

TRANSDERMAL DELIVERY OF ISONIAZID AND RIFAMPICIN BY PHEROID™ TECHNOLOGY

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Phi 4:13

*I can do all things
through Christ which
strengtheneth me.*

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This dissertation is presented in the so-called article format, which includes an introductory chapter with sub-chapters, a full length article for publication in a pharmaceutical journal and appendices containing relevant experimental data. The article contained in this dissertation is to be published in the European Journal of Pharmaceutical Sciences of which the complete guide for authors is included.

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ABSTRACT

The aim of this *in vitro* study was to investigate the feasibility of the transdermal delivery of isoniazid (INH) and rifampicin (RMP) by means of the novel Pheroid™ technology system. The application of the latter is being investigated in combination with various actives such as peptides (insulin, human growth hormone), anti-malarial drugs (chloroquine), anti-fungals (ketoconazole), local anaesthetics (lidocaine, prilocaine) as well as tuberculostatics (ethambutol, pyrazinamide etc.) *via* different administration routes at the North- West University.

Pheroid™, a stable skin-friendly carrier, comprises of a submicron (200 nm - 2 µm) emulsion type formulation for which previous studies have confirmed the ability to penetrate keratinised tissue, skin, intestinal linings, the vascular system, fungi, bacteria and even parasites. Studies involving an oral Pheroid™ formulation containing the current approved regime of four anti-tuberculosis drugs showed improved efficacy results whilst an *in vitro* analysis of bacterial growth indicated a reduction in drug resistance in multidrug resistant tuberculosis (MDR-TB) strains.

Therefore we thought it prudent to ascertain whether or not the Pheroid™ system would be able to improve the transdermal delivery of a combination of INH and RMP as a possible treatment against cutaneous tuberculosis (tuberculosis involving the skin). The latter refers to pathological lesions of the skin caused by any one of the following: *Mycobacterium tuberculosis*, *Mycobacterium bovis* or the bacilli Calmette-Guérin (BCG) vaccine. Demonstration of *M. tuberculosis* within the infected tissues by traditional acid-fast bacilli (AFB) staining, culture or polymerase chain reaction (PCR) confirms the diagnosis. CTB lesions are associated with various degrees of one or more of the following ulceration, plaque formation, hyperkeratosis or the presence of necrotic matter.

Seeing as CTB is mostly associated with systemic involvement, current treatment comprises of the standard three/four drug regimens used for pulmonary TB in general. Cases of CTB usually show improvement within 1 month of therapy with anti-TB drugs, but complete resolution is only attained after 4 - 6 months. The major drawback to current therapy is that patients not only remain a source of infection (viable organisms can still be demonstrated in the lesions), but they also suffer from

constant embarrassment due to the disfiguring nature of CTB until these lesions have healed completely. No evidence of an already existing topical formulation of this kind could be found.

Therefore *in vitro* permeation studies were conducted using vertical Franz diffusion cells and female abdominal skin as permeation membrane over a period of 12 hours. Concentrations of 5 mg/ml and 10 mg/ml for isoniazid (INH) and rifampicin (RMP) respectively, were applied to the donor phase suspended in either phosphate buffered saline (PBS) or entrapped in Pheroid™. Permeation studies were conducted at pH 5.5. *In vitro* penetration of INH and RMP were assayed directly by HPLC. Particle size distribution for rifampicin and entrapment of actives within the Pheroid™ carrier system was determined by polarized light and laser scanning microscopy (CLSM) respectively and revealed definite entrapment.

Permeation profiles obtained for INH in Pheroid™ indicated a biphasic character, whilst that obtained for RMP in Pheroid™ showed a triphasic character. The Pheroid™ delivery system proved more efficacious for delivery of both anti-tubercular drugs and resulted in greater percentage yield as well as flux values than that for a PBS solution. Furthermore, the Pheroid™ formulation was able to deliver the entrapped INH and RMP in concentrations sufficient to exceed their respective minimum inhibitory concentrations (MIC).

Keywords: Isoniazid; Rifampicin; Confocal laser scanning microscopy; Pheroid™; Topical drug delivery; Cutaneous tuberculosis; Tuberculosis

UITTREKSEL

Die doel van hierdie *in vitro*-studie was om die transdermale aflewering van isoniasied (INH) en rifampisien (RMP) met behulp van die Pheroid™ geneesmiddelafleweringsstelsel te bepaal. Die toepassing van lg. afleweringsstelsel in kombinasie met verskeie ander geneesmiddels soos peptiede (insulien, menslike groeihormoon), anti-malaria middels (chlorokien), lokale anestetika (lidokaïen, prilokaïen), anti-fungale middels (ketokonasool) asook anti-tuberkulose middels (etambutol, pirasienamied ens.) *via* verskillende toedieningsroetes, word tans aan die Noordwes Universiteit nagevors.

Pheroids™, 'n kosmeties aanvaarbare afleweringsstelsel, word geformuleer as 'n submikron (200 nm – 2 µm) tipe emulsie wat oor die vermoë beskik om verskeie skanse te penetreer naamlik, hiperkeratotiese weefsel, die vel, intestinum- en bloedvatwande asook fungi, bakterieë en parasiete. Tydens 'n studie uitgevoer met 'n orale Pheroid™ formule waarin die tans goedgekeurde vier anti-tuberkulose geneesmiddels geïnkorporeer is, is daar 'n verhoging in hul kliniese doeltreffendheid waargeneem. *In vitro* studies het verder 'n afname in geneesmiddelweerstandigheid by *M. tuberculosis* getoon.

Na aanleiding van die bg. het ons dit goedgevind om die transdermale aflewering van INH en RMP m.b.v. die Pheroid™ geneesmiddelafleweringsstelsel te bepaal ten einde 'n moontlike behandeling te ontwikkel vir kutane tuberkulose (tuberkulose van die vel). Laasgenoemde verwys na 'n patologiese toestand van die vel veroorsaak deur een van die volgende: *M. tuberculosis*, *M. bovis* of BCG vaksien. Diagnose word bevestig deur die demonstrasie van *M. tuberculosis* in die velletsels d.m.v. kweking, suur-vaste kleuring of polimerase ketting reaksie (PKR) tegniek.

Kutane tuberkulose (KTB) letsels word geassosieer met een of meer van die volgende naamlik plake, hiperkeratose, ulserasie asook weefselnekrose. Huidige behandeling van KTB behels die gebruik van die standaard orale anti-tuberkulose geneesmiddels aangesien die toestand hoofsaaklik met onderliggende pulmonale TB gepaard gaan. KTB letsels toon 'n verbetering in respons op chemoterapie met anti-tuberkulose middels na ongeveer 1 maand, met volledige opklaring na 'n tydperk van 4 – 6 maande. Die grootste tekortkoming i.v.m. die huidige vorm van behandeling is

die feit dat die pasiënt 'n bron van infeksie bly (lewende *M. tuberculosis* aanwesig in velletsels) totdat volledige genesing van die letsels plaasgevind het. Verder nog is pasiënte uitgelewer aan die misvormende effek van hierdie toestand. Geen aanduiding van 'n reeds bestaande topikale produk soos wat hier beoog word, is gevind nie.

Dus is *in vitro*-diffusie studies met behulp van vertikale Franzelle oor 'n tydperk van 12 uur uitgevoer, terwyl 'n pH van 5.5 deurentyd gehandhaaf is. Vroulike abdominale vel, verkry vanaf abdominoplastiese prosedures, is as permeasie-membraan gebruik. 'n Kombinasie van 5 mg/ml isoniasied en 10 mg/ml rifampisien, gesuspendeer in óf 'n fosfaatbuffer óf Pheroid™, is in die donorfase aangebring. Die *in vitro*-permeasie van INH en RMP is bepaal met behulp van hoëdruk-vloeistofchromatografie (HDVC). Die kristalgrootteverspreiding vir rifampisien (RMP) asook die inkorporering van aktiewe bestanddele binne-in die Pheroid™ sisteem is met lig en konfokale laserskanderingmikroskopie (KLSM), respektiewelik bepaal. Laasgenoemde mikroskopie het die teenwoordigheid van die aktiewe bestanddele in die Pheroid™ bevestig.

