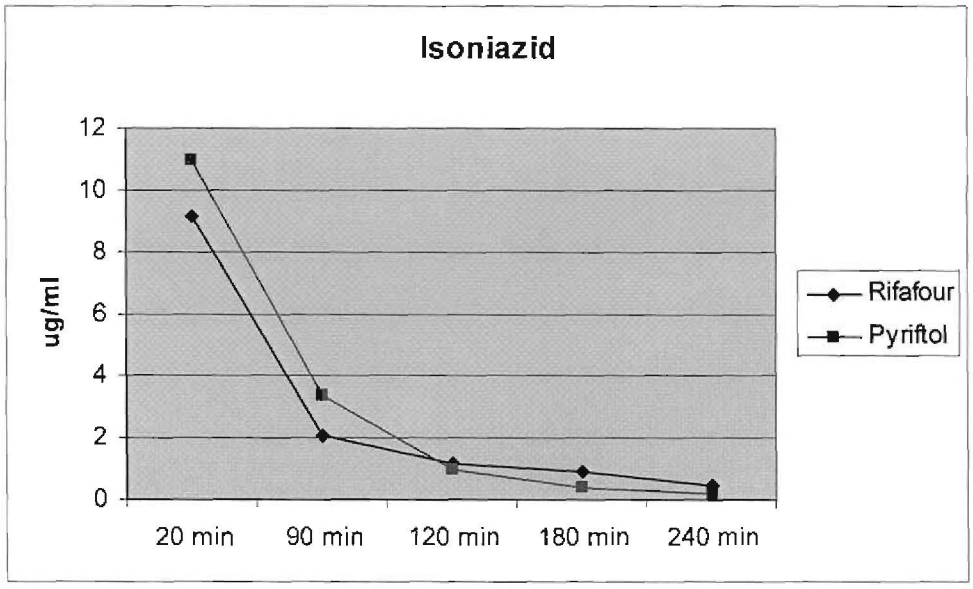

Figure 3.5: Comparative graph of plasma concentrations of isoniazid between the test formulation (Pyriftol) and Rifafour e-275®. *Reprinted with permission of L.M Nieuwoudt.*

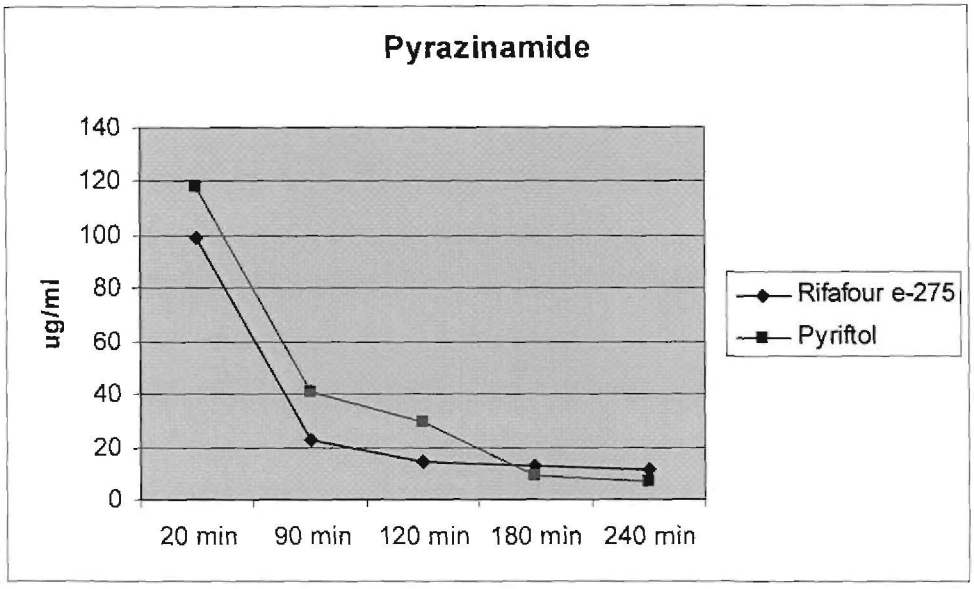

Figure 3.6: Comparative graph of plasma concentrations of pyrazinamide between the test formulation (Pyriftol) and Rifafour e-275®. *Reprinted with permission of L.M Nieuwoudt.*

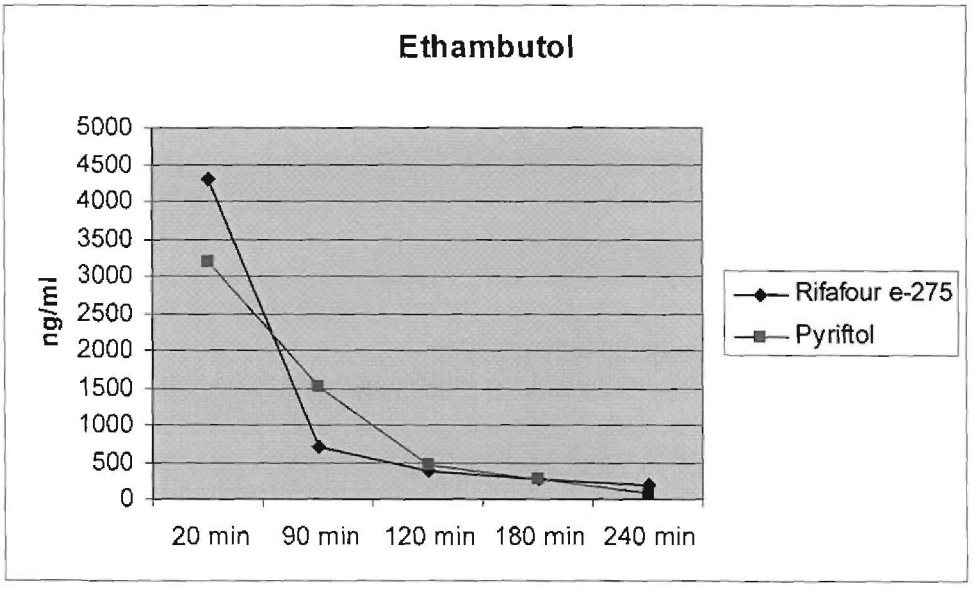

Figure 3.7: Comparative graph of plasma concentrations of ethambutol between the test formulation (Pyriftol) and Rifafour e-275®. *Reprinted with permission of L.M Nieuwoudt.*

3.5.2 Increased bioavailability of active substances

An increase in average plasma levels of rifampicin for 14 volunteers was illustrated after oral administration of combination anti-tuberculosis DOTS treatment (Grobler, 2004).

3.5.3 Reduction of Minimum Inhibitory Concentration

Studies have shown that using less of the entrapped active compound (in some cases as little as 1/40th) may still result in an effective Pheroid™ formulation. In a practical setting, this can mean a reduction of side effects and increased cost savings in treatment (Grobler, 2004).

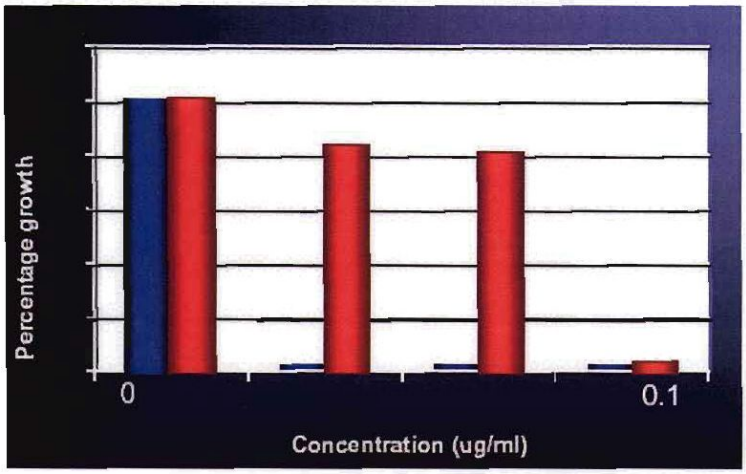

Figure 3.8: The growth of *Mycobacterium tuberculosis* (reference strain H37Rv) at various concentrations of Pheroid-entrapped as well as free isoniazid. The red bars represent free isoniazid. The blue bars indicate Pheroid™-entrapped isoniazid (Grobler, 2004).

In figure 3.8, the reduction of minimum inhibitory concentration by the Pheroid™ system is illustrated. In this figure, the blue bars represent Pheroid™-entrapped isoniazid and the red bars the free Isoniazid. In this study, growth was determined with the use of radio-active labels in a BACTEC system.

3.5.4 Maintenance of the therapeutic blood levels

From figures 3.4 - 3.7 it became clear that the plasma levels of all active drugs entrapped in Pheroids™ were kept above therapeutic dosage levels for longer when compared to Rifafour e-275®. The significance of this could be twice-weekly intermittend dosage regimes.

3.5.5 The Pro-Pheroid™ concept

With the addition of larger concentrations of polyethylene glycol (PEG) and the omission of water, pro-Pheroids™ can be formulated. The stable Pheroid™ vesicles will form once it encounters the fluid component that is found *in vivo*. This is a very important formulation tool for unstable molecules and a great advantage of the Pheroid™ system. The pro-Pheroid™ concept was used throughout this study in an attempt to increase stability of the preclinical product. This was because of the fact that rifampicin is unstable in aqueous solution (Grobler, 2004).

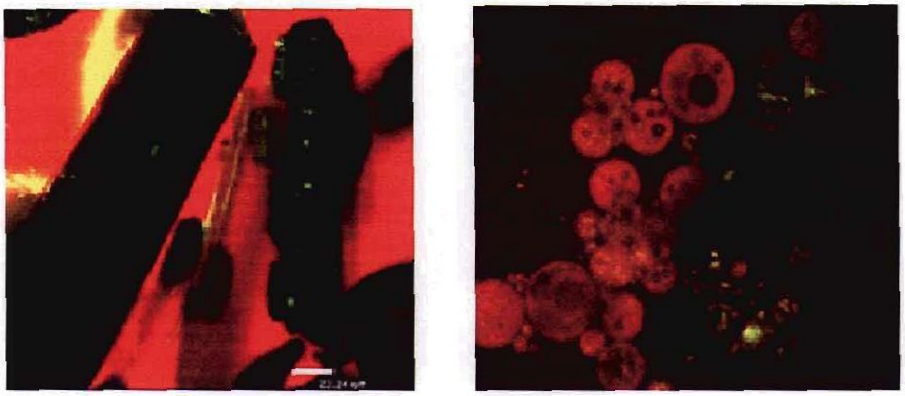

Figure 3.9: The micrograph on the left illustrates pyrazinamide in the pro-Pheroid™ formulation and the micrograph on the right shows the pyrazinamide that was entrapped in Pheroids™. *Reprinted with permission of A.F. Grobler*

3.6 Proposed mechanism of drug delivery across the mycobacterial cell wall.

Mycobacterium tuberculosis is surrounded by an outer lipid bilayer that consists of a vast amount of long fatty acids, namely mycolic acids. The mycolic acids are α-branched β-hydroxy fatty acids with chain lengths of up 90 carbon atoms. This outer membrane is the thickest biological membrane known to man. The exceptionally low permeability of this membrane renders *M. tuberculosis* intrinsically resistant to most antibiotics (Ingraham & Ingraham, 1995).

Three general pathways exist for transport of compounds across the lipid bilayer membrane namely:

- Passive diffusion (hydrophobic compounds);
- Transport through porins in the membrane (hydrophyllic compounds);
- Membrane disruption (polycationic compounds).

Similar uptake mechanisms were found in *M. tuberculosis*, but lower permeability of hydrophyllic compounds through the porins was noted. This was explained by a lower amount of porins and the exceptional length of the porins found in mycobacteria. This is problematic since three of the four current first-line anti-tuberculosis drugs, namely isoniazid, pyrazinamide and ethambutol, are hydrophyllic molecules. Thus, although these drugs are highly effective, transport into the bacterial cell might be limited (Niederweis, 2003).

Since all compounds can be entrapped into Pheroids™, this problem seems to be something of the past. The following paragraph will explain the underlying theory of Pheroid™ drug delivery.

When the drugs are entrapped within the Pheroid™ drug delivery system, the emphasis is shifted from the physicochemical properties of the foreign drug molecule to that of the fatty acids. Both the human body and the bacteria are unable to recognize the toxic drug molecule within the Pheroid™. Thus, lipophyllic pheroids™ diffuse readily through all membranes, including that of *M. Tuberculosis.* The bacteria will compete with the human cell for fatty acids used in various processes that is essential to its existence. This facilitates the uptake of the Pheroids™ by the bacterial cell. The release of active drug from the Pheroids™ rely on the metabolism of fatty acids in the mitochondria by a process called β-oxidation.

3.7 From the Concept to Reality

After achieving great results with the transdermal delivery of coal tar from Pheroid™ formulations, more effort went into the hypothesis claiming that the efficacy of oral and parental therapies can be enhanced. For tuberculosis, a cross-over bioequivalence study was done in 16 volunteers (healthy) to measure the efficiency of the Pheroid™ delivery system in which rifampicin, isoniazid, pyrazinamide and ethambutol were entrapped (Pyriftol) against the WHO-approved anti-tuberculosis commercial product (Rifafour e200®). Increased absorption of all four actives from the Pheroid™ formulation was anticipated so strongly that researchers at Meyerzall Laboratories only used 60% of the dosage used in Rifafour e-200®.

3.7.1 Objectives for the Trial

Based on the benefits of the Pheroid™ drug delivery system, the objective of this trial was to prove the following:

a) Pheroid™ can increase the bioavailability of antimicrobials in human plasma;

b) Pheroid™ can enhance absorption of antimicrobials without an increase of toxicity;

c) Pheroid™ leads to an increase in specificity for drug targets;

d) Pheroid™ can increase the circulatory time of the active drugs;

e) Pheroid™ can increase the bactericidal effect of the antimicrobials inside the target cells;

f) Pheroid™ can lessen side effects (Grobler, 2004).

3.7.2 Results for this trial

Both the C_{max} (maximum concentration the active attained) and AUC (area under the curve) for all actives was significantly higher in the case of Pyriftol. The T_{max} (time it takes to attain maximum concentration) was reached faster for rifampicin and isoniazid in Pyriftol. The T_{max} of pyrazinamide remained the same as the comparative. Although it seems that Pheroid™ does not promote the absorption of pyrazinamide, it may play a role in the transport of pyrazinamide into the blood cells and may be protective of the cell from toxic effects.

Whereas the comparative product (Rifafour e-200) never reached the accepted minimum inhibitory concentration (MIC) for rifampicin, the Pyriftol test product not only reached, but maintained its effective concentrations for up to 5 hours.

The one true advantage of Pheroids™ for tuberculosis treatment was found to be the fact that the Pheroid™ delivered more of the active drugs right at the site where it is needed – the macrophage. This was proven by using reflected light and fluorescent labeling in a confocal laser scanning microscopy study.

This trial succeeded in reaching all of its objectives and led the researchers to believe that an expansion of research efforts is justified (Grobler, 2004).

3.8 Conclusion

In this chapter an effort was made to address the key aspects around this delivery system, including previous research done at MeyerZall Laboratories in order to give a better understanding of the opportunities that the Pheroid™ propose for the future in the delivery of active pharmaceutical ingredients. The Pheroid™ has proved itself as a versatile, effective and relatively inexpensive nanoparticulate drug delivery system. All of its components are based on naturally occurring molecules and are considered as safe. Nevertheless, the exploration of this system is still young and more research is needed to expand the Pheroid™ to its full potential.

4 Introduction

The purpose of a scientific journal is a means of reporting the findings of the researcher. Various journals are available in all fields of research. The Open Drug Delivery Journal was chosen for this study as this journal publishes all research articles that involves new drug delivery systems. This study revolved around the Pheroid™ drug delivery system. A further advantage of this journal was the fact that it is available to all individuals with internet access, which will ensure a great impact factor. When writing a scientific research article, the researcher should adhere to a set of rules regarding the formatting of the article. This set of rules is known as the guide for authors. The format of the article in this chapter, was done according to a template that was available to download from the internet for the Open Drug Delivery Online Journal.

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Conference Proceedings:

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URL(Web Page):

[5] National Library of Medicine. Specialized Information Services: Toxicology and Environmental Health. http://sis.nlm.nih.gov/Tox/ToxMain.html (accessed May 23, **2004**).

Patent:

[6] Hoch, J.A.; Huang, S. Screening methods for the identification of novel antibiotics. U.S. Patent 6,043,045, March 28, **2000**.

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[7] Kirby, C.W. Thesis, University of Waterloo, **2000**.

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A preclinical evaluation of the bioavailability and stability of a pro-Pheroid™ fixed-dose combination against tuberculosis

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Abstract: Pheroids™ (a novel, nanoparticulate drug delivery system) were investigated as a promising approach to alter the pharmacokinetic profiles of the current first-line anti-tuberculosis drugs. The plasma levels of rifampicin, isoniazid, ethambutol and pyrazinamide were determined in a pilot study in which various fixed-dose combination (FDC) formulations were administered to CD57 inbred mice. Higher plasma levels were obtained in the mice to which the pro-Pheroid™ formulation was administered. A preclinical stability study was carried out on two pro-Pheroid™ FDC formulations. The formulations were subjected to temperatures ranging from 5 °C to 40 °C and humidity ranging from 60-75% over a period of 3 months. The formulation containing isoniazid (H) and pyrazinamide (Z) showed physical changes at low temperatures, while the formulation containing rifampicin (R) and ethambutol (E) showed both physical and chemical changes. It is suggested that more attention should be paid to the manufacturing of these formulations as high variability in drug content was observed. In addition, the packaging material should be of the highest quality.

Keywords: tuberculosis, bioavailability, stability, pheroid™, FDC, drug delivery

INTRODUCTION

With the rise of the HIV-pandemic and persistent global poverty, tuberculosis was declared a global emergency, claiming millions of lives annually and putting severe pressure on the socio-economic status of developing countries [1]. Fixed dose combination (FDC) products containing anti-tuberculosis drugs were introduced to the market as a means to simplify treatment and increase patient compliance [2,3]. Numerous cases of poor bioavailability have been reported for rifampicin in FDC products. Some of the reasons for the poor bioavailability given in the literature are degradation in the gastro-intestinal tract and inherent variability in absorption and metabolism upon oral administration of FDCs [4]. However, the fact that the oral route is the most advantageous of all other routes for the purpose of drug administration is emphasized. Some contributing factors within the formulation environment include raw material characteristics and changes in the crystalline forms of rifampicin, [5]. Although the identification of novel anti-TB agents remains a priority, the development of nanoparticle drug delivery systems for currently used agents may represent a promising alternative [6,7,8]. Some key technogical advances of these systems are high stability, high carrier capacity, feasibility of entrapment of both hydrophyllic and hydrophobic compounds and feasibility of variable routes of administration [8,9]. The patented Pheroid™ system is one of these nanoparticle drug delivery systems, but it appears to be even more advantageous than its counterparts. Previous studies suggested that the use of Pheroid™ technology might be able to improve drug bioavailability and reduce the dosing frequency [10]. This may resolve the problem of nonadherence to standardized treatment regimens, which is one of the major obstacles in the control of TB epidemic [9]. A preclinical study was done in our laboratories on a possible fixed-dose combination against tuberculosis based on Pheroid™ technology. This included an investigation into the drug bioavailability after administration of the Pheroid™-based formulations, as well as the stability of these formulations when exposed to accelerated climatic conditions for three months. Although the bioavailability of three of the four drugs ie. rifampicin, isoniazid and pyrazinamide was markedly improved by Pheroid™ drug delivery technology, the stability could not be determined because of formulation difficulties.

MATERIALS AND METHODOLOGY

Rifampicin (RMP/NC-020/06) and Pyrazinamide (PYZ/P-228/06) powders were obtained from Linaria Chemicals Limited (Thailand). Isoniazid (IF070501) and Ethambutol (IF070608) were obtained from D B Fine Chemicals (Pty) Limited (South Africa). All pharmaceutical active compounds were used as received (Assay: R = 101.4%; H = 99.3%; Z = 100.4%; E = 99.6%). All reference standards were obtained from Industrial Analytical, Johannesburg. Methanol was HPLC

grade and all other chemicals were analytical grade, and water appropriate for liquid chromatography was used. Pro-pheroids™ were manufactured and supplied by the Northwest University (Potchefstroom campus, South Africa).
All surfaces of the clean or new containers, measurers and dispensers that came in contact with the formulations during preparation or storage were autoclaved before use. Formulations were stored in glass containers with screw caps and the containers were covered in several layers of aluminium foil to protect the content from light.

Pre-formulation studies

A complete particle-size distribution analysis was performed on all four active pharmaceutical active ingredients. The powders were grinded with an automatic grinding apparatus (Fritsch Pulverisette) to particle sizes ranging from 25 µm – 65 µm for optimal entrapment in Pheroids™. Rifampicin was characterized in terms of polymorphic form by X-ray powder diffractometry, differential scanning calorimetry (DSC) and infrared spectrofotometry.

Manufacturing of pro-Pheroid™ fixed-dose combinations

For the pilot study, the pro-Pheroid™ formulation was optimized for administration to mice. It consisted of a large pro-Pheroid™ component to make it less viscous. A slight modification was made for the stability formulations to make it more analogous to the amount of Rifafour e-275® tablets that need to be taken in current treatment regimes. This modification entailed the pairing of rifampicin with ethambutol instead of with pyrazinamide. The pro-Pheroid component was almost halved in the stability formulations. Rifampicin and isoniazid was formulated separately because of the formation of isonicotinyl hydrazone from 3-formylrifamycin and isoniazid within the formulation environment. Briefly, formulation A was manufactured by thoroughly mixing 10 g of isoniazid and 57 g of pyrazinamide with 33 g of pro-Pheroid™. Formulation B was manufactured by thoroughly mixing 19 g of rifampicin and 39 g ethambutol with 42 g of pro-Pheroid™.

In vivo bioavailability test

The plasma levels of rifampicin, isoniazid, pyrazinamide and ethambutol were determined by means of a pilot study. Briefly, a pro-Pheroid™ formulation was administered to one group of CD57 inbred mice daily for 4 days. The current fixed-dose combination tablet (Rifafour e-275®) was administered in water to another group of mice and acted as the positive control group. Blood was collected at approximately 20 minutes after administration on day 4. To prevent bias, the plasma was assayed by the Department of Pharmacology, University of Cape Town, who used proprietary LCMS methods to determine individual drug concentration.

Accelerated stability tests

The purpose of this preclinical stability testing was to determine how the quality of the pro-Pheroid™ formulations vary with time. The stability testing protocol was designed in such a way as to mimic the packaging of the finished product *ie.* to protect it from light and moisture. Transparent glass jars, covered in several layers of aluminium foil, containing 100 g of the pro-Pheroid™ formulations were stored for 3 months in various stability chambers. The accelerated storage conditions were 5 °C, 25 °C/60 % RH, 30 °C/65 % RH and 40 °C/75 % RH as prescribed by the guidelines for stability testing of the International Conference on Harmonization (ICH), [11]. The samples stored at 40 °C and 75 % RH were of utmost importance because it reflects on the climate of tropical areas [12]. Samples were evaluated every 30 days for drug content, physical changes and microbial growth. The acceptance criteria for the stability tests were 10 percent change in assay from its initial value, or any microbial growth detected.

Microbial growth determination

Microbiological assays of the formulations were conducted to determine the presence of yeasts and moulds, *E.Coli, Staphylococcus Aureus* and *Salmonella.* The assays were performed by Consulting Microbials Laboratory, South Africa according to the microbiological limit tests described in section 61 of the 29th edition of the USP [13].

Determination of drug content in pro-Pheroid™ formulations

The formulations were assayed at the start of the study to determine the initial drug contents. The assays were repeated on the different stored samples in the stability chambers on a monthly basis for the total duration of 3 months. A previously validated HPLC method was adapted to perform the assays [14]. Prior to use, the method was validated for some performance parameters. This was necessary to ensure the suitability of the method for its intended purpose. The system consisted of a Shimadzu Prominence series HPLC equipped with a LC-20AD quaternary pump, SIL-20AC auto sampler, SPD-M20A diode array detector and Shimadzu LC Solution data acquisition and analysis software. All samples were injected onto the chromatogram in duplicate.
For rifampicin: A quantity (395 mg) of the pro-Pheroid™ formulation equivalent to about 75 mg of rifampicin was accurately weighed and dissolved in 100 ml of methanol. A standard solution was

prepared in a similar way, but rifampicin reference standard (RS) was used. Both solutions were filtered through a 0.45 µm filter and the filtrate was transferred to amber-coloured HPLC vials. The solutions were injected (without delay) into a Phenomenex Luna® (250 x 4.6 mm, 5 µm) column obtained from Separations, South Africa. Other HPLC conditions included a flow-rate of 1.0 ml/min, injection volume of 20 µl, detection wavelength of 254 nm and column oven temperature of 25 °C. The mobile phase consisted of six volumes of methanol and four volumes of a phosphate buffer pH 7.0 (potassium dihydrogen phosphate (0.01 mol/L), adjusted with sodium hydroxide (0.1 mol/L)). Although formulation B contained both rifampicin and ethambutol, no effort was made to analyse ethambutol due to the lack of absorbance at 254 nm.

For isoniazid and pyrazinamide: A quantity (55 mg) of the pro-Pheroid™ formulation equivalent to about 5.5 mg of isoniazid was accurately weighed and transferred to a 100 ml volumetric flask. Thirteen milliliters of methanol were added and the mixture was shaken for 1 minute. The flask was filled with water to volume and shaken for a further 10 minutes. A standard solution was prepared in a similar way, but isoniazid and pyrazinamide reference standards were used. Both solutions were filtered through a 0.45 µm filter and the filtrate was transferred to amber-coloured HPLC vials. The solutions were injected (without delay) into a Phenomenex Luna® (150 x 4.6 mm, 5 µm) column obtained from Separations, South Africa. Other HPLC conditions included a flow-rate of 2.0 ml/min, injection volume of 20 µl, detection wavelength of 270 nm and column oven temperature of 25 °C. The mobile phase consisted of 50 g ammonium acetate dissolved in 1000 mL water. The pH was adjusted to 5.0 with glacial acetic acid.

For ethambutol: A quantity (51 mg) of the pro-Pheroid™ formulation equivalent to about 20 mg of ethambutol was accurately weighed and transferred to a 100 ml volumetric flask. Thirteen milliliters of methanol were added and the mixture was shaken for 1 minute. The flask was filled with water to volume and the mixture was shaken for a further 10 minutes. A standard solution was prepared in a similar way, but ethambutol reference standard was used. Both solutions were filtered through a 0.45 µm filter and the filtrate was transferred to amber-coloured HPLC vials. The separation column and conditions remained the same as the assay for isoniazid and pyrazinamide. The mobile phase consisted of 50 g ammonium acetate and 0.2 g copper(II) acetate, dissolved in 1000 mL water. The pH was adjusted to 5.0 with glacial acetic acid.

RESULTS AND DISCUSSION

Characterization of rifampicin polymorphic form

From the IR results (fig. 1) it seems that the rifampicin powder used in this study showed similarities to that of rifampicin form II. Because the IR results were not conclusive, X-ray powder diffractometry (XRPD) was used. XRPD is a highly effective method of distinguishing different polymorphic forms in a solid.

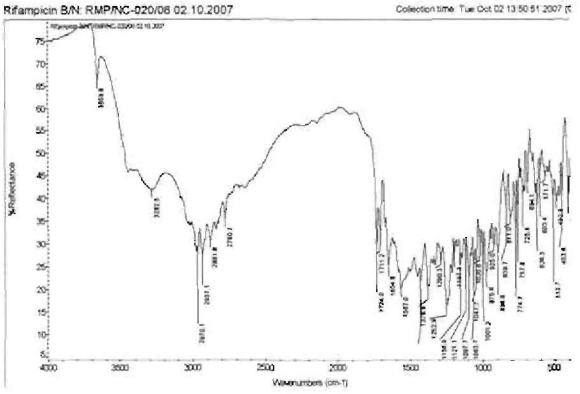

Fig (1). IR spectrum of the Rifampicin sample

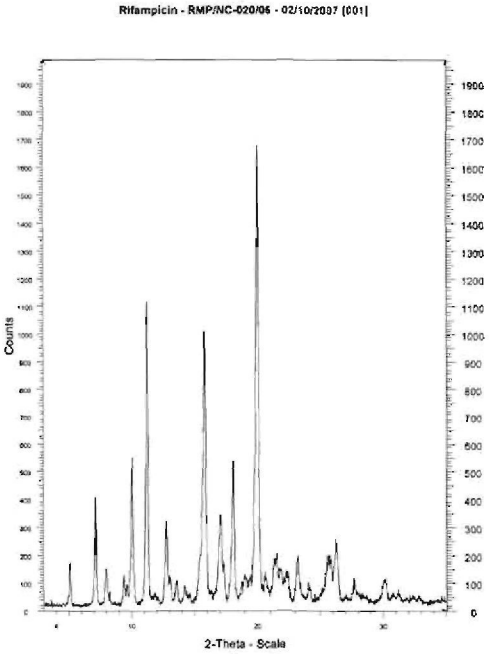

Fig (2). XRPD diffractogram of rifampicin sample

The X-ray powder diffractogram (fig. 2) of the rifampicin sample showed resemblance to rifampicin form II as described by Henwood *et al.* [5].

The peak intensity count of 1681 showed that the sample was more crystalline in comparison with peak intensity count values of below 1000 which

Henwood *et al.* [5] reported for a mixture of Polymorph II and an amorphous form. Where amorphous material is concerned, the peak intensity counts were low and the incoherent scattered radiation from the amorphous fraction of the sample adds to the general background of the powder pattern, i.e. higher background scatter signs on diffractometer tracings [5].
Slight differences in *d* spacings and corresponding relative intensities were observed between the results reported here and the results of Henwood *et al.* and could be explained by the fact that different X-ray powder diffractometers and measurement conditions were used.

Analysis of pro-Pheroid™ fixed-dose combination formulations

Formulation A had a paste-like texture and appeared oily. The colour of the formulation was described as white with a hint of grey (Fig. **3a**). Drug loading of isoniazid and pyrazinamide was 100 and 573 mg/g respectively. Formulation B formed a "plastic" layer on the surface (Fig. **3b**). When the formulation was stirred, it was found to have a soft clay-like texture. The formulation was brick red in colour. Drug loading of rifampicin and ethambutol was 186.2 and 365.8 mg/g respectively.

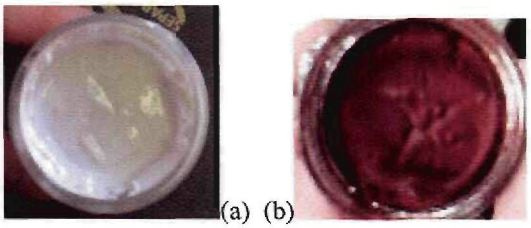
(a) (b)

Fig. (3) a and b. Photographs of formulation A (a) and B (b) on the day of manufacture.

The texture of both formulations was a direct result of a high solid to liquid ratio. The pro-Pheroid™ (oily liquid) component was limited in an attempt to lessen the amounts of capsules to be taken by patients.

In vivo bioavailability of active pharmaceutical ingredients in the pro-Pheroid™ fixed-dose formulation

An increase of about 305% in rifampicin plasma concentrations in mice were observed for the pro-Pheroid™ formulation above that found for Rifafour e-275® in water (Fig. **4**).

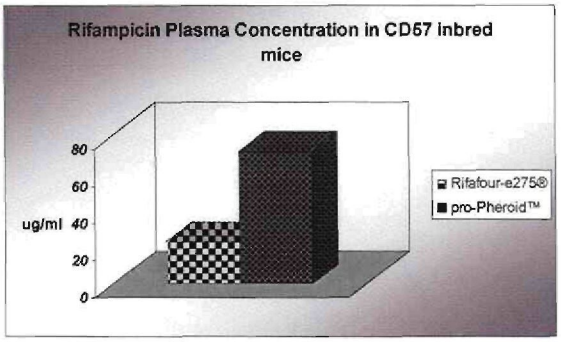

Fig. (4). Comparative bioavailability of rifampicin twenty minutes after the administration of different fixed-dose combinations.

The changes in the bioavailability of the other three active drugs are given in table **1**.

Table 1: Changes in bioavailability after administration of a pro-Pheroid™ fixed dose combination formulation, as compared to Rifafour e-275®.

Active drug compound	Increase in bioavailability (%)
Isoniazid	20
Pyrazinamide	19
Ethambutol	-21

Significant increases (20 and 19 %) in bioavailability were also observed for isoniazid and pyrazinamide for the pro-Pheroid™ formulation. The increase in plasma concentrations of rifampicin, isoniazid and pyrazinamide is ascribed to an increase in absorption across the intestinal epithelium. Although the Pheroid's mechanism for the increase in absorption is still speculative, it is hypothesized that the Pheroid™ might favour the route for passive transcellular absorption because of its lipophillic nature. The plasma concentration of ethambutol in mice showed a decrease of 21% after administration of the pro-Pheroid™ formulation, when compared with Rifafour e-275®. This decrease in plasma concentration of ethambutol could be a direct result of a shift in the time to reach peak plasma concentration (T_{max}) that were observed in previous studies with the pro-Pheroid™. A decrease in T_{max} is also supported by data from a new PK/PD study currently underway for which data are not shown here. In this case, it is hypothesized that ethambutol reached peak plasma concentrations (C_{max}) before the 20-minute time point on which plasma samples were obtained.

Accelerated stability test

Over the 90 days of the stability test, all the containers were inspected for physical changes on a monthly basis. Formulation A showed slight physical changes from the date of manufacture to

the end of the first month. A change in colour (from white to beige) was observed and the formulation lost its oily appearance. No other changes were observed between months 1 and 3. All samples of formulation A stored at 5 °C turned powder blue. Interestingly, these samples turned back to the beige colour upon exposure to room temperature. This phenomenon could be due to the ionization state of the copper ions present in the caps of the containers that were used in this study. However, a further investigation into the reasons for this observation will be undertaken. The possibility of a change of containers should also be investigated.

Considerable physical and chemical changes were observed for formulation B. Although this formulation did not show any significant changes in colour over the 3 months, changes in texture and appearance did occur. Separation of the components was evident from an oily layer that formed on top of the formulation. This separation was reversible by simply stirring the formulation and should not be problematic if a capsule dosage form was to be used. Another change that was noted included an increase of fluidity of the formulation. This could have been because of moisture uptake by ethambutol due to the hygroscopic nature of ethambutol [15,16]. Chemical changes are shown in fig. 7. The main chemical change was observed as the formation of rifampicin quinone during HPLC analysis. Unfortunately, this degradation product could not be quantitively assayed because of difficulties experienced in obtaining reference standards.

Determination of drug content

The initial drug contents for both formulations (Table 3) were found to lie within the range of 90-110% of the label claim (Table 2), with the content of ethambutol showing the highest variation.

Table 2: Label claim of drugs in test formulations

Formulation	Date of manufacture	Label claim (mg/100g)			
		R	H	E	Z
A	July 2007	-	10	-	57
B	July 2007	19	-	39	-

Table 3: Initial drug content in test formulations

Formulation	Date of manufacture	Label claim (mg/100g)			
		R	H	E	Z
A	July 2007	-	9.6	-	57.3
B	July 2007	18.6	-	36.6	-

Stability behaviour of both pro-Pheroid™ formulations over 3 months

The changes that were observed for the pro-Pheroid™ formulations are illustrated in figures **5** - **8**.

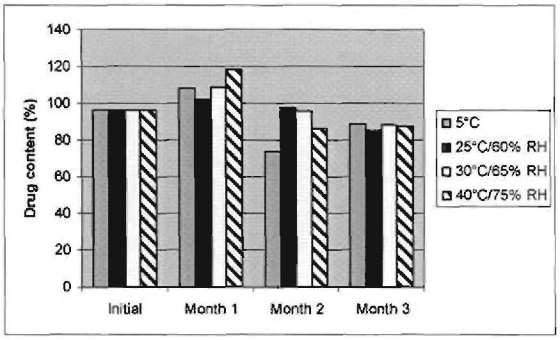

Fig. (5). Drug content (%) of isoniazid over 3 months.

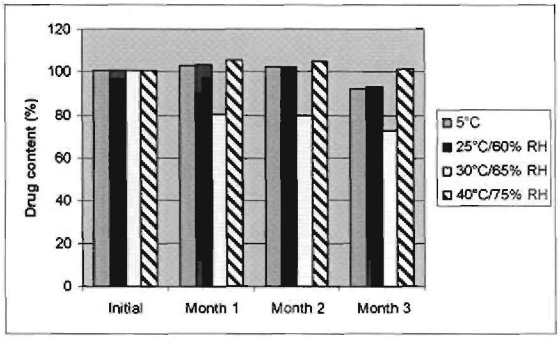

Fig. (6). Drug content (%) of pyrazinamide over 3 months

The variations that were observed in the monthly drug content determinations can be explained by one or more of the following technical inconsistencies:

1. Calibration status of analytical balances;
2. Experimental weighing error;
3. Leaking of containers;
4. Uniformity of drug content.

After considering the above-mentioned technical difficulties that were encountered, the stability issues could be addressed. All the drugs, except for rifampicin remained within 90-110% of the initial label claim after three months of storage at the various stability conditions. A significant decrease of up to 27% in pyrazinamide content was found in the sample exposed to 30°C/65% RH. The only possible explanation for this can be given as an operational malfunction of the climate chamber, but the explanation is difficult to verify as the chambers are controlled by a third party. The percent drug content of rifampicin and ethambutol are given in figures 7 and 8. Ethambutol was found to be the most stable drug with almost no variation in monthly drug content values. A significant degradation of rifampicin (about 20%) was found in the samples stored at 30°C/65% RH and 40°C/75% RH. This degradation is explained by the formation of an acidic environment upon moisture uptake by the ethambutol-containing formulation. The degradation of rifampicin is highly sensitive to both pH and the presence of water.

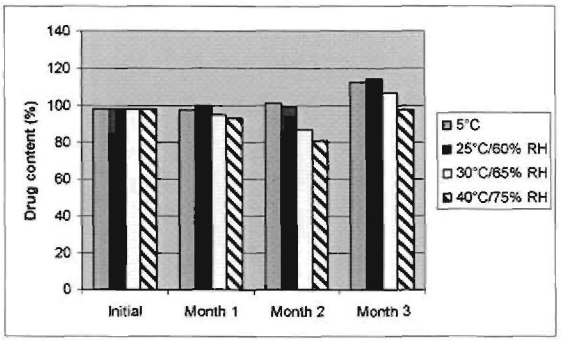

Fig. (7). Drug content (%) of rifampicin over 3 months

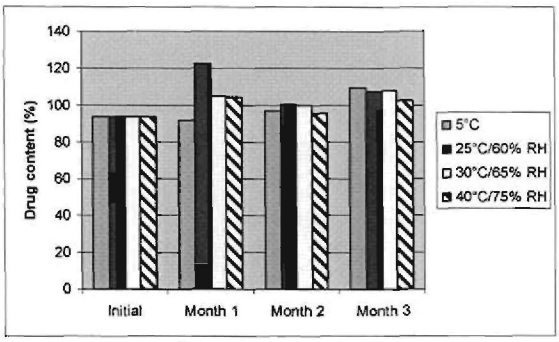

Fig. (8). Drug content (%) of ethambutol over 3 months

The extent of decomposition of R, H, E and Z in the respective formulations stored under ambient, 5°C, 30°C/60% RH and 40°C/65% RH conditions was determined as per cent of the drug concentration relative to the initial drug content. The data for formulation A and B are given in tables **4** and **5**, respectively.

Table 4. Percentage degradation of isoniazid and pyrazinamide from initial drug concentration in formulation A after storage for 3 months at accelerated stability conditions

	Decomposition with respect to initial drug content (%)	
Condition	**Isoniazid**	**Pyrazinamide**
5°C	7.6	8.2
25°C/60% RH	11.4	7.4
30°C/65% RH	7.9	27.6
40°C/75% RH	8.8	-0.7

Table 5. Percentage degradation of rifampicin and ethambutol from initial drug concentration in formulation B after storage for 3 months at accelerated stability conditions

	Decomposition with respect to initial drug content (%)	
Condition	**Rifampicin**	**Ethambutol**
5°C	-14.6	-15.5
25°C/60% RH	-16.3	-13.2
30°C/65% RH	-8.7	-14.6
40°C/75% RH	0.62	-8.8

The change limit of 5% given by the ICH guidelines for stability testing [11] was well exceeded in both the formulations within 3 months. However, this data only reflects on the quantity of active drug and not on any active metabolites that might have been present in the formulation. Thus, although the drug content was found to be out of specification, the formulation might still have sufficient antimycobacterial effectivity. Metabolites were not quantitively determined in this study because of difficulties experienced in obtaining reference standards. It is promising that the tropical conditions showed the least degradation.