Die permeasieprofiel van INH in Pheroid™ het 'n bifasige karakter getoon, terwyl dié van RMP in Pheroid™ 'n trifasige karakter getoon het. Na aanleiding van die waargenome verhoging in die gemiddelde flukswaardes asook persentasie opbrengs verkry vir beide geneesmiddels in Pheroid™ teenoor dié in PBS, blyk dit dat die Pheroid™ geneesmiddelafleweringsisteem hoogs voordelig vir transdermale aflewering van hierdie twee geneesmiddels is. Laastens is ook waargeneem dat die hoeveelheid INH en RMP afgelewer m.b.v. die Pheroid™ hul onderskeie minimum inhiberende konsentrasies (MIK's) oorskry het.

Sleutelwoorde: Isoniasied; Rifampisien; Konfokale laserskanderingmikroskopie; Pheroid™; Topikale geneesmiddelaflewering; Kutane tuberkulose; Tuberkulose

TRANSDERMAL DELIVERY OF ISONIAZID AND RIFAMPICIN WITH PHEROID™ TECHNOLOGY

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

Tuberculosis (TB) has been known to have existed since 3000 BC, but it continues to pose a significant threat to the world population even today, being one of the most common infectious diseases (Kumar & Muralidhar, 1999:494). Estimates by the World Health Organisation (WHO) reveal approximately 9 million new cases, 1.8 billion infected people and 3 million deaths annually (World Health Organization, 2006:1).

The culprit: *Mycobacterium tuberculosis*, the causative micro-organism of tuberculosis (TB). Although the lungs are the primary target of the TB disease, it can occur in almost any anatomical site or as disseminated disease. Extrapulmonary TB sites include the pleura, lymph nodes, skeleton (bones, joints and/or vertebrae), bone marrow, genito-urinary system, multiple organs (in miliary form), meninges, central nervous system, pericardia, adrenal glands, gastro-intestinal system, eyes, ears as well as the skin (Jakubowiak *et al.*, 2001:23). The latter is also referred to as cutaneous tuberculosis (CTB).

Of all the TB cases reported, only about 10% of these comprise of extrapulmonary TB and CTB makes up a very small portion of these cases (Barbagallo *et al.*, 2002:320). In a twenty year (1975-1995) retrospective survey of all patients seen at a tertiary care hospital in India, the detected incidence for CTB was 0.1% and this percentage is even less in developed countries (Kumar & Muralidhar, 1999:494). It was also found that the incidence of patients with CTB at the dermatology department in Casablanca, Morocco, over a 23-year (1981-2004) study period was 2% (Zouhair *et al.*, 2006:1).

Though these cases are rare, it has been noted over the last two decades that their numbers seem to increase proportionally with the rising incidence of pulmonary TB

due to the AIDS pandemic and the fatal synergy which exist between *M. tuberculosis* and HIV.

Other causes include the emergence of multi-drug resistant TB strains, socio-economic problems (poverty, immigration) as well as non-compliance with the current anti-TB treatment regimens (Gopinathan *et al.*, 2001:194; Maccari *et al.*, 2005:2509).

For a skin lesion to be diagnosed as true CTB, the presence of *M. tuberculosis* must be demonstrated by special staining, culture or polymerase chain reaction (PCR). Laboratory detection, however, is problematic: routine culture takes more than 4 - 6 weeks to complete and results are usually negative due to the low bacillary load present in CTB. The same goes for histopathology findings which are characteristic, but not pathognomonic and are shared by various other diseases including leprosy, sarcoidosis, leishmaniasis and subcutaneous fungal infections (Ramam *et al.*, 2007:244). This can delay the onset of anti-tuberculosis therapy, making the patient a more liable source of infection.

PCR is increasingly being utilised because of its rapidity (results available within 24 h), sensitivity and specificity (Prasad *et al.*, 2001:138). The latter can detect *M. tuberculosis*-specific DNA sequences and therefore small numbers of mycobacteria (Barbagallo *et al.*, 2002:319). The clinical presentation of CTB varies considerably and may include the following: superficial dermal granulomas around hair follicles or sweat ducts, caseation necrosis in the upper dermis, ulceration of lymph nodes (often seen in the neck), ulceration of the mouth and nose as well as inflammation of the subcutaneous fat lobules (Barbagallo *et al.*, 2002:320).

Current treatment for CTB is the same as for pulmonary TB since the majority of patients have systemic disease involvement as well. It is believed that the anti-tuberculosis regimens used for pulmonary TB should be adequate for treating the cutaneous form since the bacillary load is significantly lower than that of pulmonary TB. The downside to this is that CTB lesions only resolve after about 5 weeks treatment or longer (Ramam *et al.*, 2007:246).

Therefore the rationale behind the chosen drug combination and route of administration for this study is to provide a possible means of treatment for patients with CTB in an effort to alleviate the psychological and physical suffering inherent to

this disease. No evidence of an already existing topical formulation of this kind could be found.

The formulation of a successful transdermal product poses many challenges in its own right because the human skin effectively prohibits significant amounts of a drug to be delivered. According to Wiechers (1989:185) this is mainly due to the outermost skin layer or stratum corneum (SC). Therefore, formulators are investigating different measures to circumvent the skin's barrier function in order to ensure that sufficient quantities of the active ingredients reach the intended site of action within the skin, whether it be surface effects (e.g. sunscreens) or systemic effects (e.g. nicotine patches) (Walters, 2002:2; Zatz, 1993:11).

One such measure is to select an active with the correct physicochemical properties to permeate across the stratum corneum at an acceptable rate. Ideal physicochemical properties for effective permeation include:

- Low molecular weight (< 600 Da), when the diffusion coefficient tends to be high.
- Adequate solubility in oil as well as aqueous phases.
- A high, but balanced partition coefficient.
- Low melting point, which correlates with good ideal solubility (Barry, 2001:968).

Unfortunately, since the majority of drugs do not display these ideal characteristics they are rendered unsuitable for use in transdermal delivery unless some alternative mode can be found to lessen the barrier function of the SC. A number of these alternative measures have been developed which have shown some success namely:

- Using a more suitable pro-drug form of the parent drug.
- Iontophoresis.
- Reverse iontophoresis.
- Electro-osmosis.
- Lipid-based delivery systems (e.g. liposomes) and
- Penetration enhancers (e.g. alcohols) (Barry, 2001:970).

In this study the novel Pheroid™ system will be used as the delivery vehicle to entrap the actives, isoniazid (INH) and rifampicin (RMP), which are both poor candidates for skin permeation. The ability, if any, of the Pheroid™ to enhance INH and RMP's flux across the skin will be studied by means of an *in vitro* method using vertical Franz cells.

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CHAPTER 2: DISEASE AETIOLOGY & FACTORS AFFECTING TRANSDERMAL DELIVERY OF TOPICALLY APPLIED DRUGS

2 INTRODUCTION

A considerable amount of research has been directed towards transdermal delivery over the past two decades due to an increased interest in the factors that govern skin permeability, the various advantages it holds over conventional routes and the synthesis and development of potent topical drugs (Denet *et al.*, 2004:659; Hadgraft, 1983:255).

In addition, transdermal delivery has focused our attention on a range of skin diseases such as psoriasis, eczema, ichthyosis, acne, skin tumours and now also infectious diseases e.g. cutaneous tuberculosis (Chan & Li Wan Po, 1989:1).

However, the principal limiting factor of this route is the difficulty to control drug fluxes because of the resistance of the stratum corneum (SC) to penetration and the huge biological variability encountered in human skin (Sinha & Kaur, 2000:1131).

In the following sections attention will be given to the pathology of CTB, skin structure and function in general, advantages and limitations of the transdermal route, physiochemical factors as well as penetration enhancers that will impact the delivery of isoniazid (INH) and rifampicin (RMP).

2.1 CUTANEOUS TUBERCULOSIS

2.1.1 HISTORY

Cutaneous tuberculosis (CTB), also known as dermal tuberculosis or tuberculosis cutis, refers to pathological lesions of the skin caused by any one of the following: *Mycobacterium tuberculosis*, *Mycobacterium bovis* or the BCG vaccine (Barbagallo *et al.*, 2002:319).