Microbial growth

No strains of *E.coli*, *Salmonella spp.* or *Staphylococcus spp.* was detected in either one of the formulations. Furthermore, no yeasts and moulds were detected in either one of the

formulations. This could be ascribed to the high antibiotic drug loading in the formulation. This high antibiotic drug loading was also the reason for the omission of preservatives from both formulations.

CONCLUSION

Rifampicin, isoniazid, pyrazinamide and ethambutol were obtained from different sources for the formulation of the pro-Pheroid™ preparations used in this study. According to good manufacturing practices, the quality of these active ingredients was screened and found to be of acceptable quality. Rifampicin is seen as the most critical component in FDCs regarding its stability and bioavailability, followed by isoniazid and pyrazinamide (in decreasing order). The fact that rifampicin is classified as a type II drug by the Biopharmaceutical Classification System, and presents polymorphic variants, makes this drug less stable and more problematic in the formulation environment. It was therefore necessary to carry out the necessary production controls, of which particle size and polymorphism formed an integral part, to be able to achieve a homogeneous quality of the final product. The crystal form of the rifampicin used throughout this study was determined as form II. According to the available literature, this form is the pharmaceutical preferred form of the drug currently available on the market and shows acceptable dissolution and bioavailability characteristics.

The results of this pilot study revealed that Pheroid™ technology markedly increased the bioavailability of rifampicin in mice plasma. This confirms previous studies where higher plasma levels were obtained with a Pheroid™-based formulation that contained only 60% of the rifampicin dosage in Rifafour-e200®. Isoniazid and pyrazinamide plasma levels were also elevated considerably. Previous studies also showed an increase in the peak plasma concentration (C_{max}) of all active compounds and shortening of the time needed to obtain peak plasma concentrations (T_{max}) in humans. Unfortunately, the pilot study was unable to confirm this in mice as blood was only collected at one point in time.

This pilot study confirmed the expected outcomes and will serve as the basis for a full-scale PK/PD study in the near future. The active drug content was evaluated seven weeks after formulation and was found to be within specifications of 90-110 % of the label claim without any stability enhancing excipients being added to the formulation.

A salient finding from this study is that the barrier packaging of the pro-Pheroid™ formulations plays an important role in the stability of the active pharmaceutical ingredients. This is especially true in ethambutol-containing combinations due to the hygroscopicity of ethambutol. The uptake of moisture creates an acidic environment that leads to instability of rifampicin. The containers used in this study were not able to protect the formulations from moisture. A further shortcoming was identified as dose uniformity within the two formulations. This was evident from the marked differences occurring in the drug content between months. This calls for further intensive studies into the optimization of the manufacturing process of the pro-Pheroid™ formulations. The liquid pro-Pheroid™ component could be increased as a possible solution to dose uniformity problems. However, the pro-Pheroid™ formulations did succeed in increasing the bioavailability of rifampicin, isoniazid and pyrazinamide. This marked increase is also the reason to continue investigating this drug delivery system.

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CHAPTER 5

SUMMARY AND CONCLUSION

This study was build around the immediate action against tuberculosis. A thorough literature study was carried out on tuberculosis. Special attention was paid to the current treatment and control strategies followed to combat the disease. From this literature study, two key problems were highlighted and addressed in this study. These problems were:

- Interactions that counter the stability of drugs in fixed-dose combination products;
- Reduced *in vivo* bioavailability of rifampicin when formulated in fixed-dose combination products.

It was hypothesized that Pheroid™ drug delivery technology might be able to be the solution to these problems in the treatment of tuberculosis. This hypothesis was tested in this study.

For the hypothesis stating that the bioavailability of rifampicin might be increased by Pheroid™ technology, a pilot study was carried out in mice. A Pheroid™-based fixed-dose formulation was administered to one group of mice, whilst Rifafour e-275® was administered to another group of mice. The latter group acted as the positive control. The results from this pilot study were astounding. The Pheroid™ system led to a triple fold increase in rifampicin plasma levels compared to Rifafour e-275®. Further advantages might be observed once a complete blood profile is done.

A series of preformulation activities were performed such as particle size analysis and the characterization of the crystal form of the obtained rifampicin. Reports from available literature suggested that the crystal structure and particle sizes might be responsible for poor solubility of rifampicin. Various methods of analysis include thermal analyis, infrared spectrophotometry and x-ray diffractometry.

The formulation used in the pilot study was adapted slightly for studies in humans. Two pro-Pheroid™ formulations were manufactured on pilot scale. One of these contained isoniazid and pyrazinamide, whilst the other contained rifampicin and ethambutol. The rationale behind this formulation was the separation of isoniazid and rifampicin due to reported instability. These formulations were packaged and subjected to accelerated stability conditions over a period of three months. Unfotunately, the monthly determination of drug content was hampered by poor drug uniformity within the formulations. This was because of the high viscosity of the formulations.

In conclusion, Pheroid™-based formulations containing the current drugs of choice in the treatment of tuberculosis were prepared and tested for stability and *in vivo* bioavailability. The Pheroid™ drug delivery system did succeed in increasing bioavailability of all active ingredients, especially rifampicin. Stability of the formulations was found to rely heavily on the packaging of the product and the separation of rifampicin and isoniazid, but the hygroscopicity of ethambutol remained problematic.

It can be expected that future research will concentrate on optimizing the manufacturing process of pro-Pheroid™ formulations. This must be done to ensure drug uniformity within these formulations. Moreover, some research into the incorporation of Pheroid™-based formulations into various solid dosage forms is underway. These approaches would further improve efficacy and practicability of the Pheroid™ drug delivery system.

APPENDIX A

PHYSICO-CHEMICAL ANALYSIS AND PURITY ASSAYS OF RIFAMPICIN, ISONIAZID, ETHAMBUTOL, AND PYRAZINAMIDE ACTIVE PHARMACEUTICAL INGREDIENTS.

A.1 Introduction

Most drugs are used in a crystalline form and the physico-chemical properties of a drug can affect its solubility, bioavailability and other performance characteristics. A tendency to bioinequivalence has been recorded for various marketed rifampicin-containing products. Rifampicin shows polymorphism and it is believed to be due to numerous possibilities for hydrogen bonding, conformational changes and ionization states within the molecule. According to various references in the literature, polymorphism is one of the most influential factors regarding the bioavailability and therefore the effectiveness of treatment. By the selection of a suitable crystal form of rifampicin from the early development stages, optimum solubility and dissolution rates can be ensured (Henwood *et al.*, 2000).

The particle size of a drug also has an effect on dissolution and solubility. No evidence could be found which suggested that bigger particle sizes of rifampicin are responsible for bioavailability problems. However, smaller particle sizes are necessary for entrapment of these drugs into Pheroids™. Henwood *et al.* (2000) noted that in the case of the drug being very fine (mean particle size of <10 µm), the presence of electrostatic forces may result in lump formation which may result in delayed dissolution and poor bioavailability.

The above-mentioned studies prompted an investigation into the particle sizes and crystal forms of the raw materials used in the formulation of the pro-Pheroid™ combinations, which in turn was used in the pilot study and the accelerated stability study.

A.2 Experimental

A.2.1 Materials

All solvents and reagents were obtained from Merck, South Africa. All solvents were analytical grade, and water appropriate for liquid chromatography was used. Rifampicin (RMP/NC-020/06) and pyrazinamide (PYZ/P-228/06) powders were obtained from Linaria Chemicals Limited. Isoniazid (IF070501) and ethambutol (IF070608) were obtained from Iffect Chemphar (HK) company limited represented by D B Fine Chemicals (Pty) Limited.

A.2.2 Determination of the purity of active drug prior to formulation

The purity of all active drugs used in this study was determined by the Research Institute for Industrial Pharmacy, North West University, Potchefstroom. A compendial HPLC method was used for all assays (USP 30, 2006).

A.2.2.1 Rifampicin

For the standard solution 40 mg of rifampicin RS was accurately weighed and transferred to a 200 ml volumetric flask and dissolved in acetonitrile. The solution was sonicated for 30 seconds to ensure dissolution. 10 ml of this solution was transferred to a 100 ml volumetric flask and diluted with solvent mixture (water, acetonitrile, 1.0 M dibasic potassium phosphate, 1.0 M monobasic potassium phosphate, and 1.0 M citric acid – 640:250:77:23:10) to volume. For the assay preparation rifampicin was used in the above preparation. A mobile phase was prepared consisting of a mixture of water, acetonitrile, phosphate buffer, 1.0 M citric acid, and 0.5 M sodium perchlorate (510:350:100:20:20). This mixture was filtered through a filter of 0.7 µm or finer porosity, and degassed. The chromatograph was equipped with a 254 nm detector and a 4.6 mm x 10 cm column that contained 5 µm packing (Phenomenex Luna®, obtained from Separations, South Africa). The flow rate was 1.5 ml/min. 50 µl of the standard solution, the assay solution was injected separately into the chromatograph, and peak responses of the major peaks were measured.

A.2.2.2 Isoniazid

For the standard preparation a quantity of isoniazid RS was weighed and diluted with the mobile phase to obtain a solution of 0.36 mg per ml. For the assay preparation, about 16 mg was accurately weighed, transferred to a 50 ml volumetric flask and diluted to volume with mobile phase. The mobile phase consisted of 4.4 g of docusate sodium in 600 ml methanol to which 400 ml of water was added and the pH adjusted to 2.5 with 2 N sulfuric acid. The liquid chromatograph was equipped with a 254 nm detector and a 4.6 mm x 25 cm column (Phenomenex Luna®, Separations, South Africa) that contained packing L1. The flow rate was 1.5 ml per minute. Equal volumes (10 µl) of the standard preparation and assay preparation were injected into the chromatograph and the peak responses of the major peaks were measured.

A.2.2.3 Ethambutol

About 200 mg of ethambutol hydrochloride was weighed and dissolved in a mixture of 100 ml of glacial acetic acid and 5 ml of mercuric acetate TS. Crystal violet TS were added and titrated with 0.1 N perchloric acid VS (the colour change at the end point was from blue to blue-green). A blank determination was performed and all necessary corrections were made.

Each milliliter of 0.1 N perchloric acid was equivalent to 13.86 mg of ethambutol hydrochloride.

A.2.2.4 Pyrazinamide

About 300 mg of pyrazinamide was weighed and transferred into a Kjedahl flask. The powder was dissolved in 100 ml water and 75 ml 5 N sodium hydroxide. The flask was connected to a well-cooled condenser by means of a distillation trap and a delivery tube was dipped into 20 ml of boric acid solution (1 in 25). The mixture was boiled for 20 minutes and then boiled vigorously to complete the distillation of the ammonia. The liquid in the receiver was cooled, methyl purple TS was added and titrated with 0.1 N hydrochloric acid VS. A blank determination was done. Each milliliter of 0.1 N hydrochloric acid is equivalent to 12.31 mg of pyrazinamide.

A.2.3 Particle size analysis

The particle size distribution profiles of all active drugs were determined at the Research Institute for Industrial Pharmacy, North West University, Potchefstroom. Particle size distributions in suspension were measured with a Galai-Cis-1 particle size analyser. This analyser uses dual discipline analysis integrating laser diffraction and image analysis for particle sizing. Samples of the powder suspended in a suitable dispersing solution (glycerine) were each placed in a small cuvette and the cuvette fitted into the analyser. A small magnetic stirrer inside the cuvette prevented sedimentation of the particles during the measurement. The acquired data was used to compute means, medians and standard deviations based on the total particle population.

A.2.4 Analysis of rifampicin powder

A.2.4.1 Infrared spectrophotometry

IR spectra were recorded on a Nicolet Nexus 470-FT-IR spectrometer over a range of 600 - 4000 cm^{-1}. The diffuse reflectance method was used.

A.2.4.2 X-ray powder diffractograms

The X-ray powder diffraction profiles were obtained at room temperature with a Bruker D8 advance diffractometer (Bruker, Germany). The measurement conditions were: target, Cu: K_{β}-filter, Ni; voltage, 40 kV; current, 30 mA; divergence slit, 2 mm; anti scatter slit, 0.6 mm: detector slit, 0.2 mm; scanning speed, 2°/min (step size 0.025°, step time, 1.0 sec). Approximately 200 mg sample was loaded into an aluminium sample holder, taking care not to introduce a preferential orientation of the crystals.

A.2.4.3 Differential scanning calorimetry

DSC thermograms were recorded with a Mettler Toledo DSC822^{e}700 instrument. The measurement conditions were as follows: sample weight, approximately 2 mg; sample holder, aluminium crimp cell; gas flow, nitrogen at 30 ml/min; heating rate, 10°C per minute.

A.3.1 Results and discussion

A.3.1.1 Particle sizes of active pharmaceutical ingredients

The median particle size of rifampicin was reported to be 56.43 µm. Isoniazid powder had a median particle size of 91.81 µm, while pyrazinamide and ethambutol had median particle sizes of 40.26 µm and 40.90 µm, respectively. All powders had a diffuse particle size distribution which ranged from 2 – 200 µm (figures A.1 – A.4).