Although the lungs are the primary target of the TB disease, it can occur in almost any anatomical site or as disseminated disease. The skin, being the largest organ of the body, is therefore not excluded from infection by *M. tuberculosis* (Ngan, 2006).

Of all the TB cases reported, only about 10% of these comprise of extrapulmonary TB and CTB makes up a very small portion of these cases (Barbagallo *et al.*, 2002:320). Although these cases are rare, it has been noted over the last two decades that their numbers have increased proportionally with the rising incidence of pulmonary TB due to the AIDS pandemic and the fatal synergy which exist between *M. tuberculosis* and HIV (human immunodeficiency virus). Other causes include the emergence of multi-drug resistant TB strains, socio-economic problems (poverty, immigration) as well as non-compliance with the current anti-TB treatment regimens (Gopinathan *et al.*, 2001:194; Maccari *et al.*, 2005:2509).

2.1.2 MICROBIOLOGY

Mycobacteria are aerobic, nonsporeforming, paucibacillary, intracellular, curved rods measuring 0.2 - 0.5 by 2 - 4 μm . Their cell walls are comprised of phospholipoglycans (mycolides) and mycolic acid-rich long-chain glycolipids which do not only provide protection for the mycobacterium against lysosomal attack but also retain red basic fuchsin dye after acid rinsing (Ziehl-Neelson acid fast stain). They are not classified as Gram positive or Gram negative since they do not express the chemical characteristics of either (Meltzer, 2006).

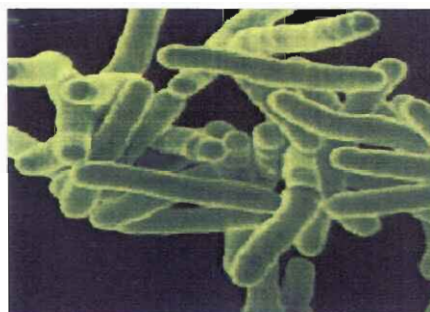


Figure 1: Scanning electron micrograph of *Mycobacterium tuberculosis* (Bishop, 2005).

M. tuberculosis forms part of the *M. tuberculosis* complex, a group of closely related organisms: *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium microti*. TB caused by the first two is very rare whilst the last does not cause human disease (Meltzer, 2006). Other non-tuberculous mycobacteria species include: *M. kansasii*, *M. scrofulaceum*, *M. marinum*, *M. fortuitum* complex, *M. leprae* and the *M. avium* complex (MAC) (Mandell & William, 1996:1167). The latter comprises of two species – *M. avium* and *M. intracellulare*, which is the most common cause for disseminated infection in immunocompromised individuals i.e. those suffering from AIDS. These patients usually develop disseminated MAC disease when their lymphocyte (CD₄) count falls below 50 cells/μl (Koirala & Harley, 2006). *M. leprae* is the causative micro-organism of leprosy (Hansen's disease) (Mandell & William, 1996:1159).

In addition to its efficacy against *M. tuberculosis*, RMP also inhibits the growth of *M. kansasii*, the majority of strains of *M. scrofulaceum*, *M. avium* and *M. intercellulare* as well as *M. leprae* (Mandell & William, 1996:1159). Its application regarding the non-tuberculous mycobacterium species falls outside the scope of this study.

2.1.3 DETECTION METHODS

CTB, being such a rare condition, often goes undiagnosed due to lack of suspicion (Arora *et al.*, 2000:72). A confirmed diagnosis depends essentially on the demonstration of *M. tuberculosis* within the infected tissues by traditional acid-fast bacilli (AFB) staining, culture or polymerase chain reaction (PCR) (Barbagallo *et al.*, 2002:320).

However, laboratory detection is problematic: routine culture takes more than 3 - 4 weeks to complete and results are usually negative due to the low bacillary load present in CTB. The same goes for histopathological findings which are characteristic, but not pathognomonic and are shared by various other diseases including leprosy, sarcoidosis, leishmaniasis and subcutaneous fungal infections (Ramam *et al.*, 2007:244). This can delay the onset of anti-tuberculosis therapy. BACTEC radiometric culture, a routinely used technique, is rapid with recovery of *M. tuberculosis* within 10-14 days but it requires expensive culture media and equipment (Meltzer, 2006:2). Another technique which is increasingly being utilised is PCR

because of its rapidity (results available within 24 h), sensitivity and specificity (Prasad *et al.*, 2001:138). The latter can detect *M. tuberculosis*-specific DNA sequences and therefore small numbers of mycobacteria (Barbagallo *et al.*, 2002:319).

According to Ramam *et al.* (2007:244) a 5-week therapeutic trial of anti-tuberculosis therapy can be undertaken in exceptional cases where CTB is suspected but a differential diagnosis could not be made. Patients who didn't respond after that time should be re-evaluated for reasons such as the onset of drug-resistant disease, inadequate therapy, non-compliance and/or a mistaken diagnosis.

Currently scientists are probing for new diagnostic techniques which will be both specific and sensitive enough to demonstrate the presence of *M. tuberculosis* within skin lesions. One such approach entails the use of immuno-histochemical staining to identify *M. tuberculosis* antigen in tissues. This is based on the observation that the mycobacterial antigen is last to disappear from tissues and can therefore be used as a marker of mycobacterial infections (Padmavathy *et al.*, 2005:31).

Thus the identification of CTB relies on the correlation of patient history, clinical findings as well as diagnostic techniques to ensure timely therapy.

2.1.4 DISEASE TRANSMISSION

TB is mainly contracted after inhalation of infectious droplets expelled by patients with pulmonary TB during coughing or sneezing. These droplets can remain airborne for several hours due to their small size (1 - 5 μm). Life-long tuberculin sensitivity generally develops within 2 -10 weeks after infection (Meltzer, 2006).

CTB can either develop as disseminated pulmonary TB or by direct skin inoculation with *M. tuberculosis*. CTB affects patients across the world, with a male-to-female ratio of 1.35 to 1 and occurs predominantly, but not exclusively, in young adults (up to age 30) (Meltzer, 2006).

Factors that govern the development of this disease include pathogenicity of the infecting organism, route of infection, the patient's prior sensitisation to TB and the patient's own cell-mediated immunity (Barbagallo *et al.*, 2002:320).

2.1.5 CLASSIFICATION

CTB is classified as either true CTB or tuberculids. The former is characterised by granulomatous inflammation with varying degrees of necrosis or vasculitis plus the demonstration of *M. tuberculosis*. The latter has a similar morphology to true CTB, but *M. tuberculosis* however, is not demonstrated in these lesions (Barbagallo *et al.*, 2002:320).

Previously CTB was classified in terms of the morphology of the skin lesions. These however, had many shortcomings seeing that they did not take into account the extent of systemic organ involvement or immunological aspects of the disease (Kumar & Muralidhar, 1999:496). Recent contributions of Kumar and Muralidhar (1999:496) and Barbagallo *et al.* (2002:320) regarding the classification of true CTB now include the route of infection together with the patient's immune status in order to assist physicians in selecting the most appropriate anti-tuberculous regimen.

They further distinguish true CTB as being acquired either endogenously or exogenously (Tables 1 - 3) (Kumar & Muralidhar, 1999:496; Barbagallo *et al.*, 2002:320). Endogenous infection is found in patients who were previously infected either by contiguous extension (extension from an underlying focus), hematogenous (acute miliary TB) or lymphatic spread. Exogenous infection occurs after direct inoculation of the organism into the skin of a susceptible person (Barbagallo *et al.*, 2002:321).