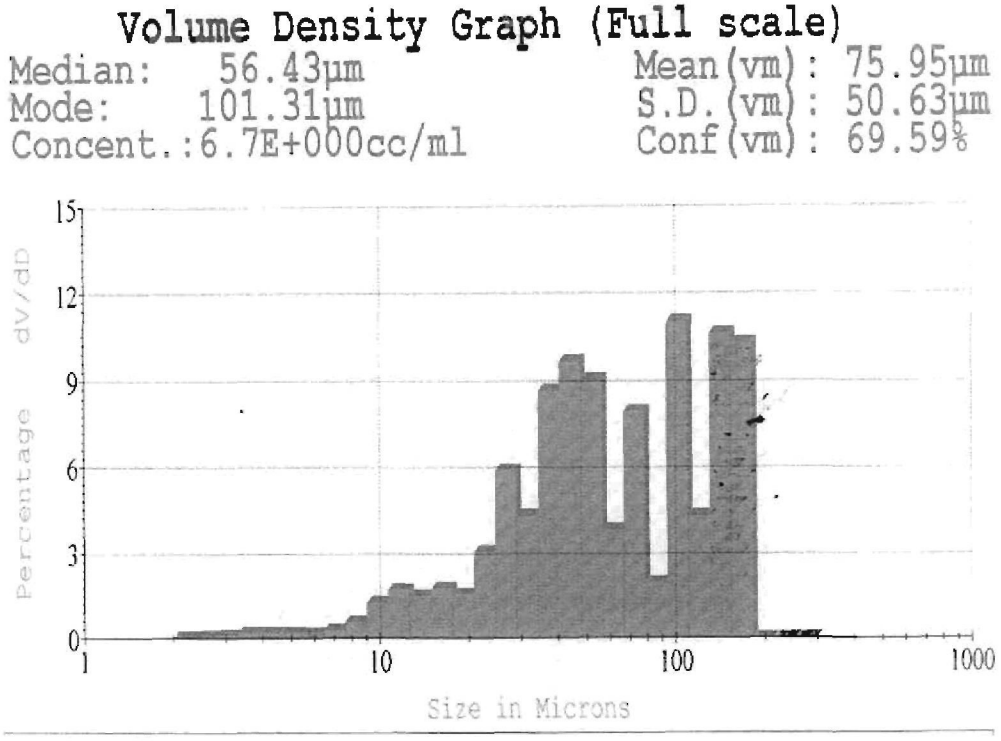


Figure A.1: Particle size distribution profile of rifampicin

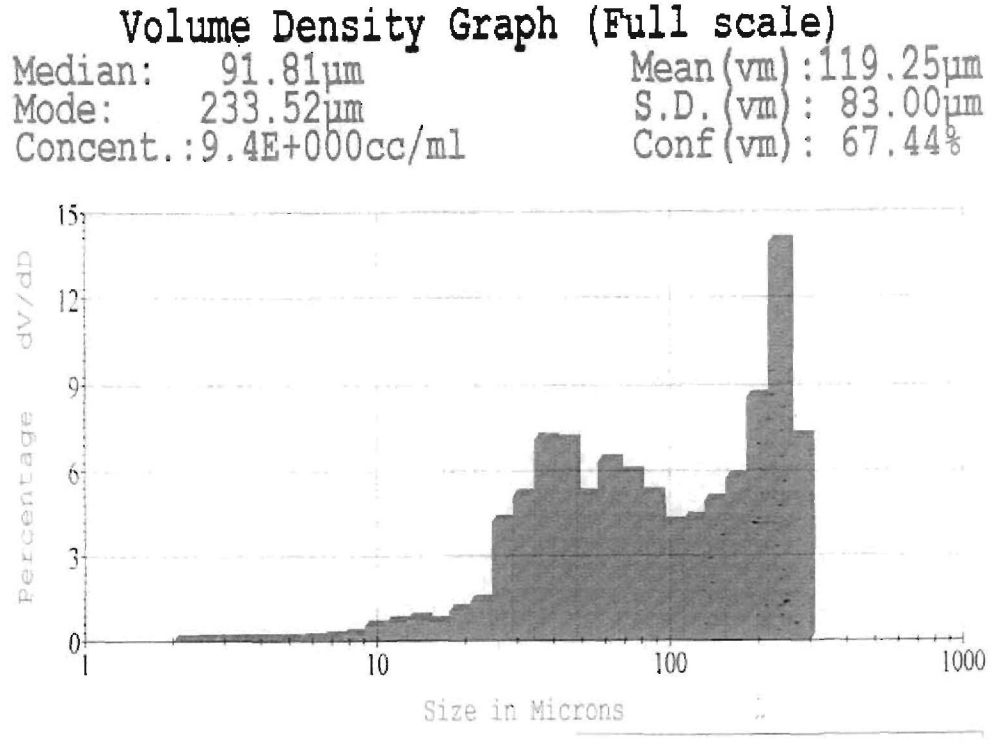


Figure A.2: Particle size distribution profile of isoniazid

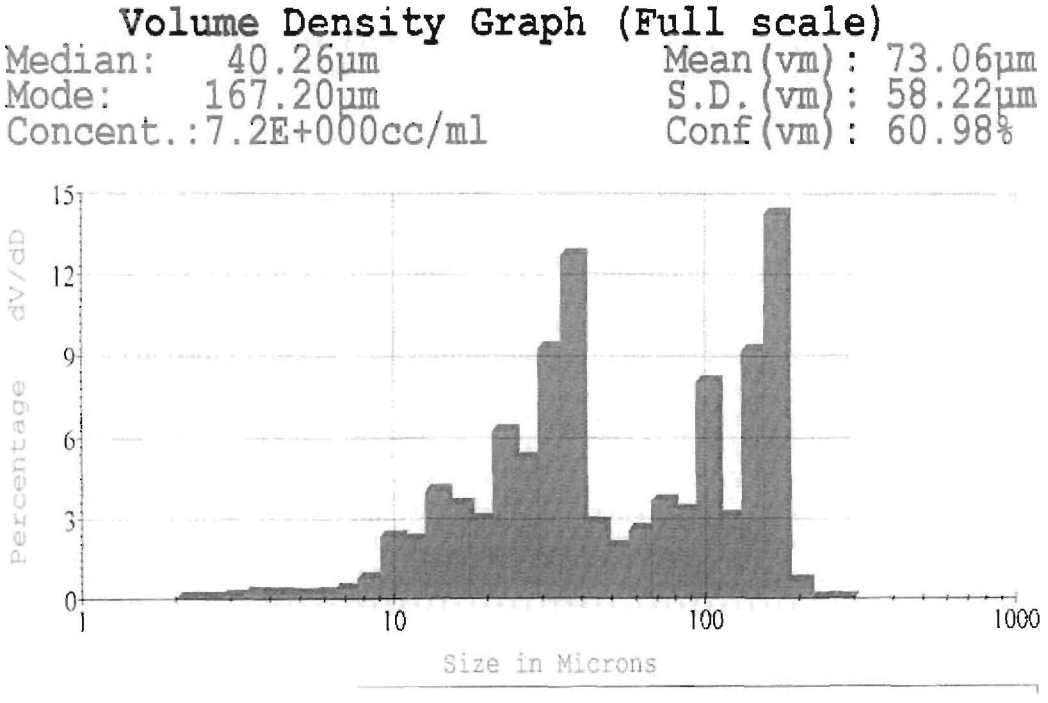


Figure A.3: Particle size distribution profile of pyrazinamide

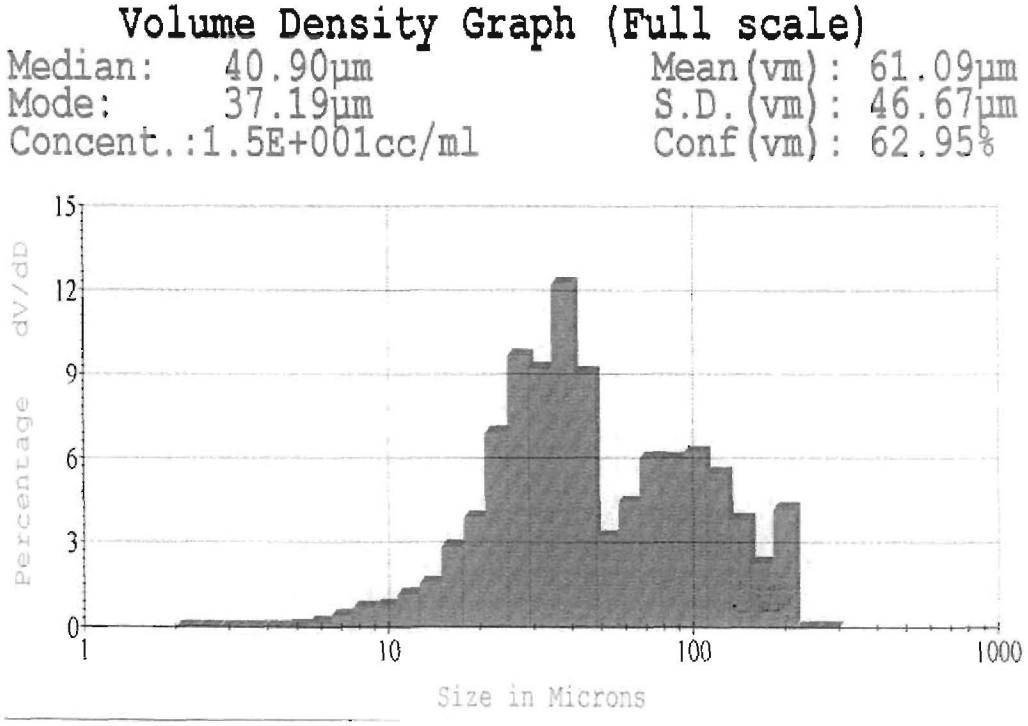


Figure A.4: Particle size distribution profile of ethambutol

A.3.1.2 Purity of active pharmaceutical ingredients used in formulation

All raw materials used in this study had to conform to quality standards and purity of the substances was assayed according to methods described in section A.2.2. The results are summarized in table A.1.

Table A.1: Assay values for raw materials

Active ingredient	Assay (%)
Rifampicin	101.4
Isoniazid	99.3
Pyrazinamide	100.4
Ethambutol	99.6

All APIs conformed to USP assay values and identity was confirmed by the known infrared spectra.

A.3.1.3 Characterization of rifampicin polymorphic form

Reports of the characterization of rifampicin crystals by using IR spectroscopy were found in the literature. Therefore, an IR spectrum (figure A.5) of a rifampicin sample was obtained and compared. From the IR results it seems that the rifampicin powder used in this study showed similarities to that of rifampicin form II. Because the IR results were not conclusive, X-ray powder diffractometry (XRPD) was used. XRPD is a highly effective method of distinguishing different polymorphic forms in a solid.

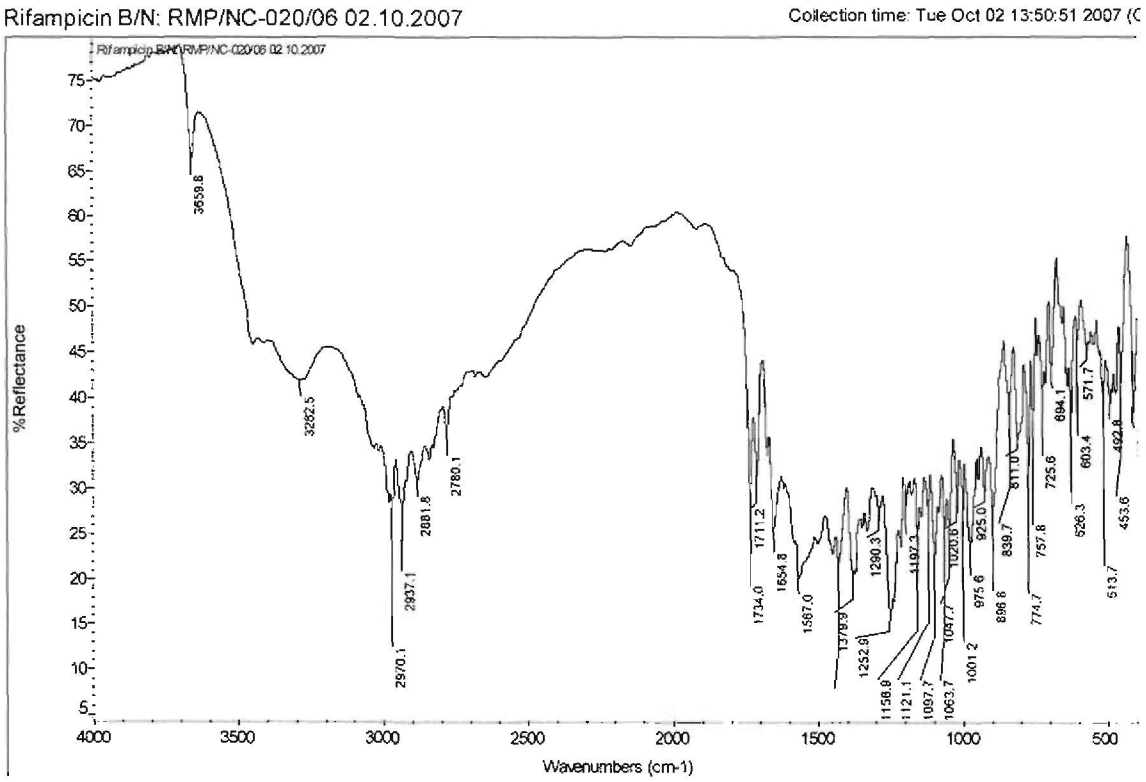


Figure A.5: IR spectrum of the Rifampicin sample

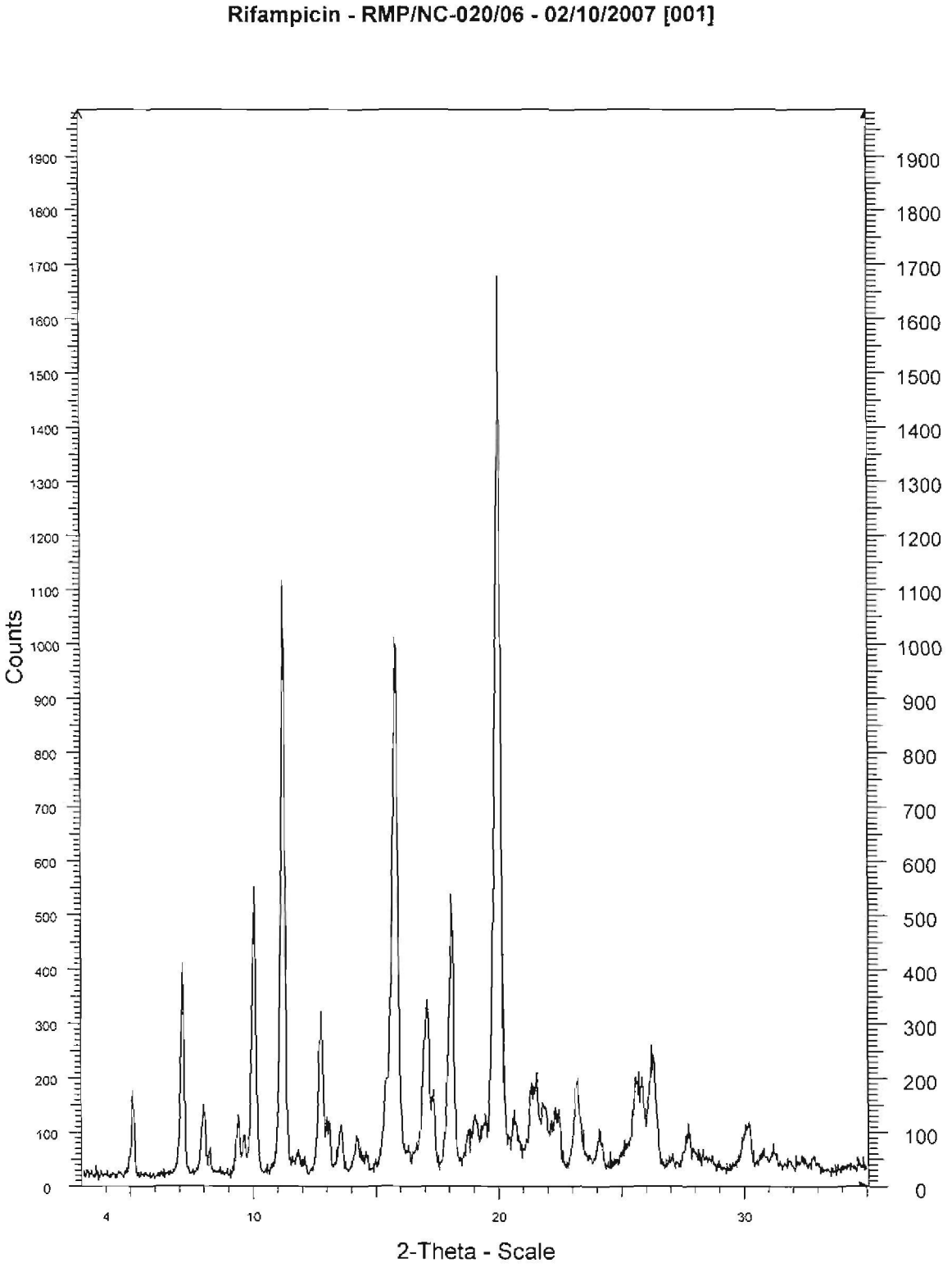


Figure A.6: XRPD diffractogram of rifampicin sample

Table A.2: Intensity values (I/Io) at the main X-ray diffraction peak angles (°2θ) of the rifampicin sample

Main peaks	°2θ	(I/Io x 100)
1	5.07	10.3
2	7.10	24.3
3	7.93	8.9
4	8.20	4.0
5	10.02	32.7
6	11.21	66.4
7	12.75	19.1
8	13.56	6.6
9	14.19	5.4
10	15.81	60.1
11	17.08	20.3
12	18.10	31.9
13	19.03	7.7
14	20.04	100
15	20.64	8.3
16	21.36	11.2
17	23.21	11.4
18	25.67	11.4
19	25.85	11.9
20	26.31	14.4

The X-ray powder diffractogram (fig. A.6) of the rifampicin sample showed resemblance to rifampicin form II as described by Henwood *et al.* (2000).

The peak intensity count of 1681 showed that the sample was more crystalline in comparison with peak intensity count values of below 1000 which Henwood *et al.* (2000) reported for a mixture of Polymorph II and an amorphous form. Where amorphous material is concerned,

the peak intensity counts were low and the incoherent scattered radiation from the amorphous fraction of the sample adds to the general background of the powder pattern, i.e. higher background scatter signs on diffractometer tracing. Slight differences in *d* spacings and corresponding relative intensities were observed between the results reported here and the results of Henwood *et al.* (2000) and could be explained by the fact that different X-ray powder diffractometers and measurement conditions were used.

Differential scanning calorimetry (DSC) was used as another means to characterize the rifampicin powder and the DSC thermogram is shown in figure A.7. Although slight differences in peak sizes and positions were observed, the general appearance of the thermogram was comparable with that reported for form II (Henwood *et al.*, 2000). The sample showed an endotherm at 189 °C – 192 °C, which corresponded to the melting temperature of rifampicin. The temperature, at which decomposition occurs, was observed to be around 250 °C.

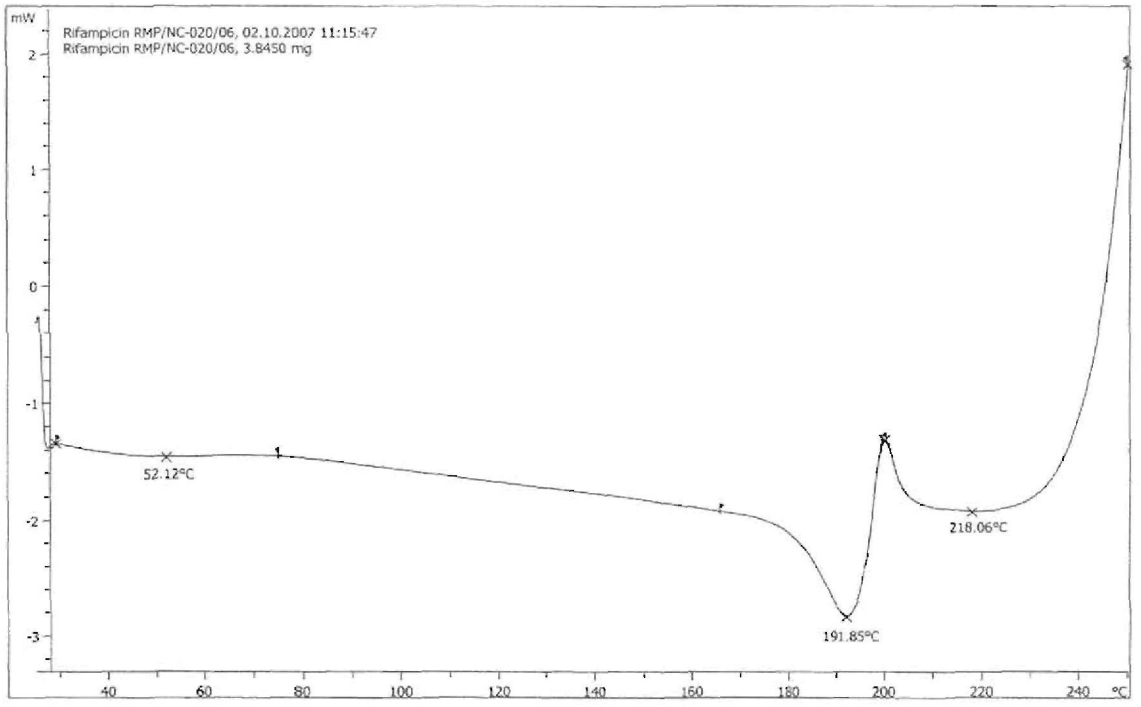


Figure A.7: DSC thermogram of rifampicin sample

APPENDIX B

PILOT STUDY TO DETERMINE BLOOD PLASMA LEVELS OF A PRO-PHEROID™ FORMULATION IN CD 57 INBRED MICE AND THE EVALUATION OF PRODUCT STABILITY SEVEN WEEKS AFTER MANUFACTURING.

B.1 Introduction

When formulating a fixed dose combination against tuberculosis, acceptable bioavailability must be proven. This is accentuated by the fact that rifampicin shows considerable decomposition and thus decreased bioavailability when formulated in combination with isoniazid, ethambutol and pyrazinamide.

Our hypothesis is that the absorption of anti-tuberculosis drugs can be greatly improved by using Pheroid™ technology. Pheroid™ is a patented carrier system (North-West University) that actively transports drug actives through various physiological barriers (refer to chapter 3). This investigation was the first step in testing this hypothesis in animals. The mouse was selected as model for all animal studies because of several reasons given below.

Orme (2003) has found that the inbred mouse provided the largest amount of information of all of the several animal models that were tested, in terms of predicting mechanisms of immunity that is similar to those found in humans. This conclusion followed the realization that extensive similarities exist between mice and humans in terms of the basic immune response.

The physical size of the mouse poses some challenges for *in vivo* experimental work and significant physiological variations may occur between individual mice. This pilot study was used to determine important parameters (such as number of experimental groups and number of mice per group to be used) in the planning of a larger future study.

B.2 Objectives

The aim of this study was to:

- Determine the number of experimental groups and number of mice per group to plan a statistically accurate pharmacokinetic and pharmacodynamic (PK/PD) study;
- Determine and correct any potential problems in the execution of the methods that will be used to conduct future *in vivo* studies with mice;
- Gain a better understanding of the potential of Pheroid™ to act as absorption enhancer and drug carrier system for anti-tuberculosis drug delivery;

- Act as indication of the physical and chemical stability of the candidate pro-Pheroid™ combination product against tuberculosis.

B.3 Experimental design

B.3.1 Chemicals and drugs

All solvents were analytical grade and were obtained from Sigma-Aldrich in South Africa. Water appropriate for liquid chromatography was used. Rifampicin (RMP/NC-020/06) and Pyrazinamide (PYZ/P-228/06) powders were obtained from Linaria Chemicals Limited. Isoniazid (IF070501) and Ethambutol (IF070608) were obtained from D B Fine Chemicals (Pty) Limited, South Africa.

B.3.2 Animals

Black CD57 inbred mice of both sexes (weighing 26-28 g) were obtained from South African Vaccine Producers, Sandringham, South Africa. Animals were housed in the Animal Research Centre at the Potchefstroom campus of the North-West University under normal temperature, humidity and light conditions. The animals were fed a standard pellet diet and water *ad libitum*.

B.3.3 Preparation of test formulations

Control: One Rifafour® tablet was dissolved in distilled water.

Pro-Pheroid™ test formulation: pro-Pheroid™ was manufactured by the Unit of Drug Research and Development, North-West University, Potchefstroom. Two Pro-Pheroid™ formulations each containing two drugs (Rif/Pyr) and (Inh/Eth) were prepared separately. The formulations were mixed in 1:2 ratios before administration.

B.3.4 Investigation into the suitability of the pro-Pheroid™ formulation for oral gavage in mice

Syringes, fitted with gavage needles, were filled with the two separate pro-Pheroid formulations and each formulation was tested for its ability to pass through the needle.

B.3.5 *In vivo* bioavailability study from pro-Pheroid™

Mice were divided into two groups, each of three or four animals. The doses of R, H, Z and E used in mice were 11.5, 5.7, 30.7 and 21.1 mg/kg, respectively. Group B acted as positive control and received Rifafour® (dissolved in water); group C (negative control) received the pro-Pheroid™ formulation. These test products were administered via oral gavage on a daily

basis for four days. All dosages were further administered on the same time each morning. The mice were sacrificed 20 minutes after administration on the fourth day.

B.3.6 Plasma collection and analysis

The mice were decapitated and whole blood was collected into Eppendorf™ tubes. All tubes were immediately put on ice until they could be centrifuged. The whole blood was centrifuged for 7 minutes at 4000 rpm. Plasma were separated and transferred to cryotubes for storage in liquid nitrogen. Rifampicin undergoes rapid oxidation to 3-formyl-rifampicin and 1-amino-4-methyl-piperazine. It was therefore critical that blood samples were collected, centrifuged and the plasma samples were stored within 15 minutes. The frozen cryotubes were transported on dry ice to the University of Cape Town (South Africa) where the samples were assayed.

B.3.7 HPLC determination of APIs in pro-Pheroid™ formulation

The HPLC method used to determine the drug content of APIs in the pro-Pheroid™ formulations were adapted from an existing validated method (Redelinghuys, 2006). This method was revalidated in our laboratories (Appendix C).

For isoniazid, pyrazinamide and ethambutol:

A pro-Pheroid™ sample amount equivalent to 100 mg ethambutol (from the formulation containing E and H) was transferred to a 500 ml volumetric flask. The pro-Pheroids™ were dissolved with methanol to release the active drugs. The released drugs were dissolved in 400 ml extra pure water by ultrasonic shaking for about 15 minutes. The solution was diluted to 500 ml. A standard solution was prepared by dissolving 27.3 mg isoniazid reference standard (RS), 145.5 mg pyrazinamide RS and 100 mg ethambutol RS in water. 20 µl of solution was injected onto a 5 µm particle size, reverse phase, C-18 Phenomenex Luna® column (15 cm x 4.6 mm).

The mobile phase consisting of ammonium acetate, copper (II) acetate and water (pH 5.0) was pumped at a flow rate of 2.0 ml/min. Detection was at 270 nm.

For Rifampicin:

A pro-Pheroid™ sample equivalent to 150 mg rifampicin (from the formulation containing R and Z) was dissolved in 100ml methanol. This solution was filtered and 5 ml was diluted to 10 ml with methanol. A standard solution was prepared by dilution of 150 mg of rifampicin RS as described above. 20 µl of solution was injected onto a 5 µm particle size, reverse phase, C-18 Phenomenex Luna® column (25 cm x 4.6 mm).

The mobile phase consisting of a v/v mixture of 6 volumes phosphate buffer (pH 7) and 4 volumes of methanol was pumped at a flow rate of 1 ml/min. Detection was at 254 nm.

B.4 Results and discussion

B.4.1 Pre-formulation of pro-Pheroid™ formulation for mice

The viscosity of the formulation containing rifampicin and pyrazinamide was too high and was therefore unable to pass through the gavage needle. An additional 6.76 g of pro-Pheroids™ were added to the formulation. The latter formulation was found to be suitable for the successful administration to mice. The initial drug concentrations of the formulations are given in table B.3.

B.4.2 Pilot bioequivalence study

Table B.1: Drug plasma levels in individual mice

Active ingredient	Drug Plasma concentration (µg/ml)							
	Negative Control		Rifafour e-275®		Pro-Pheroid™			
	1	2	1	2	1	2	3	4
Rifampicin	0	0	7.2	2.06	59.2	80.9	74.9	20
Isoniazid	0	0	3.63	1.35	9.03	9.73	14.2	3.4
Ethambutol	0	0	1.3	-	1.78	4.89	2.88	1.605
Pyrazinamide	0	0	32.7	14.5	104	140	111	35.5

In table B.1 the pro-Pheroid™ formulation showed a major improvement in terms of bioavailability over the Rifafour® group. At closer inspection of each mouse's individual data, the following was noted. Mouse B2 had lower plasma levels than B1 and C4 is lower than C1-C3. This was consistent with the INH, ETH and PYR results.

However, it was discovered that a faulty dosage calculation resulted in the overdosage of the mice that were administered the pro-Pheroid™ formulation. Data was correlated to the corresponding Rifafour e-275® data and are given in table B.2.

Table B.2: Correlated drug plasma levels

Active ingredient	Mean Drug Plasma concentration (µg/ml)			
	Negative Control	Rifafour e-275®	Pheroid™	Percentage enhancement ratio (%)
Rifampicin	0	23.49	71.6	205%
Isoniazid	0	9.15	10.98	20%
Ethambutol	0	4.3	3.18	-26%
Pyrazinamide	0	99.37	118.3	19%

In this data set, C4 was not included in calculations because of a huge variation from the rest of the mice. The reasons for this were considered one of the following:

1. C4 could have received Rifafour® instead of the pro-Pheroid™ formulation, because the values corresponds to that group B.

2. Administration was not staggered, which allowed more time to pass between administration and decapitation. Therefore, C4 could have passed the maximum drug concentration (C_{max}) by the time the blood was collected.

Because of the procedure followed, the wrong administered formulation was ruled out as the reason for C4 being lower than the rest. Therefore, the time of blood collection was the most plausible cause of the low plasma level found for mouse C4. This was confirmed with the value of B2 also being lower than that of B1. The figures below (figure B.1 – B.4) show plasma concentrations calculated for comparative administered dosages of the active compounds.

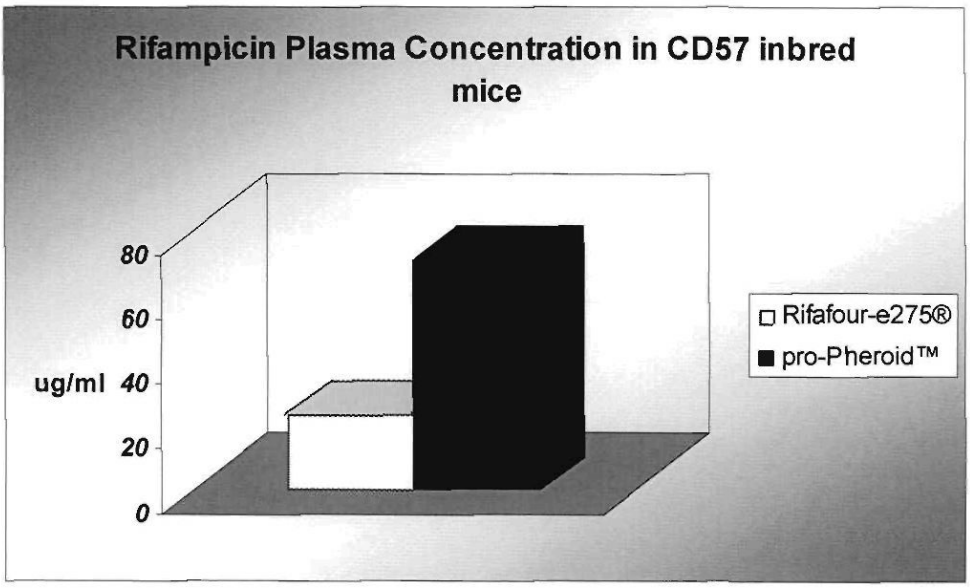


Figure B.1: Rifampicin plasma concentrations at t=20 minutes

The pro-Pheroid™ formulation showed a 3 times increase in plasma levels of rifampicin at t=20 min when compared to Rifafour-e275®.

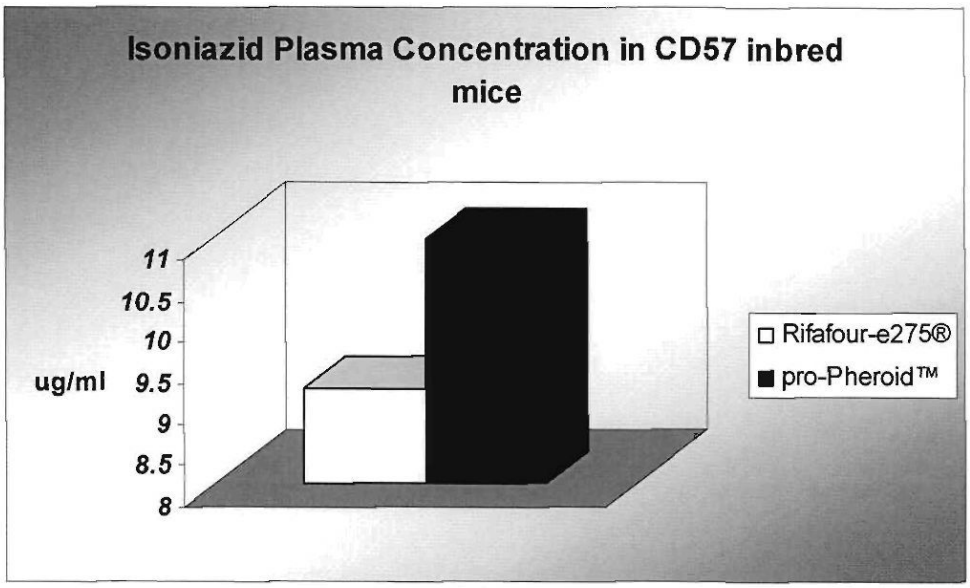


Figure B.2: Comparative isoniazid plasma concentrations at t=20 minutes

The comparative isoniazid plasma levels were increased at t=20 min in the case of the pro-Pheroid™ formulation.

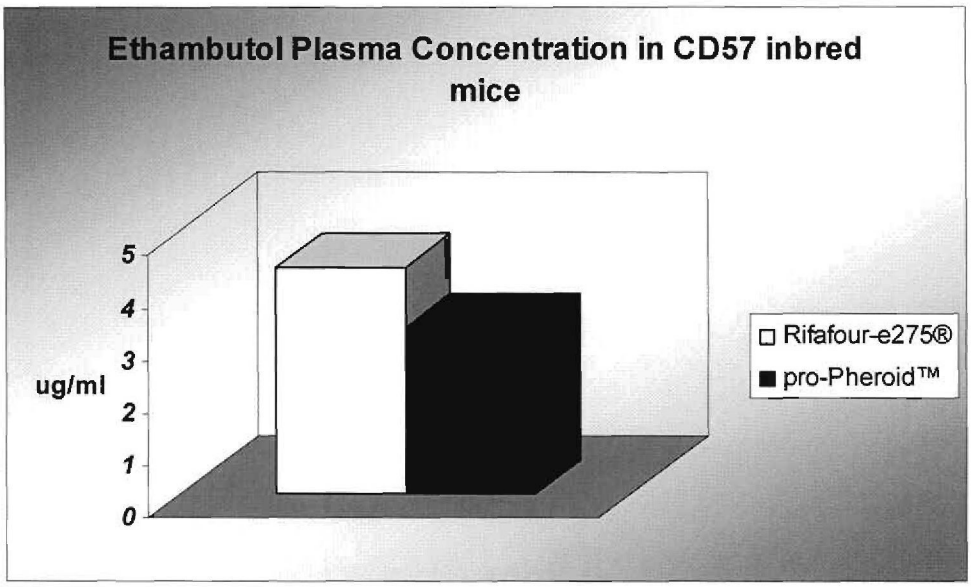


Figure B.3: Comparative ethambutol plasma concentrations at t=20 minutes

The plasma levels of ethambutol were found to be slightly less at t=20 min with the pro-Pheroid™ formulation than with the Rifafour®. The plasma levels of ethambutol have given a clear indication that the maximum concentration of the drug may have been passed at 20 minutes. It is hoped that a further study with more points in time will be able to confirm this to be the true.

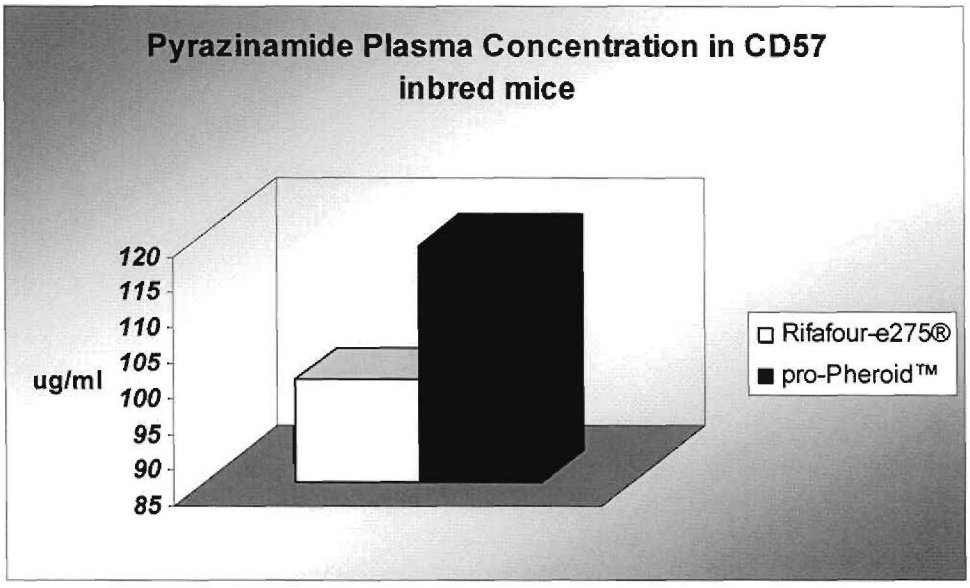
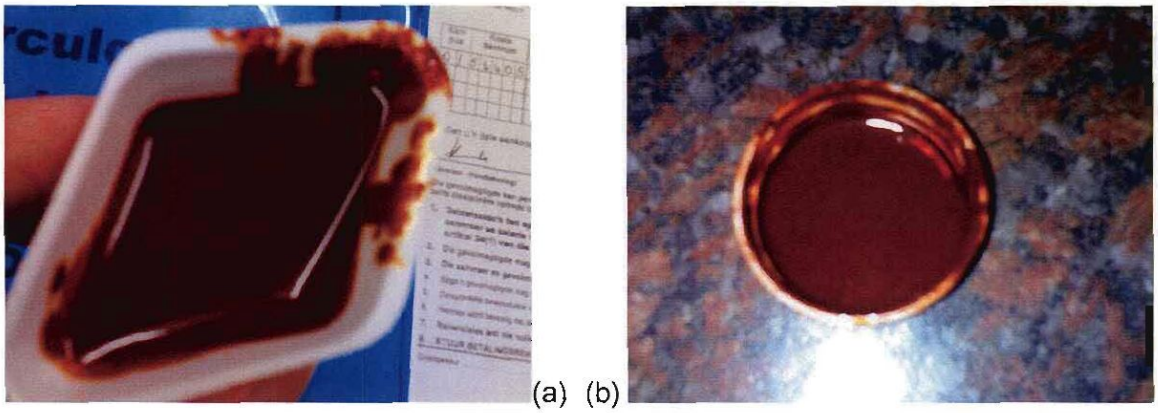


Figure B.4: Pyrazinamide plasma concentrations at t=20 minutes

In the case of pyrazinamide, an elevation of plasma levels were found at t=20 min with the pro-Pheroid™ formulation.

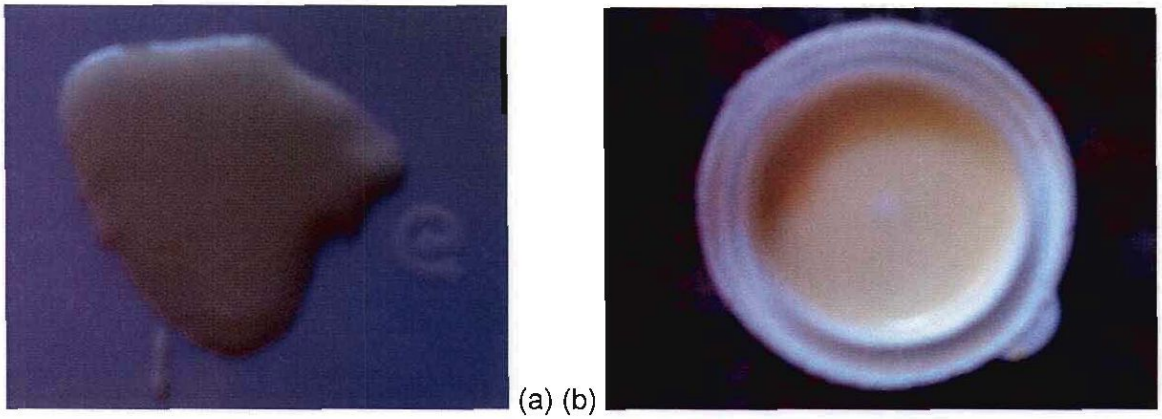
B.4.3 Stability of the pro-Pheroid™ formulations

Both pro-Pheroid™ formulations were closely observed for a change in the stability that may have occurred during the pilot study. Figures B.5 and B.6 shows the respective formulations on the date of manufacture and seven weeks thereafter.



(a) (b)

Figure B.5: Figure **(a)** shows the pro-Pheroid™ formulation containing rifampicin and pyrazinamide on day of manufacture. Figure **(b)** show the same formulation after 49 days of storage at 5°C.This formulation kept its characteristic brick-red colour and no changes in colour or appearance was observed.



(a) (b)

Figure B.6: Figure **(a)** shows the pro-Pheroid™ formulation containing isoniazid and ethambutol on day of manufacture. Figure **(b)** show the same formulation after 49 days of storage at 5°C.

This formulation kept its characteristic beige colour and no changes in colour or appearance were observed.

Furthermore, the drug content was determined for all active compounds in both formulations after 49 days of storage at 5°C. These values are given in table B.3.

Table B.3: Initial drug concentrations in pro-Pheroid™ formulations

API	Initial drug concentration (mg/ml)	Drug concentration after 49 days of storage at 5°C (mg/ml)
Rifampicin	126.4	125.6
Pyrazinamide	280	303.5
Isoniazid	106.2	104.4
Ethambutol	354.4	333.5

The drug content of both formulations remained between specifications of 90-110 % of the initial values and no significant changes in physical appearance were visible.

APPENDIX C

PRECLINICAL INVESTIGATION INTO THE STABILITY OF TWO PRO-PHEROID™ FIXED DOSE COMBINATIONS

C.1 Introduction

As mentioned in chapter 2, fixed-dose combination (FDC) products have several distinct advantages over single drug formulations in the treatment of tuberculosis. However, serious concern was expressed on the utility of these products due to quality problems. Chapter 2 dealt with the behaviour and properties of the current four first-line drugs used in tuberculosis treatment. With all factors taken into account, two pro-Pheroid™ formulations were manufactured and a formal stability study was designed according to a set of guidelines published by the International Conference on Harmonization (ICH) (ICH, 2003). Three different stability storage conditions are given as long term (25°C/60% RH), intermediate (30°C/65% RH) and accelerated (40°C/75% RH) conditions. The pro-Pheroid™ formulations were subjected to all three conditions for 6 months. Due to time constraints, this dissertation will only report the findings of the first three months. This stability study covered the physical, chemical and microbiological attributes of both pro-Pheroid™ formulations. A stability indicating analytical method was used to determine the drug content in the formulations. According to ICH guidelines, all analytical procedures must be fully validated (ICH, 2003).

Validation of an analytical procedure is a process used to ensure that the performance characteristics of the specific procedure meet the requirements for the intended applications (USP, 2007). In this study a compendial analytical method was used and hence it was only needed to revalidate certain validation parameters. Transfer of a validated analytical method serves to establish that the specific method can be transferred from one laboratory to another without negatively affecting the performance characteristic of the method. All the procedures are documented and serve as evidence. Results should be compared to acceptance criteria and where the results are not satisfactory, it is necessary to find the source of the problem and take corrective action. Thus, this process is necessary to verify that the method is suitable for the purpose for which it was intended and that acceptable results will be obtained by the individual that will be using the validated method. This appendix reports on the transfer validation of the HPLC method used in the drug content determination as well as the stability aspects that were investigated.

C.2 Definitions of validation test parameters

C.2.1 Specificity

Specificity can be defined as the ability to distinguish the analyte from any other components that may be present, such as impurities and degradation products. Lack of specificity of an analytical method may however be compensated for by other supporting analytical procedures (USP, 2007).

C.2.2 Linearity and Range

The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written (USP, 2007).

C.2.3 Precision

The precision of an analytical method gives the degree of agreement among individual test results when the procedure is applied to multiple samplings of a homogenous sample.

C.3 Origin of the method

The method used throughout this study was developed and validated by Redelinghuys (2006) as part of a doctoral thesis. The method is suitable for the determination of rifampicin, isoniazid, ethambutol and pyrazinamide from 4 fixed dose combination products.

C.4 Chromatographic conditions

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

Column: Phenomenex Luna C-18 (150 mm x 4.6 mm, 5 µm)

Mobile phase: 50 g ammonium acetate weighed into a 1000 ml volumetric flask, dissolved and made up to volume with water. The pH was adjusted to 5.0 with glacial acetic acid. Mix 940 ml of this solution with 60 ml methanol.

Flow rate: 2.0 ml/min

Injection volume: 20 µl

Detection: 270 nm

Retention times: About 2.9 and 3.7 minutes for isoniazid and pyrazinamide, respectively.

Solvent: 4 % methanol solution and water

C.5 Preparation of sample solutions

- Accurately weigh an amount of the pro-Pheroid™ formulation equivalent to 5.4 mg of isoniazid (about 54.6 mg of the pro-Pheroid™ formulation).
- Transfer the formulation to a 100 ml volumetric flask, and dissolve in 3.2 ml methanol.
- Add about 77 ml of water and shake well for about 15 minutes. Make up to volume with water.
- Filter the solution through a 0.45 µm filter, discarding the first few milliliters of the filtrate.
- Use amber-coloured glass HPLC vials and analyze by means of HPLC.

C.6 Preparation of the standard solution

- Accurately weigh 5.46 mg isoniazid reference standard and 29.1 mg pyrazinamide reference standard into a 100 ml volumetric flask.
- Dissolve in about 80 ml of water.