Table 1: True CTB: exogenous infection (Barbagallo *et al.*, 2002:320; Kumar & Muralidhar, 1999:499)

DISEASE STATE	CTB VARIANT	ROUTE OF INFECTION
Localised	Tuberculous chancre	Direct inoculation
	Tuberculosis verrucosa cutis (TBVC)	Direct inoculation
	Lupus vulgaris (some cases)	Direct inoculation

Table 2: True CTB: endogenous infection (Barbagallo *et al.*, 2002:321; Kumar & Muralidhar, 1999:499)

DISEASE STATE	CTB VARIANT	ROUTE OF INFECTION
Disseminated	Lupus vulgaris (most cases)	Hematogenous, lymphatic, contiguous
	Miliary TB	Hematogenous
	Metastatic tuberculosis abscess (tuberculosis gumma)	
	Papulonecrotic tuberculid (most cases)	
	Scrofuloderma	Contiguous
	Orificial tuberculosis	Contiguous, autoinoculation

Table 3: The tuberculids (Barbagallo *et al.*, 2002:325)

CUTANEOUS VARIANT	INFECTION SITES	ROUTE OF INFECTION
Lichen scrofulosorum	Lymph nodes and bones	Hematogenous
Erythema induratum of Bazin	Predominantly on posterolateral aspect of the calves, occasionally on thighs and upper extremities (affects more women than men)	Hematogenous
Nodular granulomatous phlebitis	Subcutaneous nodules along the leg veins of the anterior and medial side of the leg	Hematogenous
Papulonecrotic tuberculid (some cases)	-	Hematogenous

A disseminated disease state (Table 2) refers to any organ involvement besides the already existing CTB. This is of importance to prescribers, for if a three course regimen were to be prescribed without knowledge of possible underlying systemic involvement, failure would be likely due to the high occurrence of drug resistant organisms (Kumar & Muralidhar, 1999:499).

Though the present classification of CTB is more comprehensive, it is clear that some CTB variants can not be limited to exogenous or endogenous spread alone e.g. lupus vulgaris. The latter is generally spread *via* an endogenous source, but occasionally it can occur exogenously or even develop in tuberculid lesions, thus crossing between the different forms of CTB (Barbagallo *et al.*, 2002:321).

2.1.6 PATHOPHYSIOLOGY OF EXOGENOUSLY ACQUIRED TRUE CTB

2.1.6.1 TUBERCULOUS CHANCER

Tuberculous chancre (primary lesion) develops after direct infection of the skin or mucous membranes with *M. tuberculosis* in an individual who was not previously infected with TB or immunised with the *M. bovis* strain bacilli Calmette-Guérin (BCG) vaccine (Meltzer, 2006).

The organism does not cross intact skin, but gains access to the internal body *via* abrasions/injuries to the face and limbs - especially children. Cases of tuberculous chancre have been reported following mouth-to-mouth resuscitation, tattooing, ritual circumcision, ear and nose piercing, sexual intercourse, tooth extraction and after ingestion of milk contaminated with *M. bovis*. Also treatment of malignant melanoma with the BCG vaccine has been reported to cause primary inoculation TB (Barbagallo *et al.*, 2002:321; Meltzer, 2006).

An inflammatory reddish-brown papule appears after approximately 2 - 4 weeks at the inoculation site which then breaks down to form a painless, shallow, firm, non-healing ulcer with a granular base. The edges, often studded with pustules, may be red, blue and undermined (Figure 2). Painless regional lymphadenopathy manifests after 3 - 8 weeks. The chancre represents the primary tuberculous complex of the skin and is the cutaneous analogue to the Ghon complex (primary pulmonary TB) (Barbagallo *et al.*, 2002:321; Meltzer, 2006).



Figure 2: Tuberculous chancre
(Barbagallo *et al.*, 2002:322).

After 3 - 6 weeks the lesion becomes more granulomatous with an increased number of epithelioid cells and a decreased amount of tubercle bacilli. Without anti-tuberculosis treatment the primary lesion heals with scarring after 1 - 3 months. However, the latter may occasionally evolve into tuberculosis verrucosa cutis, lupus vulgaris or scrofuloderma (breakdown of lymph nodes with extension to overlying skin) (Barbagallo *et al.*, 2002:321; Meltzer, 2006; Ngan, 2006).

2.1.6.2 TUBERCULOSIS VERRUCOSA CUTIS (TBVC)

TBVC occurs after direct infection with *M. tuberculosis* at sites of skin trauma in individuals with an intact immune system but who have been previously infected with *M. tuberculosis* or *M. bovis* (Barbagallo *et al.*, 2002:321).

Contraction of this disease normally follows accidental exposure to tuberculous tissue in high-risk groups and is therefore commonly seen in physicians, pathologists, medical students and post-mortem workers. Even farmers and veterinarians may become reinfected with TB due to exposure to tuberculous cattle. TBVC lesions usually occur on the knees, elbows, hands, feet and buttocks (Meltzer, 2006).

Lesions present as single, asymptomatic, red-brown papules. The latter resembles a common wart with clefts and fissures on its surface from which it may exude pus and keratinous material (Figure 3). It has a firm periphery with a soft centre and lymphadenopathy is only associated if secondary bacterial infection occurs (Barbagallo *et al.*, 2002:322).



Figure 3: Tuberculosis verrucosa cutis
(Barbagallo *et al.*, 2002:322).

Biopsy of the lesion indicates hyperkeratosis and papillomatosis of the epidermis. Tuberculoid granulomas with moderate necrosis are found within the dermis. These lesions may persist for several years but can heal spontaneously with scarring in the absence of treatment (Barbagallo *et al.*, 2002:322; Meltzer, 2006; Ngan, 2006).

2.1.7 PATHOPHYSIOLOGY OF ENDOGENOUSLY ACQUIRED TRUE CTB

2.1.7.1 LUPUS VULGARIS

Lupus vulgaris is a benign, chronic and progressive form of CTB and occurs in patients who show tuberculin sensitivity. The majority of lesions originate mainly by hematogenous, lymphatic or contiguous spread of an endogenous source. Occasionally it is acquired exogenously by BCG vaccination, after primary inoculation or in scars of old scrofuloderma (Barbagallo *et al.*, 2002:321; Meltzer, 2006).

In Western countries 90% of lesions affect the head and neck area in which case mucus membranes can also be involved. In tropic and subtropic regions the distribution of lesions are largely found on the lower extremities and buttocks (Figure 4a – d) (Barbagallo *et al.*, 2002:322).

Lupus vulgaris lesions have four distinct clinical presentations:

- Plaque form → small sharply defined brown-red papules with an almost gelatinous consistency (also called apple-jelly nodules). Scaling sometimes occur on the lower legs. Scarring is irregular and the edges may be hyperkeratotic.
- Hypertrophic (nodular) form → displays large soft tumour nodules which are generally found on ear lobes. Ulceration and scarring is basically absent.
- Ulcerating form → characterised by scarring and crust formation over areas of necrosis. Cartilage (nose, ears) within the infection site is gradually destroyed (lupus vorax), but bone tissue is usually spared. Conjunctival, nasal and buccal mucosae may be involved by extension.
- Vegetative form → this form comprises of popular and nodular forms; necrosis and ulceration with minimal scarring (Meltzer, 2006).



Figure 4(a): Lupus vulgaris of the face (Barbagallo, 2002:323).



Figure 4(b): Lupus vulgaris developing in a BCG scar (Mlika *et al.*, 2006).



Figure 4(c): Lupus vulgaris of the ear (Ngan, 2006).

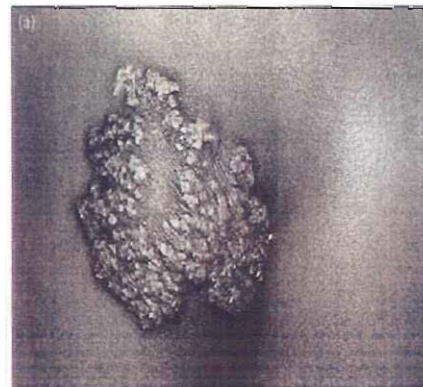


Figure 4(d): Lupus vulgaris of the buttock (Ramam *et al.*, 2005:123).

Histologic findings indicate typical granulomatous tubercle mainly in the upper dermis. There is minimal caseation necrosis and the tuberculosis bacilli concentration is very low. Lesions can remain for years if undiagnosed causing distinct disfigurement, scarring and ultimately lead to the formation of carcinomas and sarcomas (Barbagallo *et al.*, 2002:323). Patients with lupus vulgaris and concomitant pulmonary TB have a 4- to 10-fold higher mortality than those with pulmonary TB alone (Meltzer, 2006).