- Shake well for about 15 minutes and make up to volume with water.
- Analyze by means of HPLC.

C.7 Calculations

The pro-Pheroid™ formulation contained:

Isoniazid	10g/100g
Pyrazinamide	57g/100g
Pro-Pheroid™	33g/100g

The prepared sample solution contained 55 µg/ml isoniazid and 311.2 µg/ml pyrazinamide.

The equation that was used for the calculation of content:

$$\text{mg/sample} = \frac{\text{Sample peak area x std. mass (mg) x std. potency (\%) x average sample mass (g)}}{\text{Standard peak area x weighed sample mass (g) x 100}}$$

C.8 Transfer validation test procedures

C.8.1 Specificity

C.8.1.1 Method

- Prepare a solution from the placebo pro-Pheroid™ formulation similar to preparing the sample solution (See section C.4).
- Prepare a sample solution as described in section C.4 and dilute 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
- Incubate the prepared solutions overnight in closed containers at 40 °C / 75 % RH to bring about degradation.
- Analyze by means of HPLC.

C.8.2 Linearity and Range

C.8.2.1 Method

- Prepare a standard solution as described in section C.5.

- Inject variable volumes to obtain standards from 10 – 150 % of the expected sample concentration.

C.8.3 Precision

C.8.3.1 Intra-day precision

C.8.3.1.1 Method

- Prepare sample solutions (section C.4) in triplicate by weighing amounts equivalent to 80 %, 100 % and 120 % of 27.3 mg of isoniazid.
- Analyze in duplicate by means of HPLC.

C.8.3.2 Inter-day precision

C.8.3.2.1 Method

- Use the same sample prepared above (at 100 % of the sample concentration).
- Analyze this sample in triplicate on two more occasions on different days.

C.8.4 System repeatability

C.8.4.1 Method

- Inject a sample six times consecutively.

C.9 Acceptance criteria

A summary of acceptance criteria is given in table C.1 for the entire scope of test procedures used in this study.

Table C.1: Acceptance criteria for validation test procedures

Parameter	Acceptance criteria
Specificity	The placebo should not generate any peaks that will interfere with the determination of any of the active ingredients. If extra peaks are formed under stress conditions, it should be discernible from those of the active ingredients.
Linearity and Range	Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.
Precision	Relative standard deviation (RSD) of 2.0 % or less.
System repeatability	The peak area and retention times should have an RSD of 2.0 % or less.

C.10 Transfer validation results

C.10.1 Specificity

The chromatogram of the placebo (Figure C.1) was examined for any peaks that may have interfered with those of the active ingredients. No interfering peaks were found. All the extra peaks formed under stress conditions (Figure C.2 – C.5) were found to be discernible from the active ingredients.

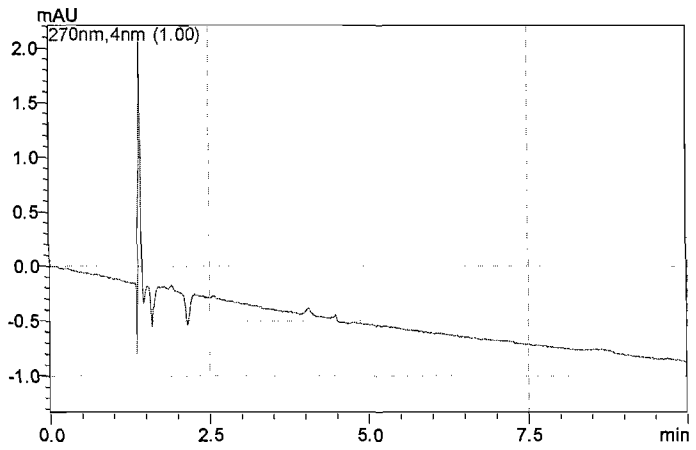


Figure C.1: Chromatogram of a placebo solution.

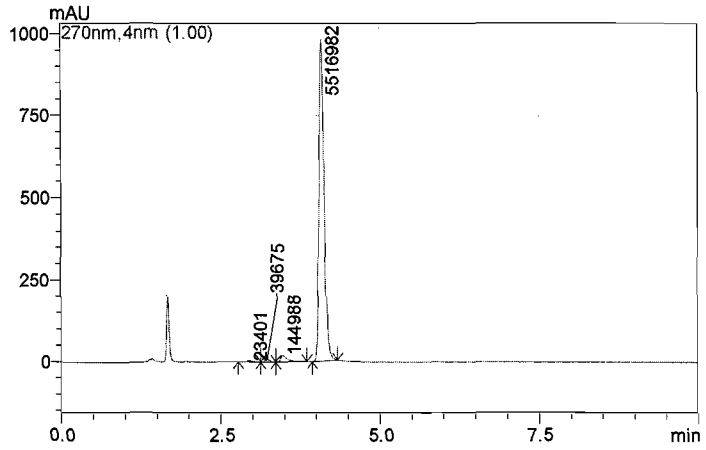


Figure C.2: Sample solution stressed overnight in 0.1 M hydrochloric acid at 40 °C.

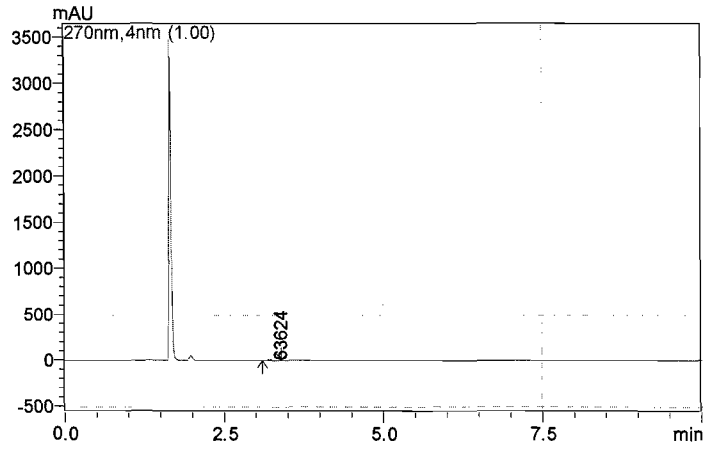


Figure C.3: Sample solution stressed overnight in 0.1 M sodium hydroxide at 40 °C.

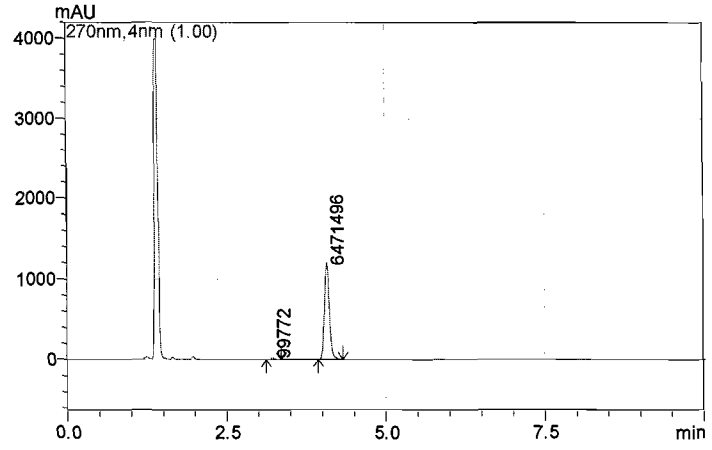


Figure C.4: Sample solution stressed overnight in 10% hydrogen peroxide at 40 °C.

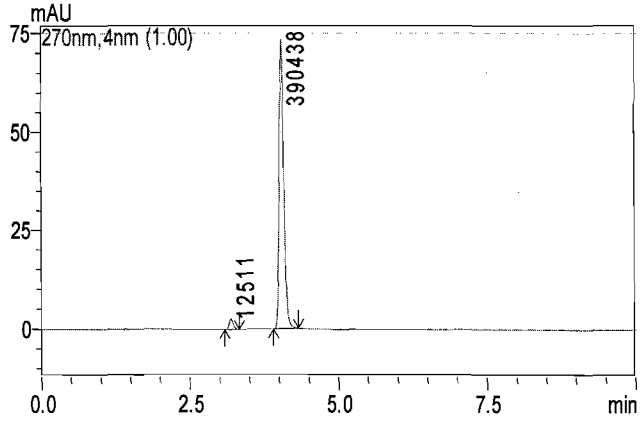


Figure C.5: Standard solution containing isoniazid and pyrazinamide.

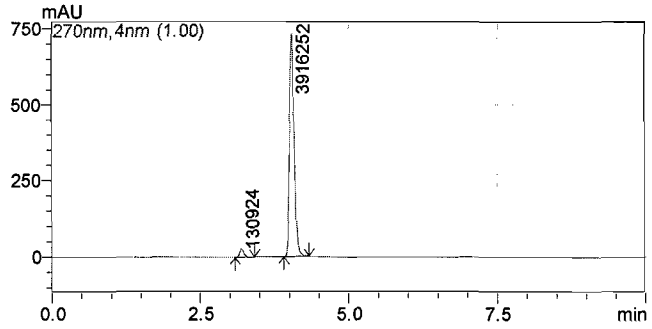


Figure C.6: Sample solution containing isoniazid and pyrazinamide.

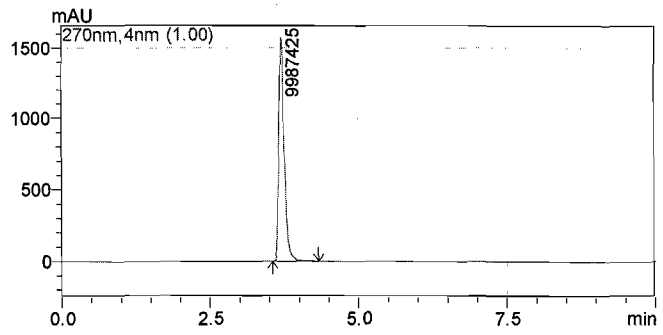


Figure C.7: Standard solution containing pyrazinamide.

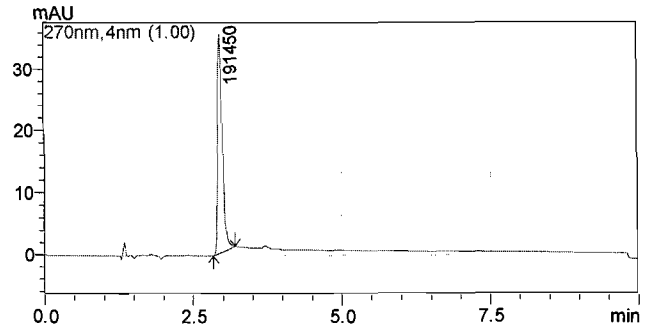


Figure C.8: Standard solution containing isoniazid.

C.11 ISONIAZID

C.11.1 Specificity

Table C.2: Validation results for isoniazid to determine method specificity

Stress condition (40 °C)	% Degradation of isoniazid
0.1 M Hydrochloric acid	86.4
0.1 M Sodium Hydroxide	78.2
10 % Hydrogen Peroxide	65.8

Conclusion

Degradation ranging from 65% to 86% occurred in samples mixed with 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide. None of the additional peaks that were formed under stress conditions interfered with those of the active ingredients. Thus, specificity of the method conformed to the set acceptance criteria in table C.1.

C.11.2 Linearity and Range

Table C.3: Validation results for isoniazid to determine linearity and range

µg/ml	Mean Area
5.5	92829
11.5	192813
16.5	298122
22.5	407789
27.5	516623
33.5	625244
38.5	756374
44.5	877015
49.5	963331
55.5	1106954
60.5	1222947
66.5	1354181

Table C.4: Regression statistics of isoniazid results of linearity and range

R squared	0.9988	**Lower 95 %**	**Upper 95 %**
Intercept	-37530	-58320	-16740
Slope	20810	20290	21320

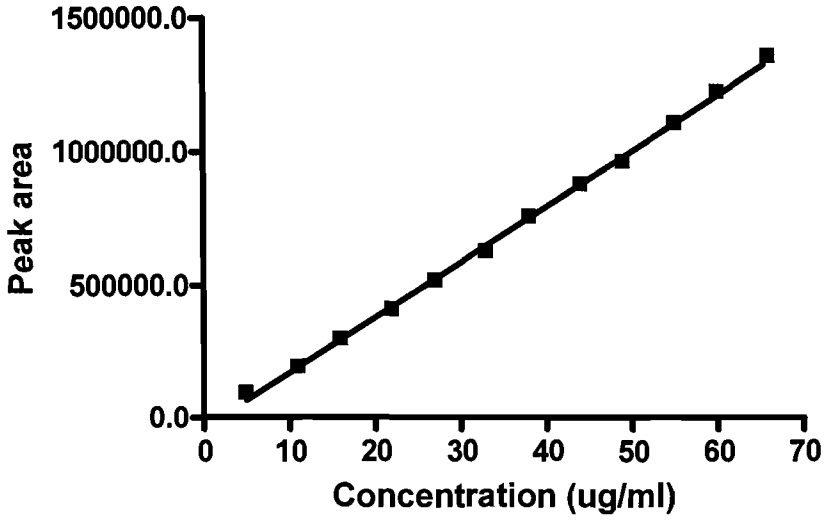


Figure C.9: Linear regression graph for isoniazid to determine linearity and range.

Conclusion

The method is linear over the concentration range 5 – 65 µg/ml as shown in figure C.9. The regression coefficient is 0.9988.

C.11.3 PRECISION

C.11.3.1 Intra-day precision

Table C.5: Validation results for isoniazid to determine intra-day precision

Sample mass (mg)	Mean Area	Recovery (%)
40.2	106070	97.6
45.5	121683	99.0
39.7	107154	99.9
46.7	129408	102.5
55.3	146295	97.9
53.4	140820	97.6
68.8	184584	99.3
66.7	178665	99.1
53.4	177243	101.8
	Mean	99.4
	SD	1.8
	RSD %	1.8

Conclusion

Intra-day precision was satisfactory with a RSD of 1.8 %.

C.11.3.2 Inter-day precision

Inter-day precision results are given in table C.6.

Table C.6: Validation results for isoniazid to determine inter-day precision

	Day 1	Day 2	Day 3	Between days
	143195	189576	172631	143436
	146295	191210	173975	190837
	140820	191725	173265	173290.3
Mean	143436.7	190837	173290.3	169188
SD	2241.7	916.1	548.9	19567
RSD %	1.6	0.5	0.3	11.6

Inter-day precision did not adhere to the set acceptance criteria (RSD % ≤ 2.0). However, for the purpose of this study it was acceptable due to the fact that the complete assay was done on the same day.

Table C.7: Validation results for isoniazid to determine system repeatability

	Area	Retention time (minutes)
	216398	2.97
	216650	2.98
	215168	2.98
	214253	2.98
	214295	2.98
	214275	2.98
Mean	215173	2.98
SD	1105.1	0.00
RSD %	0.5	0.13

System repeatability was found to be well within acceptance limits with RSD % of peak area and retention times being 0.5 and 0.13 respectively. The retention time for isoniazid was 2.98 minutes. The system was considered suitable for the purpose of this study.

C.12 PYRAZINAMIDE

C.12.1 Specificity

Table C.8: Validation results for pyrazinamide to determine method specificity

Stress condition (40 °C)	% Degradation of pyrazinamide
0.1 M Hydrochloric acid	53.6
0.1 M Sodium Hydroxide	100.0
10 % Hydrogen Peroxide	45.6

Conclusion

Degradation ranging from approximately 45% to 100% occurred in samples mixed with 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide. None of the additional peaks that were formed under stress conditions interfered with those of the active ingredients. Thus, specificity of the method conformed to the set acceptance criteria in table C.1.

C.12.2 Linearity and Range

Table C.9: Validation results for pyrazinamide to determine linearity and range

µg/ml	Mean Area
29	4571757
58	9113139
87	13826480
116	20008800
146	24699720
175	32063270
204	34946200
233	37248420
262	39481180
291	41464950
320	43199190

Table C.10: Regression statistics of pyrazinamide results of linearity and range

R squared	0.99	Lower 95 %	Upper 95 %
Intercept	-87.16	-155.6	-40.31
Slope	108300	87780	128700

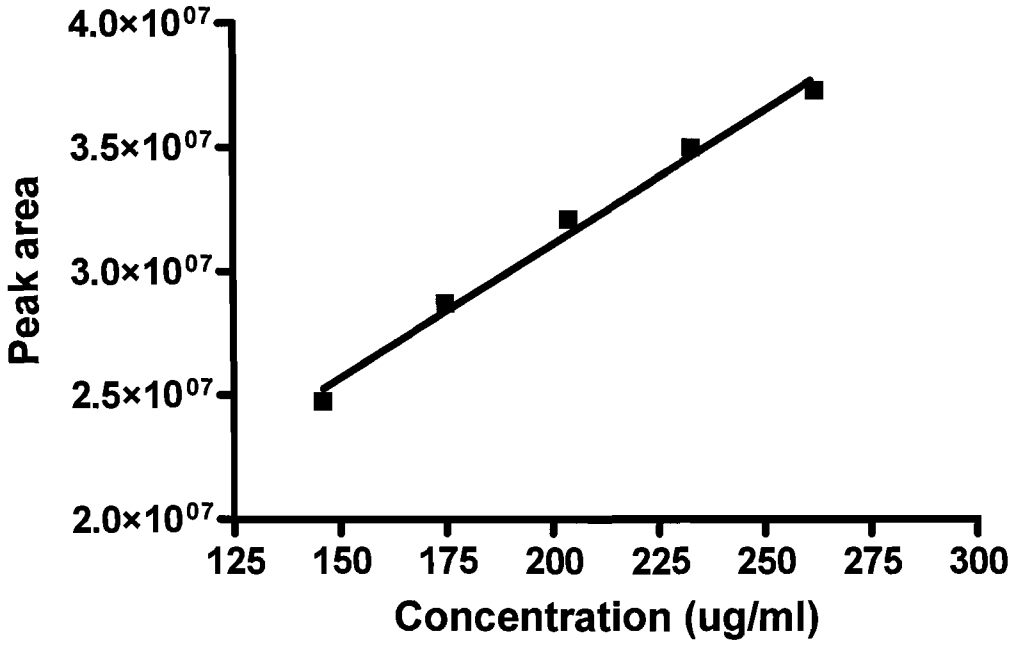


Figure C.10: Linear regression graph for pyrazinamide to determine linearity and range

Conclusion

The method is linear over the concentration range 142 – 264 µg/ml as shown in figure C.10. The regression coefficient is 0.9895.

C.12.3 PRECISION

C.12.3.1 Intra-day precision

Table C.11: Validation results for pyrazinamide to determine intra-day precision

Sample mass (mg)	Mean Area	Recovery (%)
40.2	8376323	99.5
45.5	9265594	97.3
39.7	7899640	95.0
46.7	9705568	99.3
55.3	11174017	96.5
53.4	11261956	100.7
68.8	13911276	96.6
66.7	13593628	97.3
64.4	13010963	96.5
	Mean	97.6
	SD	1.8
	RSD %	1.9

Conclusion

Intra-day precision was satisfactory with a RSD of 1.9 %.

C.12.3.2 Inter-day precision

Inter-day precision results are given in table C.12.

Table C.12: Validation results for pyrazinamide to determine inter-day precision

	Day 1	Day 2	Day 3	Between days
	11054923	11070658	10764861	1163632
	11174017	11066200	10779052	11067976
	11261956	11067069	10795433	10779782
Mean	11163632	11067976	10779782	11003797
SD	84838.3	1929.6	12491.6	163145
RSD %	0.76	0.02	0.12	1.48

Inter-day precision did conform to acceptance criteria and the method was therefore suitable for its intended use.

Table C.13: Validation results for pyrazinamide to determine system repeatability

	Area	Retention time (minutes)
	11110411	3.76
	11097579	3.77
	11093168	3.77
	11095626	3.77
	11097775	3.77
	11096732	3.77
Mean	11098549	3.77
SD	5523.3	0.01
RSD %	0.05	0.14

System repeatability was found to be well within acceptance limits with RSD % of peak area and retention times being 0.05 and 0.14 respectively. The retention time for pyrazinamide was 3.77 minutes. The system was considered suitable for the purpose of this study.