2.1.7.2 ACUTE DISSEMINATED MILIARY TB

Also known as *tuberculosis cutis miliaris acuta generalisata* or *tuberculosis cutis miliaris disseminata*, this once extremely rare condition has gradually been observed to re-emerge in patients where HIV/AIDS co-infection exists.

It arises from hematogenous spread of tubercle bacilli from a primary infection (pulmonary, meningeal) to various organs including the skin (High *et al.*, 2004:S111). The concentration of tubercle bacilli is high which coincides with the vast infection (Ngan, 2006:1). According to High *et al.* (2004:S111) the emergence of multi-drug resistant *M. tuberculosis* strains and the extreme immunosuppression caused by HIV/AIDS are the principal factors responsible for the re-occurrence of this disease.

Miliary TB lesions have a wide distribution and can appear on the thighs, buttocks, genitalia and exterior surfaces of the limbs, but rarely in facial regions. Lesions manifest as small (pinpoint) purpuric papules topped with minuscule vesicles. These vesicles rupture in due course to form a crust with umbilication. A white depressed scar with brownish halo develops on healing after about 1 - 4 weeks.

Histopathologic examination shows micro abscesses with superficial tissue necrosis and non-specific inflammation (Meltzer, 2006:6). Patients infected with this TB variant are generally gravely ill and prognosis is poor and many of these patients die even if diagnosed or treated (Ngan, 2006:1).

2.1.7.3 SCROFULODERMA

Scrofuloderma arises from contiguous involvement of the skin overlying tuberculous foci such as lymph nodes (cervical lymph nodes in particular), bone, joints or epididymis (Zouhair *et al.*, 2006:3). It is often seen in conjunction with pulmonary TB. Ingestion of *M. bovis* contaminated milk is a frequent cause, resulting in an oral or tonsillar primary lesion which progresses to cervical adenitis and consequently breakdown of overlying skin (Meltzer, 2006).

Initially lesions appear as firm, painless, red-brown sub-cutaneous nodules overlying an infected lymph node which harden and then degrade. Ulceration follows and purulent or caseous material may drain from it (Fig. 5). Acid-fast bacilli are found in the purulent discharge, lower dermis and walls of the ulcer (Barbagallo *et al.*, 2002:323; Meltzer, 2006).



Figure 5: Scrofuloderma (Barbagallo *et al.*, 2002:323).

In some cases lupus vulgaris may develop in close proximity of healing scrofuloderma. The latter may heal spontaneously, a process which takes years whilst leaving the patient with unsightly scars (Meltzer, 2006).

2.1.7.4 METASTATIC TUBERCULOSIS ABSCESS (TUBERCULOUS GUMMA)

This disease is a variant of scrofuloderma and is the result of mycobacterial spread from a primary focus, to the skin *via* the bloodstream in tuberculin-sensitive patients. It commonly occurs in malnourished children or severe immunosuppressed individuals (Barbagallo *et al.*, 2002:324).

Tuberculous gumma (TG) lesions mainly develop on the trunk, extremities or head. It presents as single or multiple, painless, subcutaneous abscesses which then progress to form fistulas and/or ulcers (similar to scrofuloderma). This condition may exist in the presence or absence of underlying organ tuberculosis (Meltzer, 2006).

Histological findings indicate granulomatous inflammation with occasional pronounced inflammatory infiltrate within the deep dermis and subcutaneous tissue (Meltzer, 2006).

2.1.7.5 TUBERCULOSIS CUTIS ORIFICIALIS (ORIFICIAL TUBERCULOSIS)

This condition is due to autoinoculation of *M. tuberculosis* into the periorificial (mouth, nose, anus) skin or mucous membranes from sites draining active TB and occurs in patients with advanced pulmonary, intestinal or genitourinary TB. Orificial TB is mostly observed in older males (Barbagallo *et al.*, 2002:324; Meltzer, 2006).

Sites generally affected by orificial TB include the tongue (tip and lateral margins), hard and soft palate, lips, perianal skin, vulva, urinary meatus and glans penis.

Oral involvement usually follows mycobacterial spread from pharyngeal or laryngeal TB. Intestinal and genitourinary TB give rise to anal and vulvar (females) involvement respectively (Barbagallo *et al.*, 2002:324).

Lesions are reddish papules that progress into painful, soft, circular/irregular ulcers with undermined edges and a 'punched-out' appearance (Figure 6). Tubercles with acid-fast bacilli are present in the dermis and ulcer walls. Histologic examination reveals an ulcer in the upper dermis together with caseous granuloma located in the deep dermis (Barbagallo *et al.*, 2002:324).



Figure 6: Orificial tuberculosis
(Barbagallo *et al.*, 2002:324).

Orificial TB serves as an indicator of advanced internal disease. Therefore even if anti-tuberculous therapy is initiated, the patient's prognosis is poor and these lesions generally do not respond to therapy (Barbagallo *et al.*, 2002:324; Meltzer, 2006).

2.1.7.6 PAPULONECROTIC TUBERCULID

Usually tubercle bacilli cannot be demonstrated by culture or seen on tissue examination, but for most cases DNA could be detected with PCR. Therefore most cases of papulonecrotic tuberculid are considered true CTB whilst a small handful of cases are considered tuberculids (Barbagallo *et al.*, 2002:325).

Mainly children and young adults are observed to be affected by this condition. It occurs as chronic, recurrent, symmetric, asymptomatic eruption of clusters of dusky red pea-sized papules which are often crusted or ulcerated. These papules may also

present with pustules. These lesions appear on the exterior aspects of extremities (elbows, knees, buttocks and lower trunk). Involution takes place spontaneously after 6 - 8 weeks, leaving pitted scars (Figure 7).

A diagnostic aid to papulonecrotic tuberculid is the presence of active lesions adjacent to areas of scarring (Barbagallo *et al.*, 2002:325; Meltzer, 2006).



Figure 7: Papulonecrotic tuberculid
(Barbagallo *et al.*, 2002:324).

Histological examination shows a wedge-shaped region of necrosis of the upper dermis extending into the epidermis. Granulomatous infiltrate or leukocytoclastic vasculitis may also be present. These lesions resolve remarkably with anti-tuberculous therapy (Barbagallo *et al.*, 2002:325).

2.1.8 TUBERCULIDS

The exact correlation between TB and tuberculids is unclear. Tuberculids often afflict individuals who:

- Are in relative good health.
- Have positive tuberculin sensitivity.

- Have tuberculous involvement (mostly inactive) of visceral organs or lymph nodes.
- Test negative during culture/staining for *M. tuberculosis* in infected tissue.
- Have lesions that heal with initiation of anti-TB medication (Meltzer, 2006).

Histological findings regarding tuberculids all show granulomatous inflammation together with vasculitis and/or necrosis of various degrees. Earlier investigators suggested that tuberculids arise from hypersensitivity reactions due to *M. tuberculosis* toxins, but recent demonstration of mycobacterial DNA by PCR methods have since led to the opinion that tuberculids are caused by hematogenous spread of bacilli from an infectious focus from somewhere else (Barbagallo *et al.*, 2002:325; Meltzer, 2006).

Tuberculids are further subdivided into two groups:

- a) True tuberculids → lichen scrofuloderma and papulonecrotic tuberculid.
- b) Facultative tuberculids → erythema induratum of Bazin.

M. tuberculosis is believed to play a prominent role in the etiology of the former because papulonecrotic tuberculid lesions may evolve into lupus vulgaris from which *M. tuberculosis* have been demonstrated by culture. It therefore supports the idea that tuberculids have a tuberculous origin. In case of the latter, *M. tuberculosis* forms part of a variety of etiologic factors (Barbagallo *et al.*, 2002:325).

2.1.8.1 LICHEN SCROFULOSORUM (TRUE TUBERCULID)

This condition is characterised by numerous, asymptomatic, perifollicular, greyish-white lichenoid papules arranged in groups on the trunk (Figure 8). For the most part children and young adults with underlying TB are affected (Barbagallo *et al.*, 2002:326).

Superficial tuberculoid granulomas surrounding hair follicles and sweat ducts without caseation necrosis can be observed during histology. No acid-fast bacilli could be demonstrated (Barbagallo *et al.*, 2002:326; Meltzer, 2006).



Figure 8: Papules of lichen scrofulosorum (Barbagallo *et al.*, 2002:326).

Over the course of weeks the lesions become more extensive before slowly regressing after some months to several years without scarring. Lesions may recur. This condition responds to anti-TB treatment, but not to the same extent as seen in other tuberculids (Meltzer, 2006).

2.1.8.2 ERYTHEMA INDURATUM OF BAZIN (FACULTATIVE TUBERCULID)

Erythema induratum is a common, persistent condition associated with active or past TB. Pre-existing erythrocyanotic circulatory disease may also serve as a predisposing factor to develop this type of tuberculid (Meltzer, 2006).

Lesions are few and erupt as tender, hardened, inflammatory cutaneous or subcutaneous nodules which may ulcerate in the posterolateral aspect of the calves of woman's legs - especially those with heavy legs (Figure 9). The thighs, upper extremities and men (< 10% of cases) are much less affected.

Histologically inflammation of subcutaneous fat lobules, vasculitis and tuberculous granulomas with caseation necrosis are shown. These lesions respond slowly to anti-TB therapy and recurrences are frequent, favouring colder seasons (Barbagallo *et al.*, 2002:326).



Figure 9: Erythema induratum
(Barbagallo *et al.*,
2002:327).

According to Barbagallo *et al.* (2002:326) patients are tuberculin positive and culture negative for mycobacteria. However, recent identification of *M. tuberculosis* DNA by means of PCR has led to the conclusion that as diagnostic techniques improve, the classification of CTB as true CTB or tuberculids may become subject to change.

2.1.8.3 NODULAR GRANULOMATOUS PHLEBITIS

As this condition is uncommon and the tuberculi recently identified, it appears to be an initial phase hypersensitivity reaction to *M. tuberculosis* and is probably an entity quite distinct from other tuberculids (Barbagallo *et al.*, 2002:326).

Lesions appear as nonulcerating, subcutaneous nodules situated along the leg veins of the anterior and medial sides of the leg. Histological investigation indicates epithelioid cell granulomas in the cutaneous veins' walls (Barbagallo *et al.*, 2002:326).

2.1.9 PREVALENCE OR INCIDENCE OF DIFFERENT TYPES OF CUTANEOUS TUBERCULOSIS

A fifteen year (1981 - 1995) retrospective study conducted by Yates and Ormerod (1997:483) revealed that scrofuloderma (55.3%) was the most common form of CTB

prevalent in the Blackburn district (U.K.), followed by lupus vulgaris (12.8%), tuberculous gumma (8.5%) and tuberculids (21.3%).

A similar trend was apparent from a retrospective study (Jan. 1981 - Dec. 2004) done by Zouhair *et al.* (2006:1) for which the major types of CTB identified were scrofuloderma and tuberculosis gumma (72%), lupus vulgaris (12%), tuberculosis cutis verrucosa (7%), tuberculids (6%), orificial TB (1%) and tuberculous chancre (1%).

However, in India, lupus vulgaris (55%) was the most frequent CTB variant encountered and thereafter scrofuloderma (26.8%), tuberculosis verrucosa cutis (6%), tuberculous gumma (5.4%) and tuberculids (6.8%) (Kumar & Muralidhar, 1999:495). The same trend was also identified by a South African study in which scrofuloderma was less frequently encountered than lupus vulgaris (Yates & Ormerod, 1997:488).

Therefore, it is evident that some types of CTB are more prevalent than others within a specific population and that the distribution may also differ from region to region.

2.1.10 DIFFERENTIAL DIAGNOSIS

Since both true CTB and tuberculids show a wide variety of morphological presentations which are often similar to other dermal conditions, a differential diagnosis is imperative to ensure timely initiation of chemotherapy. Table 4 lists the variants of CTB and other dermal diseases they should be differentiated from.

Table 4: CTB variants to be differentiated from other dermal diseases (Meltzer, 2006).

CTB VARIANT	DIFFERENTIATE FROM
TRUE CTB	
Primary inoculation TB (Tuberculous chancre)	Ulceroglandular complexes and mycobacterioses
TB verrucosa cutis	North American blastomycosis, chromoblastomycosis, ioderma and bromoderma, chronic vegetative pyoderma, verruca vulgaris, verrucous carcinoma, verrucous atypical mycobacterial infection and verrucous lupus vulgaris
Miliary TB of the skin	Differentiate these small, noncharacteristic, erythematous papular or purpuric lesions from drug reactions
Scrofuloderma	Supportive lymphadenitis with sinus tract formation e.g. blastomycosis and coccidioidomycosis
TB cutis orificialis	Glossitis, apothecosis, deep fungal infections
Lupus vulgaris	Lupoid rosacea, deep fungal or atypical mycobacterial infection, chronic granulomatous disease, granulomatous rosacea, Wegener granulomatosis
Erythema induratum	Nodular panniculitides (Weber-Christian disease) and nodular vasculitides (e.g. syphilitic gumma, nodular pernio)
Papulonecrotic tuberculid	Differentiate from other papulonecrotic entities e.g. leukocytoclastic vasculitis, lymphomatoid papulosis, papular eczema, prurigo simplex with neurotic excoriation
TUBERCULIDS	
Lichen scrofulosorum	Keratosis spinulosa, lichenoid sarcoid and lichenoid secondary syphilis

2.1.11 CURRENT TREATMENT REGIMENS FOR CUTANEOUS TUBERCULOSIS

In view of the fact that CTB is mostly associated with systemic involvement, current treatment comprises of the standard three/four drug regimens. It is proposed that since the bacillary load within CTB lesions is low, standard oral treatment should be adequate. Duration of treatment must be sufficient to eradicate all viable organisms and prevent recurrent infection or drug resistance (Barbagallo *et al.*, 2002:327).

There exist 4 categories of anti-TB treatment regimes depending on the localisation of disease, degree of TB infection, sputum smear result and history of previous treatment. Tables 5 and 6 describe the various categories with corresponding anti-tuberculosis treatment regimens respectively.

Table 5: Treatment categories with their corresponding anti-TB treatment regimens (Jakubowiak *et al.*, 2001: 41).

TREATMENT CATEGORIES	TREATMENT REGIMEN
Category I	Regimen i (new smear-positive regimen)
Category II	Regimen ii (smear-positive retreatment)
Category III	Regimen iii (smear-negative regimen)
Category IV	Chronic cases (sputum-positive after supervised retreatment). Treatment regimen given in specialised centres.

Table 6: Treatment regimens administration scheme (Jakubowiak *et al.*, 2001:46).

TB TREATMENT REGIMEN	INITIAL PHASE (2 MONTHS)	CONTINUATION PHASE (4 MONTHS)
I	E,INH, RMP, PZ or S, INH, RMP, PZ	INH & RMP
II	S, INH, RMP, PZ & E or 1 month INH, RMP, PZ & E	5 months INH, RMP & E
III	INH, RMP & PZ	INH & RMP
IV	Regimes used for drug resistance management.	

*E = ethambutol, INH = isoniazid, RMP = rifampicin, PZ = pyrazinamide, S = streptomycin

The administration of anti-TB drugs is divided into two phases. Their objectives being:

- Initial (intensive) phase → to decrease the number of tubercle bacilli in actively multiplying sub-populations, therefore bringing about a rapid decrease in bacterial load.
- Continuation (sterilising) phase → to eradicate remaining organisms or significantly decrease the number of bacilli in semi-dormant subpopulations (Jakubowiak *et al.*, 2001:40).

Kumar and Muralidhar (1999:499) suggested that for patients with tuberculids, tuberculosis verrucosa cutis, lupus vulgaris and smear-negative scrofuloderma, a three-drug regimen be used. In the case of smear-positive patients as well as those

presenting with the remaining types of CTB a four-drug regimen is recommended. Barbagallo *et al.* (2002:327) reported that patients who contracted CTB by accidental inoculation e.g. healthy laboratory personnel, responded well to treatment. Their lesions healed within 3 months of therapy with INH, RMP and E. Furthermore, the patients were advised to continue with therapy for another 2 months until their lesions have completely resolved.