C.13 ETHAMBUTOL

C.13.1 Chromatographic conditions

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

Column: Phenomenex Luna C-18 (150 mm x 4.6 mm, 5 µm)

Mobile phase: 50 g ammonium acetate and 0.2 g copper(II) acetate weighed into a 1000 ml volumetric flask, dissolved and made up to volume with water. The pH was adjusted to 5.0 with glacial acetic acid. Mix 940 ml of this solution with 60 ml methanol.

Flow rate: 2.0 ml/min

Injection volume: 20 µl

Detection: 270 nm

Retention times: About 11.5 minutes for ethambutol

Solvent: 4 % methanol solution and water

C.13.2 Preparation of sample solutions

- Accurately weigh an amount of the pro-Pheroid™ formulation equivalent to 20 mg of ethambutol (about 51.3 mg of the pro-Pheroid™ formulation).
- Transfer the formulation to a 100 ml volumetric flask, and dissolve in 3.2 ml methanol.
- Add about 77 ml of water and shake well for about 15 minutes. Make up to volume with water.
- Filter the solution through a 0.45 µm filter, discarding the first few milliliters of the filtrate.
- Use amber-coloured glass HPLC vials and analyze by means of HPLC.

C.13.3 Preparation of the standard solution

- Accurately weigh 100 mg ethambutol reference standard and transfer to a 100 ml volumetric flask.
- Dissolve in about 80 ml of water.
- Shake well for about 15 minutes and make up to volume with water.
- Analyze by means of HPLC.

C.13.4 Calculations

The pro-Pheroid™ formulation contained:

Ethambutol	39g/100g
Rifampicin	19g/100g
Pro-Pheroid™	42g/100g

The prepared sample solution contained 200 µg/ml ethambutol and 97.5 µg/ml rifampicin as impurity.

The following equation was used for the calculation of content:

$$\text{mg/sample} = \frac{\text{Sample peak area x std. mass (mg) x std. potency (\%) x average sample mass (g)}}{\text{Standard peak area x weighed sample mass (g) x 100}}$$

C.13.5 Transfer validation test procedures

C.13.5.1 Specificity

C.13.5.1.1 Method

- Prepare a solution from the placebo pro-Pheroid™ formulation similar to preparing the sample solution (See section C.12.2).
- Prepare a sample solution as described in section C.12.2 and dilute 1:1 with 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
- Incubate the prepared solutions overnight in closed containers at 40 °C / 75 % RH to bring about degradation.
- Analyze by means of HPLC.

C.13.5.2 Linearity and Range

C.13.5.2.1 Method

- Prepare a standard solution as described in section C.12.3.
- Inject variable volumes to obtain standards from 10 – 150 % of the expected sample concentration.

C.13.5.3 Precision

C.13.5.3.1 *Intra-day precision*

C.13.5.3.1.1 Method

- Prepare sample solutions (section C.12.2) in triplicate by weighing amounts equivalent to 80 %, 100 % and 120 % of 100 mg of ethambutol.
- Analyze in duplicate by means of HPLC.

C.13.5.3.2 Inter-day precision

C.13.5.3.2.1 Method

- Use the same sample prepared above (at 100 % of the sample concentration).
- Analyze this sample in triplicate on two more occasions on different days.

C.13.5.4 System repeatability

C.13.5.4.1 Method

- Inject a sample six times consecutively.

C.14 Transfer validation results

C.14.1 Specificity

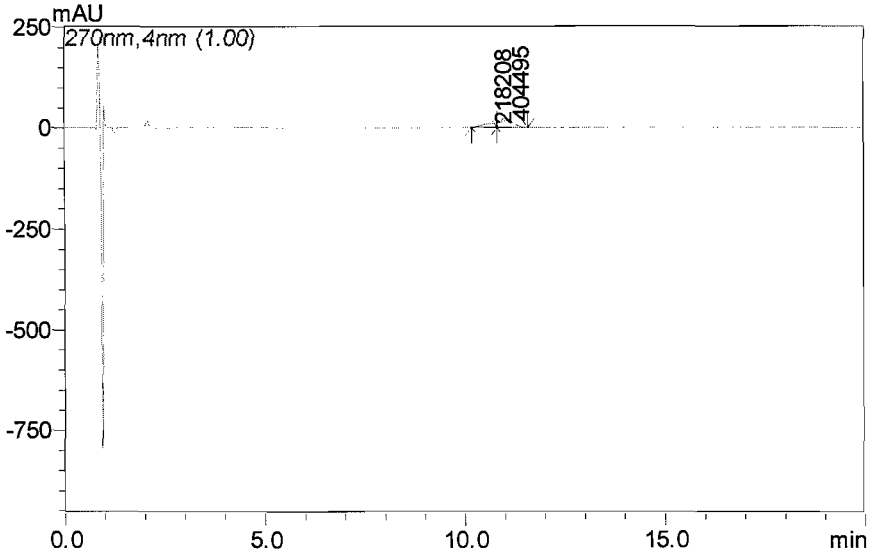


Figure C.11: Sample stressed overnight in 0.1 M hydrochloric acid at 40 °C.

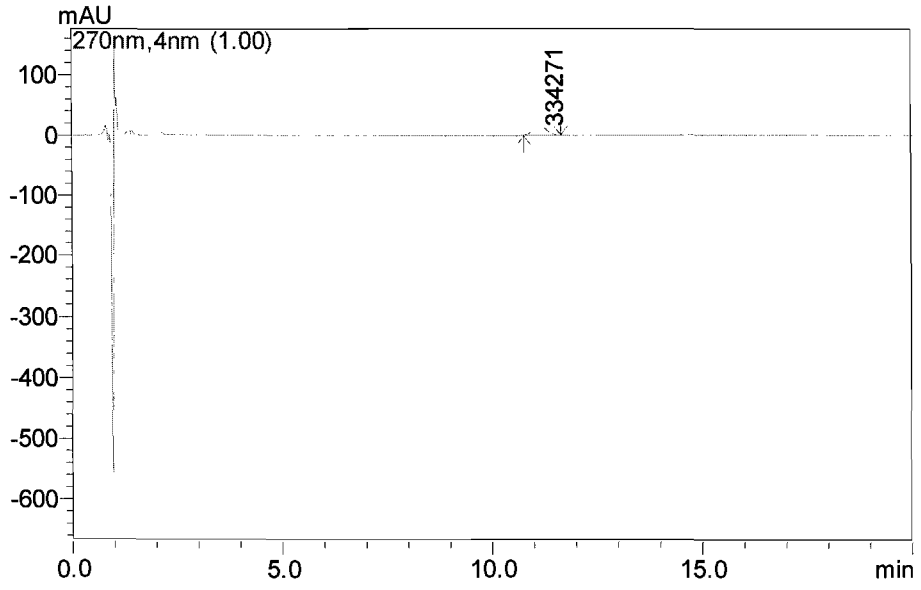


Figure C.12: Sample stressed overnight in 0.1 M sodium hydroxide at 40 °C.

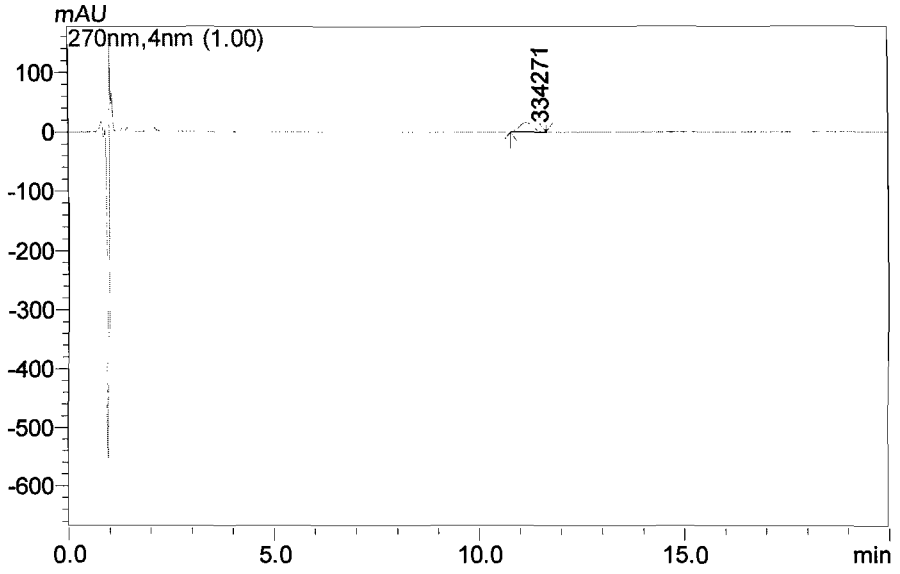


Figure C.13: Sample stressed overnight in 10 % hydrogen peroxide at 40 °C.

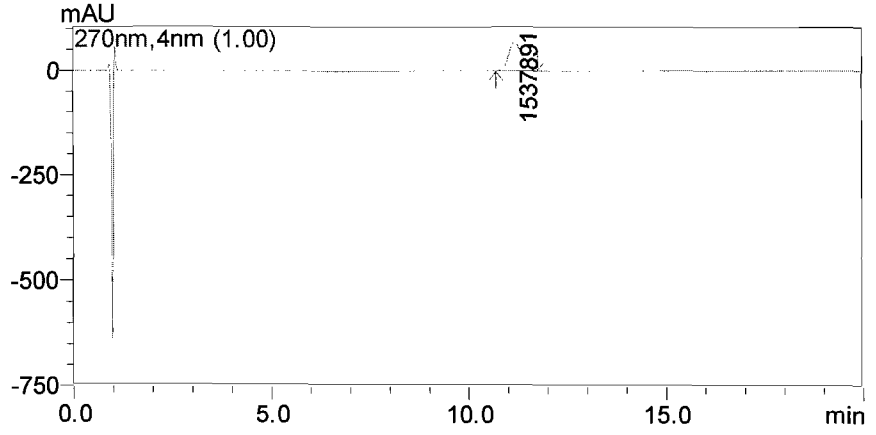


Figure C.14: Standard solution containing ethambutol.

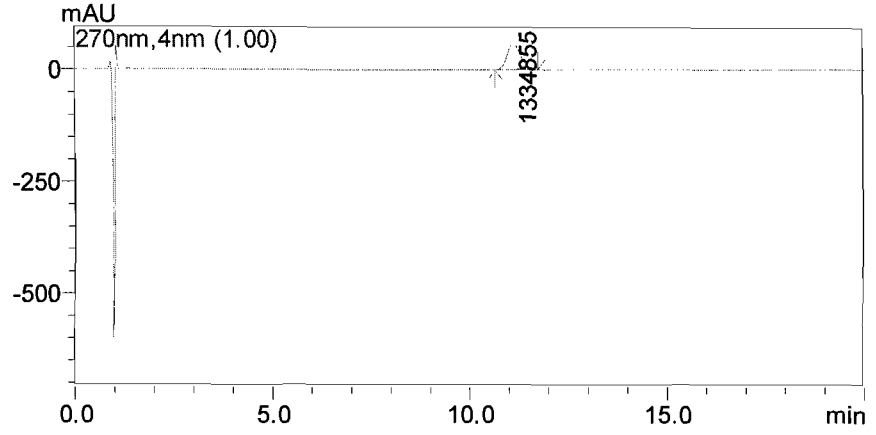


Figure C.15: Sample solution containing ethambutol.

Table C.14: Validation results for ethambutol to determine method specificity

Stress condition (40 °C)	% Degradation of ethambutol
0.1 M Hydrochloric acid	74.6
0.1 M Sodium Hydroxide	79.0
10 % Hydrogen Peroxide	63.2

Conclusion

Degradation ranging from approximately 63% to 79% occurred in samples mixed with 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide. None of the additional peaks that were formed under stress conditions interfered with those of the active ingredients. Thus, specificity of the method complied with the set acceptance criteria in table C.1.

C.14.2 Linearity and Range

Table C.15: Validation results for ethambutol to determine linearity and range

µg/ml	Mean Area
20	643405
40	1320502
60	2007428
80	2690249
100	3420942
120	4090400
140	4699952
160	5397853
180	6021747
200	6656683
220	6991663
240	8163321

Table C.16: Regression statistics of ethambutol results of linearity and range

R squared	0.9974	**Lower 95 %**	**Upper 95 %**
Intercept	-1.061	-6.557	4.096
Slope	33130	31940	34320

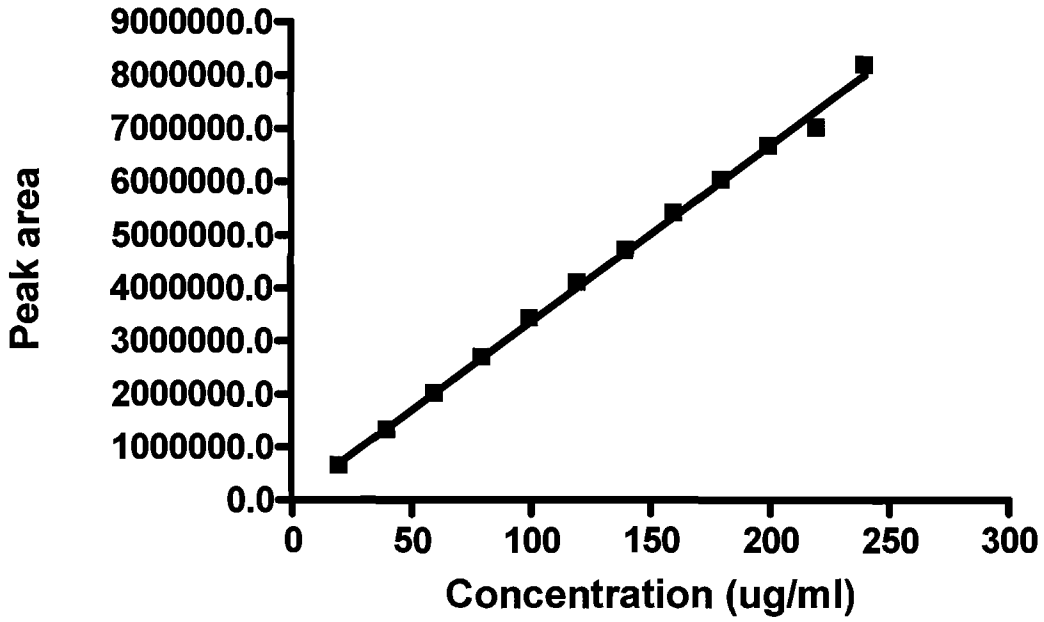


Figure C.16: Linear regression graph for ethambutol to determine linearity and range

Conclusion

The method is linear over the concentration range 20 – 240 µg/ml as shown in figure C.6. The regression coefficient is 0.9974.

C.14.3 PRECISION

C.14.3.1 Intra-day precision

Table C.17: Validation results for ethambutol to determine intra-day precision

Sample mass (mg)	Mean Area	Recovery (%)
59.0	1543084	108.4
43.7	1106082	104.9
46.7	1198011	106.4
50.8	1293820	105.6
51.6	1331218	107.0
50.1	1280627	106.0
61.3	1590935	107.6
63.2	1605483	105.3
65.1	1648053	105.0
	Mean	106.2
	SD	1.2
	RSD %	1.1

Conclusion

Intra-day precision was satisfactory with a RSD of 1.1 %.

C.14.3.2 Inter-day precision

Inter-day precision results are given in table C.18.

Table C.18: Validation results for ethambutol to determine inter-day precision

	Day 1	Day 2	Day 3	Between days
	1293820	1422047	1448834	1301888
	1331218	1429637	1428714	1428714
	1280627	1434459	1433592	1436971
Mean	1301888	1428714	1436971	1389191
SD	21427.2	5109.0	8643.4	61824.4
RSD %	1.6	0.4	0.6	4.5

The inter-day precision of this method for ethambutol was not satisfactory (RSD % > 2.0). This was because the detection of ethambutol is dependant on a complex that is formed with copper ions in the mobile phase. This complex could vary in quantity and retention times between days. Therefore, the inter-day precision was ignored for the purpose of this study. All ethambutol assays were done on the same day to ensure valid results.

Table C.19: Validation results for ethambutol to determine system repeatability

	Area	Retention time (minutes)
	1422047	11.50
	1429637	11.55
	1434459	11.56
	1435579	11.54
	1443419	11.54
	1434900	11.54
Mean	1433340.2	11.54
SD	6474.7	0.02
RSD %	0.5	0.2

System repeatability was found to be well within acceptance limits with RSD % of peak area and retention times being 0.5 and 0.2 respectively.

C.15 RIFAMPICIN

C.15.1 Chromatographic conditions

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

Column: Phenomenex Luna C-18 (250 mm x 4.6 mm, 5 µm)

Mobile phase: Six volumes of methanol mixed with 4 volumes of phosphate buffer. The phosphate buffer was prepared by dissolving 0.680 g potassium dihydrogen phosphate in 500 ml water. The pH was adjusted to 7.0 with sodium hydroxide (0.1 mol/L).

Flow rate: 1.0 ml/min

Injection volume: 20 µl

Detection: 254 nm

Retention times: About 41.2 minutes for rifampicin

Solvent: Methanol

C.15.2 Preparation of sample solutions

- Accurately weigh an amount of the pro-Pheroid™ formulation equivalent to 75 mg of rifampicin (about 394.7 mg of the pro-Pheroid™ formulation).
- Transfer the formulation to a 50 ml volumetric flask, and dissolve in methanol by shaking for 5 minutes.
- Make up to volume with methanol.
- Filter the solution through a 0.45 µm filter, discarding the first few milliliters of the filtrate.

- Transfer 5 ml of the filtered stock solution to a 10 ml volumetric flask, and fill to volume with methanol.

- Use amber-coloured glass HPLC vials and analyze by means of HPLC.

C.15.3 Preparation of the standard solution

- Accurately weigh 75 mg rifampicin reference standard a 50 ml volumetric flask.

- Dissolve in methanol.

- Shake well for about 5 minutes and make up to volume with methanol.

- Filter the solution through a 0.45 µm filter, discarding the first few milliliters of the filtrate.

- Transfer 5 ml of the filtered stock solution to a 10 ml volumetric flask, and fill to volume with methanol.

- Analyze by means of HPLC.

C.15.4 Calculations

The pro-Pheroid™ formulation contained:

Rifampicin	19g/100g
Ethambutol	39g/100g
Pro-Pheroid™	42g/100g

The prepared sample solution contained 750 µg/ml rifampicin and 1539 µg/ml ethambutol as impurity.

The equation that was used for the calculation of content:

$$\text{mg/sample} = \frac{\text{Sample peak area x std. mass (mg) x std. potency (\%) x average sample mass (g)}}{\text{Standard peak area x weighed sample mass (g) x 100}}$$

C.16 Transfer validation test procedures

Due to the rapid degradation of rifampicin in solution, fresh standard and sample solutions was prepared for every injection into the chromatogram. No injections was done in duplicate for the same reason.

C.16.1 Specificity

C.16.1.1 Method

- Prepare a solution from the placebo pro-Pheroid™ formulation similar to preparing the sample solution (See section C.13.2).
- Prepare a sample solution as described in section C.13.2 and dilute 1:1 with 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
- Incubate the prepared solutions overnight in closed containers at 40 °C / 75 % RH to bring about degradation.
- Analyze by means of HPLC.

C.16.2 Linearity and Range

C.16.2.1 Method

- Prepare five standard solutions with different concentrations ranging from 50 % to 150 % of the expected sample concentration (as described in section C.13.3).
- Inject each of these solutions (freshly prepared) immediately into the chromatograph.

C.16.3 Precision

C.16.3.1 Intra-day precision

C.16.3.1.1 Method

- Prepare sample solutions (section C.13.2) in triplicate by weighing amounts equivalent to 80 %, 100 % and 120 % of 27.3 mg of rifampicin.
- Analyze one concentration at a time by means of HPLC.

C.16.3.2 Inter-day precision

C.16.3.2.1 Method

- Use the same sample prepared above (at 100 % of the sample concentration).
- Analyze this sample in triplicate on two more occasions on different days.