However, information regarding CTB is still needed with regards to the response to anti-TB therapy. This is according to research done by Ramam *et al.* (2005:121) which concluded that cases of CTB showed improvement within 1 month of therapy with anti-TB drugs, but complete resolution was only attained after 4 - 6 months. The major drawback of current therapy is that patients not only remain a source of infection (viable organisms can still be demonstrated), but they also suffer from constant embarrassment due to the disfiguring nature of CTB until these lesions have healed completely. Surgical excision is sometimes used, especially in the management of scrofuloderma, localised tuberculosis verrucosa cutis lesions and lupus vulgaris. Cryotherapy or electrocautery may also be used to destroy lupoid nodules in scarred areas (Barbagello *et al.*, 2002:327).

Though no evidence of an already existing topical formulation containing either INH or RMP, or a combination of the two substances could be found, a surprising discovery made by Clark and Lealand (1978) comprised the topical application of a 0.5 - 10% by weight concentration of INH in a carrier base as treatment for psoriasis. They tested various concentrations of INH dispersed in hydrophilic ointment U.S.P. with or without added betamethasone valerate (0.5%) on 15 patients suffering from psoriasis. The formulation proved to be surprisingly effective towards clearing the psoriatic lesions. Their study, however, did not provide any quantification as to the actual amount of applied INH that penetrated the skin or lesions.

Hoover *et al.* (1993:111) explored the antimicrobial activity of rifaximin, a topical rifampicin derivative. *In vitro* evaluation of rifaximin against numerous organisms indicated that it had a broad antimicrobial spectrum similar to that of rifampicin, including activity against the *Mycobacterium* complex. In contrast to rifampicin, it is poorly absorbed by the oral route and therefore the authors anticipated its potential for use as a topical agent (1 - 5% concentrations) for the treatment of bacterial vaginosis and some sexually transmitted diseases (STDs).

Their study also did not include *in vitro* permeation measurements or any reference to CTB. No further evidence for a topical formulation involving specifically RMP could be found. In view of the above we thought it prudent to investigate the dermal penetration ability of INH and RMP in 0.5% and 1.0% concentrations respectively; which if proven successful, may give rise to the development of a topical formulation to serve as an adjunctive to systemic therapy, which in turn might lead to a more rapid cure for CTB lesions.

As mentioned earlier, CTB lesions show a wide variety of morphologic presentations which can affect one or more regions within the skin itself. The following section provides background information regarding the basic physiology of skin together with factors influencing transdermal drug delivery.

2.2 SKIN STRUCTURE

The skin is a unique moiety that forms the interface between the body and hostile outside environment (Verma *et al.*, 2003:141). Being the outermost layer, it was at first thought to be biochemically and chemically inert, but experimental findings have since demonstrated that it is in fact a metabolically active tissue (Ziboh & Chapkin, 1988:81). In essence the skin consists of three distinct layers: epidermis (outer non-viable and inner viable epidermis), dermis and subcutaneous fat.

2.2.1 AVASCULAR, NON-VIABLE EPIDERMIS (OUTERMOST SKIN LAYER) STRATUM CORNEUM (SC)

The SC consists of 10 - 25 layers of dead, flattened, hexagonal cells (corneocytes) filled with the fibrous protein, keratin, which is produced by the underlying viable epidermis. Corneocytes are embedded in a matrix of intercellular lipids. The intercellular lipids are composed of ceramides, cholesterol and free fatty acids (Van Hal *et al.*, 1996:89). This gives rise to the hydrophilic-lipophilic nature of the SC which dictates that only substances which have both hydrophilic and lipophilic properties will be able to readily permeate through the SC (Schalla & Schaefer, 1981:42). It is this layer that is recognised as the rate-limiting barrier with regards to solute permeation.

2.2.2 AVASCULAR, VIABLE EPIDERMIS (VE)

Located directly beneath the SC, about 150 µm thick, it consists mainly of 4 layers: the stratum lucidum, stratum granulosum (granular layer), stratum spinosum (spinous layer) and stratum basale (basal layer). The latter two layers are often referred to as the stratum germinativum. The SC is constantly being renewed by cells generated in the basal layer of the VE, which undergo many morphological changes as they migrate towards the skin surface. Also located within the basal layer are:

- Melanocytes - responsible for producing melanin (protection against UV rays).
- Langerhans cells - which act in the skin's immune response.
- Merkel cells - which play a mechanosensory role (Walters, 2002:7-11).

2.2.3 VASCULAR DERMIS (CORIUM)

The dermis lies between the epidermis and the subcutaneous fat. It mainly comprises of the proteins collagen and fibrillin, which provide for the skin's strength and elasticity. The dermis contains connective tissue, blood vessels, lymph vessels and nerves. It poses no resistance to the penetrant which allows for rapid permeation into the systemic circulation, resulting in the so-called 'sink' conditions. Therefore the penetrant concentration within the dermis remains minimal whilst maximising the concentration gradient across the epidermis (Barry, 1983:7-10).

2.2.4 SUBCUTANEOUS FAT (HYPODERMIS/ SUBCUTIS)

This layer is found underneath the dermis. The fat is stored in adipocytes (fat cells) which form a network of lobules that are supported by fibrous tissue. The thickness of this layer varies from one anatomical site to another. Its most vital functions include insulation against temperature differences, protection (shock absorber) of internal organs and energy storage (Abraham *et al.*, 1995:8).

2.2.5 SKIN APPENDAGES

The following appendages are associated with the skin:

- Hair follicles together with their sebaceous glands - the latter provides lubrication and waterproofing.
- Eccrine sweat glands - temperature regulation.
- Apocrine sweat glands - responsible for body odour.
- Nails which protect the terminal phalanx (Hunter *et al.*, 1995:14-16).

The hair follicles, eccrine and apocrine sweat glands are situated within the dermis.

2.3 FUNCTIONS OF THE SKIN

The skin performs a wide spectrum of functions which are given in Table 7:

Table 7: Main functions of the skin (Barry, 1983:14; Hunter *et al.*, 1995:5)

○ Mechanical function -	to contain body fluids and tissues.
○ Protective functions -	to protect the body from potential harmful external stimuli namely UV radiation, chemicals, particles, antigens, microbes, heat, mechanical shock and serves as an electrical barrier.
○ Sensory function -	to receive and register external stimuli such as pressure, pain or heat (specialised nerve endings).
○ Regulate body temperature (blood vessels and eccrine sweat glands).	
○ Removal of chemical waste (glandular excretions).	
○ Synthesis and metabolism of compounds (e.g. vitamin D synthesis).	
○ Regulate blood pressure (vasodilatation/ vasoconstriction).	
○ To attract members of the opposite sex (apocrine secretions).	
○ Psychosocial display for individual identification.	

2.4 PERCUTANEOUS ABSORPTION AND ROUTES OF PENETRATION

Two major pathways have been suggested for solute permeation through human skin namely the transepidermal and transappendageal routes.

2.4.1 TRANSEPIDERMAL ROUTE

The transepidermal route refers to drug transport through the SC *via* two different types of permeation mechanisms namely the transcellular and intercellular routes.

2.4.1.1 TRANSCELLULAR ROUTE

This route concerns the movement of a solute directly through the cells and intercellular spaces of the SC. The intercellular spaces consist of lipid bilayers arranged in multilaminated sheets (Van Hal *et al.*, 1996:89). They contain straight, densely packed, saturated hydrocarbon chains that alternate with watery channels which form the polar pathways through the bilayer. It is mainly water, ions and uncharged hydrophilic molecules that transverse the SC *via* these polar pathways. The intracellular spaces mainly comprise of corneocytes (cornified cells) anchored to the intercellular lipid. Extraction of intercellular lipids enhances solute permeation, therefore the lipid matrix is considered to be responsible for most of the barrier function of the SC (Wiechers, 1989:186).

2.4.1.2 INTERCELLULAR ROUTE

The intercellular route describes the movement of a permeant only through the intercellular lipid region. Due to the cellular arrangement of the lipid bilayers, it will result in any diffusing molecule taking this route, to follow a very arduous pathway through the membrane. Because this route is largely favoured by lipophilic substances we assume that it should provide the primary permeation pathway for RMP across the SC (Zatz, 1993:16).