C.16.4 System repeatability

C.16.4.1 Method

- Inject a sample six times consecutively

C.17 Transfer validation results

C.17.1 Specificity

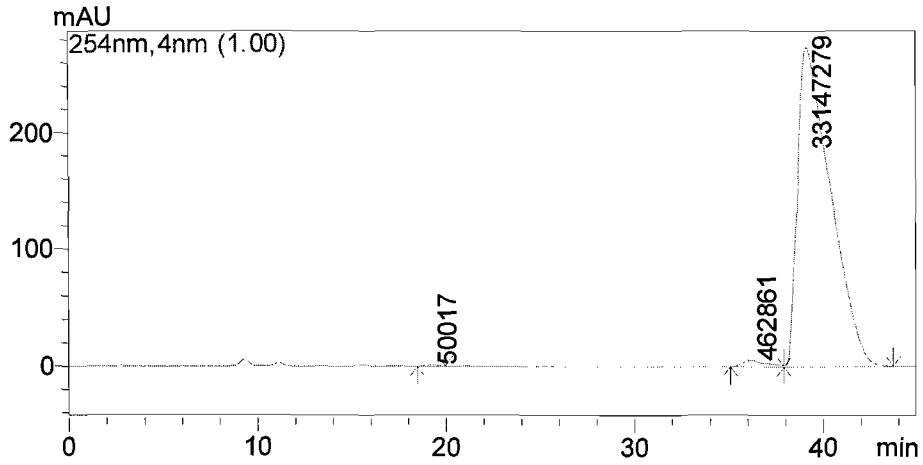


Figure C.17: Standard solution containing rifampicin

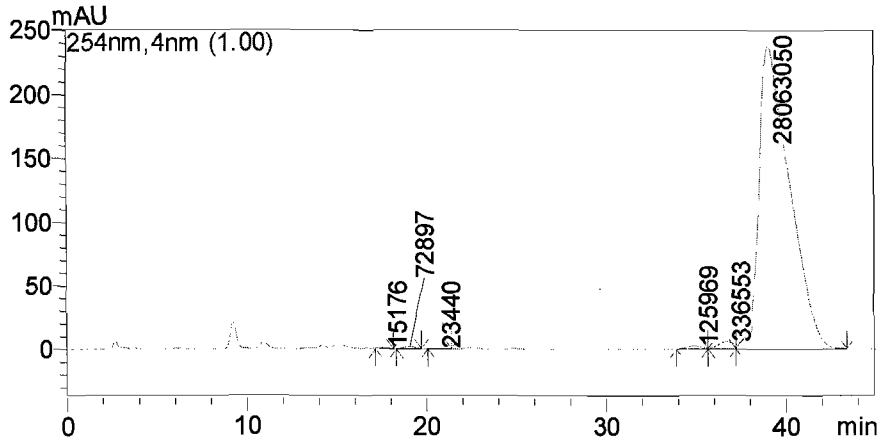


Figure C.18: Sample solution containing rifampicin

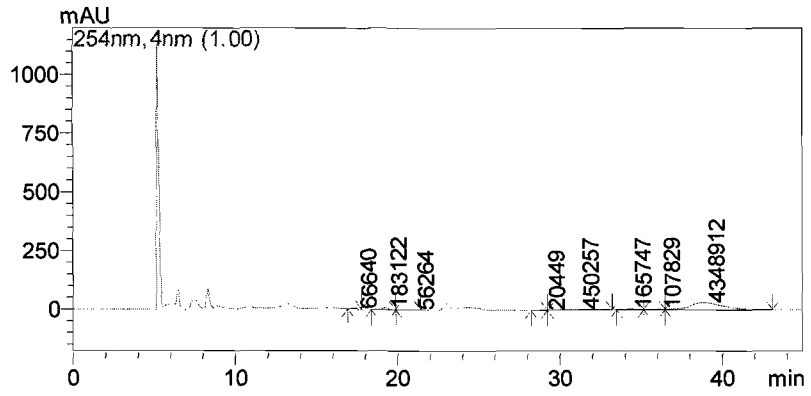


Figure C.19: Sample solution stressed overnight in 0.1 M hydrochloric acid at 40 °C

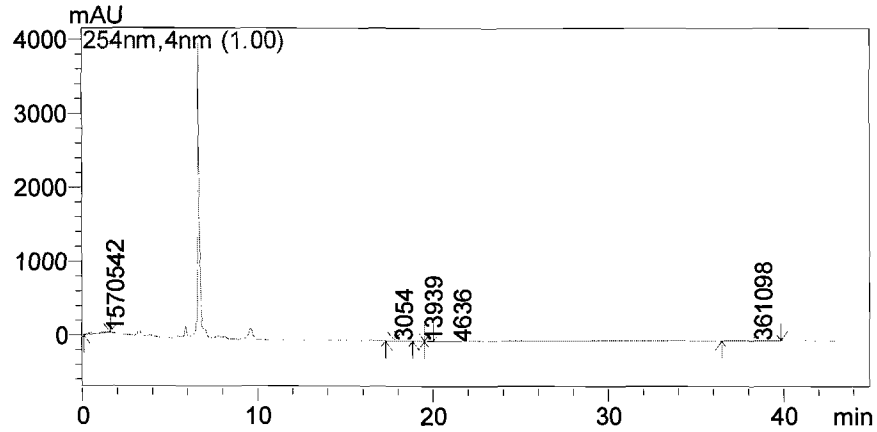


Figure C.20: Sample solution stressed overnight in 0.1 M sodium hydroxide at 40 °C

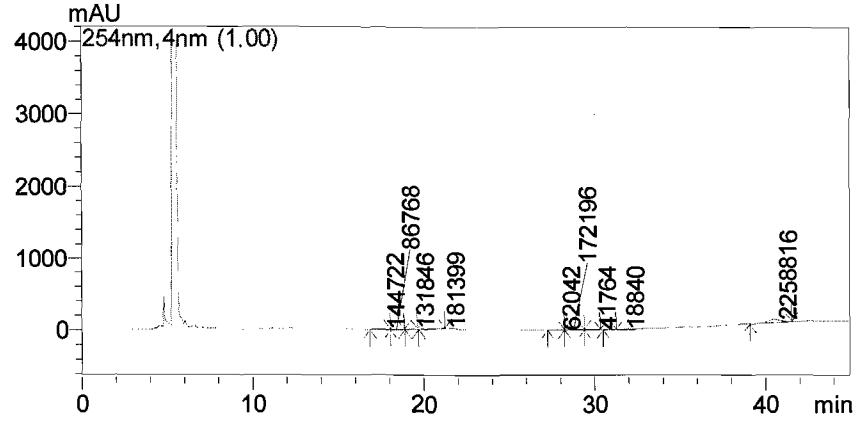


Figure C.21: Sample solution stressed overnight in 10 % hydrogen peroxide at 40 °C

Table C.20: Validation results for rifampicin to determine method specificity

Stress condition (40 °C)	% Degradation of rifampicin
0.1 M Hydrochloric acid	95.5
0.1 M Sodium Hydroxide	99.8
10 % Hydrogen Peroxide	97.2

Conclusion

Degradation ranging from approximately 95% to 100% occurred in samples mixed with 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide. None of the additional peaks that were formed under stress conditions interfered with those of the active ingredients. Thus, specificity of the method conformed to the set acceptance criteria in table C.1.

C.17.2 Linearity and Range

Table C.21: Validation results for rifampicin to determine linearity and range

µg/ml	Mean Area
375	17500134
562	27289411
750	32676390
975	43893591
1125	52120739

Table C.22: Regression statistics of rifampicin results of linearity and range

R squared	0.9921	Lower 95 %	Upper 95 %
Intercept	-16.81	-176.5	99.95
Slope	44810	37460	52170

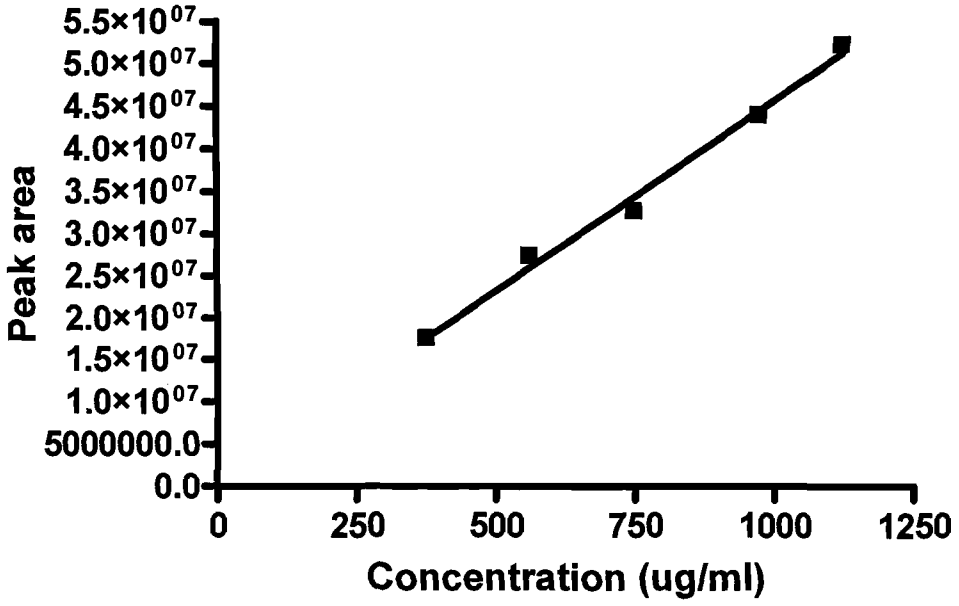


Figure C.22: Linear regression graph for rifampicin to determine linearity and range

Conclusion

The method is linear over the concentration range 375 – 1125 µg/ml as shown in figure C.22. The regression coefficient is 0.9921.

C.17.3 PRECISION

C.17.3.1 Intra-day precision

Table C.23: Validation results for rifampicin to determine intra-day precision

Sample mass (mg)	Mean Area	Recovery (%)
315	22650694	87.3
220	14596001	80.5
318	24976544	95.3
402	34799731	105.0
400	33987232	103.1
398	32549795	99.2
474	39944503	114.3
481	42231325	119.1
477	41712126	118.6
	Mean	102.5
	SD	13.5
	RSD %	13.2

Conclusion

Intra-day precision was not satisfactory with a RSD of 13.2 %, but was accepted based on instability and time constraints. The assay of rifampicin in the intended study will require fresh preparations prior to injection into the chromatogram.

C.17.3.2 Inter-day precision

Inter-day precision results are given in table C.24.

Table C.24: Validation results for rifampicin to determine inter-day precision

	Day 1	Day 2	Day 3	Between days
	34799731	24539731	23811891	32549795
	33987232	24269896	23686253	24091891
	32549795	24091891	23531054	23531054
Mean	33778919	24300506	23676399	26724246
SD	930268.2	184106.7	114862.8	4125642.9
RSD %	2.75	0.75	0.5	15.43

The inter-day precision was also not satisfactory, but was accepted on the condition that fresh samples will always be prepared and all assays must be done on the same day.

C.17.4 System repeatability

Table C.25: Validation results for rifampicin to determine system repeatability

	Area	Retention time (minutes)
	24539731	41.25
	24269896	41.33
	24091891	41.45
	23811891	41.56
	23686253	41.58
	23531054	41.63
Mean	23988452	41.5
SD	347737.9	0.14
RSD %	1.45	0.33

System repeatability was found to be well within acceptance limits with RSD % of peak area and retention times being 1.45 and 0.33 respectively. The retention time for rifampicin was 41.5 minutes. The system was considered suitable for the purpose of this study.

C.18 Results of the stability study

A significant change for a drug product is defined as:

1. A 5% change from the initial assay value;
2. Failure to meet acceptance criteria for appearance, physical attributes and functionality test (e.g. colour and phase seperation);
3. Microbiological growth

C.18.1 Physical appearrance

Some physical changes did occur at one or more of the four time points at accelerated storage conditions. These changes are discussed thoroughly in chapter 4 and can be seen in figure C.23 and C.24.

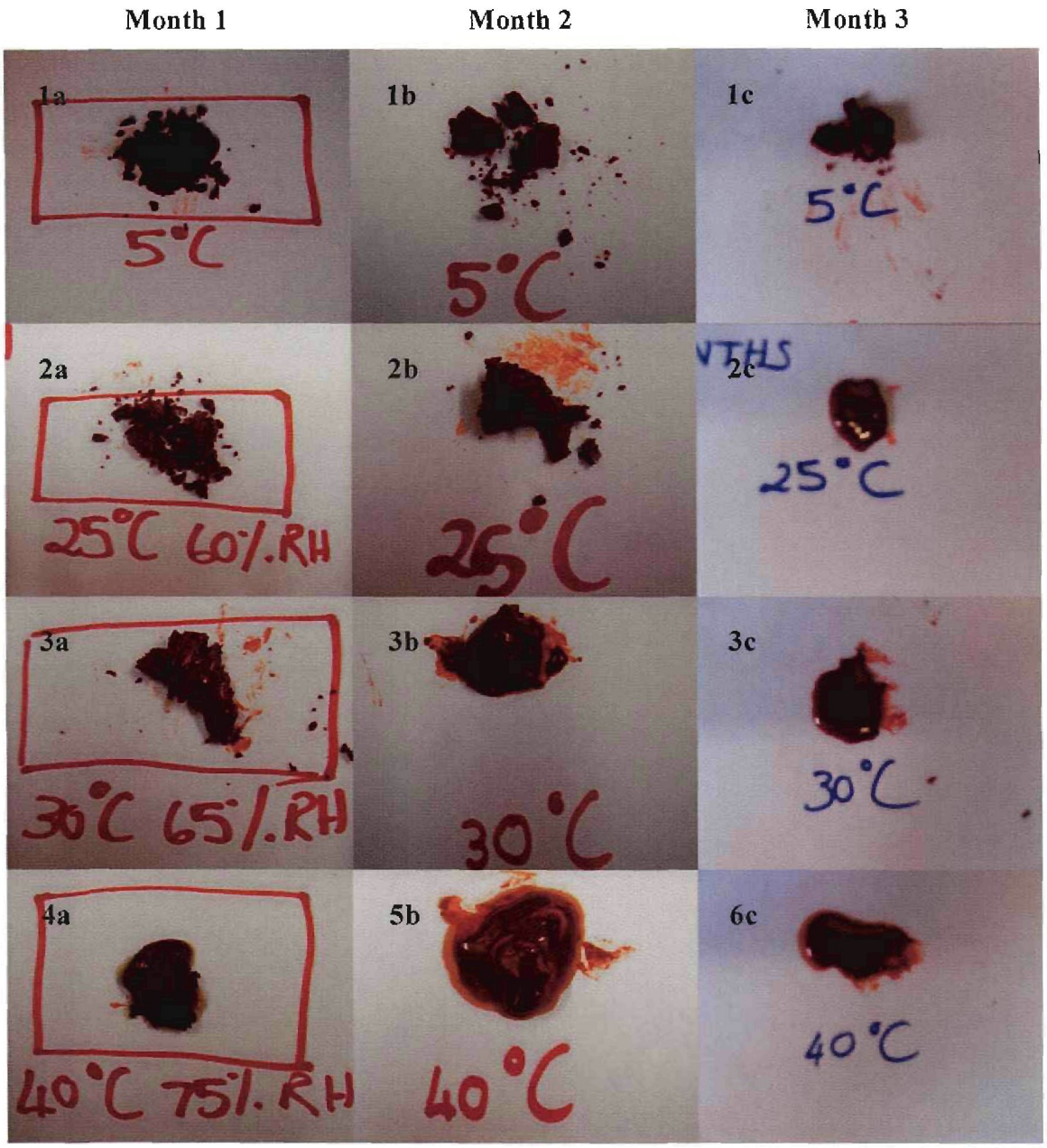


Figure C.23: Observed physical changes of Formulation B over 3 months at various accelerated stability conditions

Figure C.24: Observed physical changes of Formulation A over 3 months at accelerated stability conditions.

C.18.2 Microbiological growth

The results of the microbiological limit tests are given below in table C.26 and C.27.

Table C.26: Microbiological growth in Formulation A

Microbiological limit test	Storage condition	Initial	1 Month	2 Month	3 Month
Staphylococcus aureus	5 °C	Not detected	Not detected	Not detected	Not detected
	25 °C ; 60% RH	Not detected	Not detected	Not detected	Not detected
	30 °C ; 65% RH	Not detected	Not detected	Not detected	Not detected
	40 °C ; 75% RH	Not detected	Not detected	Not detected	Not detected
Esterichia Coli	5 °C	Not detected	Not detected	Not detected	Not detected
	25 °C ; 60% RH	Not detected	Not detected	Not detected	Not detected
	30 °C ; 65% RH	Not detected	Not detected	Not detected	Not detected
	40 °C ; 75% RH	Not detected	Not detected	Not detected	Not detected
Salmonella spp.	5 °C	Not detected	Not detected	Not detected	Not detected
	25 °C ; 60% RH	Not detected	Not detected	Not detected	Not detected
	30 °C ; 65% RH	Not detected	Not detected	Not detected	Not detected
	40 °C ; 75% RH	Not detected	Not detected	Not detected	Not detected
Total plate count	5 °C	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	25 °C ; 60% RH	<10 cfu/g	160 cfu/g	<10 cfu/g	<10 cfu/g
	30 °C ; 65% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	40 °C ; 75% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Table C.26 continued: Microbiological growth in Formulation A

Microbiological limit test	Storage condition	Initial	1 Month	2 Month	3 Month
Yeast count	5 °C	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	25 °C ; 60% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	30 °C ; 65% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	40 °C ; 75% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mould count	5 °C	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	25 °C ; 60% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	30 °C ; 65% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	40 °C ; 75% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Table C.27: Microbiological growth in Formulation B

Microbiological limit test	Storage condition	Initial	1 Month	2 Month	3 Month
Staphylococcus aureus	5 °C	Not detected	Not detected	Not detected	Not detected
	25 °C ; 60% RH	Not detected	Not detected	Not detected	Not detected
	30 °C ; 65% RH	Not detected	Not detected	Not detected	Not detected
	40 °C ; 75% RH	Not detected	Not detected	Not detected	Not detected
Esterichia Coli	5 °C	Not detected	Not detected	Not detected	Not detected
	25 °C ; 60% RH	Not detected	Not detected	Not detected	Not detected
	30 °C ; 65% RH	Not detected	Not detected	Not detected	Not detected
	40 °C ; 75% RH	Not detected	Not detected	Not detected	Not detected
Salmonella spp.	5 °C	Not detected	Not detected	Not detected	Not detected
	25 °C ; 60% RH	Not detected	Not detected	Not detected	Not detected
	30 °C ; 65% RH	Not detected	Not detected	Not detected	Not detected
	40 °C ; 75% RH	Not detected	Not detected	Not detected	Not detected
Total plate count	5 °C	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	25 °C ; 60% RH	<10 cfu/g	160 cfu/g	<10 cfu/g	<10 cfu/g
	30 °C ; 65% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	40 °C ; 75% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Table C.27: Microbiological growth in Formulation B

Microbiological limit test	Storage condition	Initial	1 Month	2 Month	3 Month
Yeast count	5 °C	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	25 °C ; 60% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	30 °C ; 65% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	40 °C ; 75% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mould count	5 °C	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	25 °C ; 60% RH	<10 cfu/g	160 cfu/g	<10 cfu/g	<10 cfu/g
	30 °C ; 65% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
	40 °C ; 75% RH	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

C.18.3 Drug content determination

The drug content in both formulations were determined and are given in table C.28.

Table C.28: Changes in drug content within two pro-Pheroid™ based FDC formulations over three months

	Active Drug	Storage condition	Initial	Month 1	Month 2	Month 3
Formulation B	Rifampicin	5 °C	98	97.5	101.5	112.6
		25°C; 60% RH	98	99.8	99.2	114.3
		30°C; 65% RH	98	94.7	86.7	106.7
		40°C; 75% RH	98	93.4	80.6	97.4
	Ethambutol	5 °C	93.8	92.0	96.6	109.3
		25°C; 60% RH	93.8	122.3	100.8	107.2
		30°C; 65% RH	93.8	105.1	100.0	108.4
		40°C; 75% RH	93.8	104.1	95.7	102.6
Formulation A	Isoniazid	5 °C	96.3	108.3	74	88.7
		25°C; 60% RH	96.3	101.9	97.4	84.9
		30°C; 65% RH	96.3	108.6	95.6	88.4
		40°C; 75% RH	96.3	118.0	86	87.5
	Pyrazinamide	5 °C	100.6	103.0	102.4	92.3
		25°C; 60% RH	100.6	103.2	102.6	93.2
		30°C; 65% RH	100.6	80.4	79.9	73.1
		40°C; 75% RH	100.6	105.7	105.1	101.2

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