Although the physicochemical properties of a permeant ultimately determine which pathway is favoured above the other, the total amount of drug that penetrates the SC is the sum of both contributions (Wiechers, 1989:186).

2.4.2 TRANSAPPENDAGEAL ROUTE

This route can be described as the shunt route by which a diffusing molecule penetrates the skin *via* eccrine sweat ducts and along hair follicles with their associated sebaceous glands, therefore bypassing the SC (Hadgraft, 1983:255-256). The transappendageal route may be of some importance to ionic or large polar

compounds. Unfortunately, as shunts account for less than 1% of the total body surface area of human skin, the transepidermal route is expected to be the principal pathway for most substances (Zatz, 1993:15).

2.5 ADVANTAGES AND LIMITATIONS OF TRANSDERMAL DRUG DELIVERY

The most important advantages and limitations associated with transdermal drug delivery are discussed in sections 2.5.1 and 2.5.2 respectively.

2.5.1 ADVANTAGES

Topical drug delivery has become an attractive alternative because it provides some biopharmaceutical benefits over the oral route such as:

- Drug delivery at the site of action.
- Avoidance of hepatic first-pass metabolism.
- Avoidance of problems relating to stomach emptying, pH effects, food intake and enzyme deactivation associated with passage through the intestinal tract.
- Continuous controlled drug input (for drugs with short half lives) - less frequent dosing, thus improved patient compliance.
- Elimination of pulsed entry into systemic circulation - decreased side effects (Walters, 2002:4).

2.5.2 LIMITATIONS

As with any other administration route, the delivery of solutes *via* the skin is also laden with difficulties. These include:

- The variability in amount of absorbed drug due to site, disease, age and species differences.
- The skin's "first-pass" metabolism effect.
- Reservoir capacity of the skin.

- Irritation, allergic sensitization, hyper pigmentation and other toxicities caused by topical products.
- Heterogeneity and inducibility in both the turnover and metabolism of the skin.
- Inadequate definition of bioequivalence criteria.
- An incomplete understanding of the technologies that may be used to facilitate or retard cutaneous absorption (Walters, 2002:4).

2.6 FACTORS THAT INFLUENCE THE PERCUTANEOUS ABSORPTION OF SUBSTANCES

The amount of drug that transverse the skin after topical application is determined by bio-medical factors, physicochemical properties of the penetrant and vehicle as well as dosing conditions.

2.6.1 BIO-MEDICAL FACTORS

2.6.1.1 REGIONAL SKIN SITES

A considerable amount of variation in SC thickness exists from one anatomical site to another. Solute permeability can be ranked as follows: genitals > head areas > trunk > limbs > plantar regions of the palms and feet, depending on the nature of the penetrant used (Zatz, 1993:13). For the purpose of this study, only abdominal skin from Caucasian female patients obtained after abdominoplastic surgery will be used in an effort to diminish the permeation variability between skin sites.

2.6.1.2 SKIN AGE

The skin of neonates, young children and the elderly is regarded to be more permeable than that of adults. In neonates and infants this is because the skin structures and SC are not yet fully developed. Little evidence exists about permeability in the elderly, but atrophy and dry skin conditions are frequent features that may result in a decreased barrier function. A diminished dermal blood supply in old age may also contribute to permeation variations (Wiechers, 1989:188; Schalla & Schaefer, 1981:58). CTB affects patients from as early as childhood (< 10 years) to those well above 60 years of age, with the majority of cases occurring in adolescents and adults up to $\pm$ 50 years (Kumar & Muralidhar, 1999:494). Therefore if it could be

demonstrated that INH and RMP are able to penetrate the SC of adult skin, it can be assumed that the same will be true for children and/or the elderly.

2.6.1.3 SKIN CONDITION

The skin can fulfil its barrier function on condition that it remains intact (Wiechers, 1989:188). CTB may affect different skin layers which alter the degree of barrier function of the skin. In some instances, according to the disease pathology, the SC might lack entirely, which would allow for easy passage of the topical applied anti-tubercular drugs. However as the skin heals, its barrier function will be restored, which may decrease the penetration of the applied drugs to some extent. CTB lesions are furthermore associated with various degrees of one or more of the following: ulceration, plaque formation, hyperkeratosis or the presence of necrotic matter (Barbagallo *et al.*, 2002:321). All of these may influence the diffusion of INH and RMP in the Pheroid™ vehicle to the intended sites on the tubercle bacilli.

2.6.1.4 HYDRATION

The normal SC contains 15 - 20% (dry weight) water. When exposed to high humidity i.e. soaking or occlusion (prevention of transepidermal water loss) the SC water content is increased. According to Barry (1987:89-90) the insertion of water molecules loosen the lipid packing by bonding to the lipid polar heads, thereby increasing the fluidity of the lipid domains. Water also competes for hydrogen bonding sites on the protein chains thus reducing interactions between them. This mechanism might explain why hydration promotes the permeation of both polar (i.e. INH) and non-polar (i.e. RMP) substances.

2.6.1.5 SKIN METABOLISM

Microbial flora (dermatophytes, yeasts) and enzymes from glandular secretions that are present on the skin surface have a negligible effect on the stability of a topically applied drug (Täuber, 1981:142). However, metabolism (also referred to as “first-pass” metabolism of the skin) of up to 5% of candidate topical drugs can occur within the viable skin layers. Substances known to be metabolised by skin enzymes include steroid hormones, chemical carcinogens and some drugs. Dermal enzymatic activity can be a determining factor when investigating the efficacy of topical pro-drugs (Barry, 2002:510). For the purpose of this *in vitro* study, diffusion experiments

will be conducted with excised epidermis as diffusion membrane. Since the viability of the epidermal layer is not maintained it can be assumed that metabolism within the skin will be negligible (Zatz, 1993:21).

2.6.2 PHYSICOCHEMICAL FACTORS

2.6.2.1 PARTITION COEFFICIENT (P)

The partition coefficient, sometimes also abbreviated as K, is a measure of the distribution of molecules between octanol and water. For transdermal studies it serves as an indication of the distribution of penetrant molecules between the vehicle and biological membrane (SC). The partition coefficient of a substance is the factor that dictates which possible pathway the permeant will follow through the skin (Williams, 2003:36).

Hydrophilic substances will preferentially permeate *via* the transcellular route. For more hydrophilic and charged molecules ($\log P < 1$) the transappendageal route may also be of significance. The $\log P$ value for INH = -0.64, therefore it can be assumed that INH will transverse the skin *via* the transcellular and transappendageal routes (Drugbank, 2007).

Predominantly lipophilic substances ($\log P > 3$) such as RMP ($\log P = 3.714$) will penetrate the SC almost exclusively by the intercellular route. Molecules with intermediate partition ($\log P$ of 1 to 3) will largely depend on the intercellular pathway to transverse the SC although a small amount might use the transcellular route (Williams, 2003:36; Drugbank, 2007).

2.6.2.2 DIFFUSION COEFFICIENT (D) / DIFFUSIVITY

The diffusion coefficient dictates the rate and ease with which a permeant crosses a membrane under specified conditions. It is a kinetic property, usually expressed in units of area per time (cm^2/s or cm^2/h) (Smith, 1990:31; Williams, 2003:223).

If the temperature is held constant, the magnitude of the diffusion coefficient is dependant upon the properties of the drug and the diffusion medium as well as the interactions between them. Drug binding of highly lipophilic substances with the SC may result in an increased lag time with corresponding decrease in diffusivity (Barry,

2002:512). For the hydrophilic INH, binding with the SC seems unlikely but the same might not be true for the lipophilic RMP.

2.6.2.3 MOLECULAR SIZE AND SHAPE

An inverse relationship exists between the absorption rate and the molecular weight of a permeant. Smaller molecules will therefore penetrate the SC faster (have a higher diffusivity) than larger molecules (Williams, 2003:36). This is because large molecules encounter steric hindrance of motion in the available pathways for diffusion (Smith, 1990:28). For drugs with a molecular weight of 100-500 and even up to 5000, there seems to be no major influence on the absorption rate (Wiechers, 1989:190). From the above is clear that INH (MW = 137.14) falls within the ideal molecular weight range for transdermal delivery and should therefore penetrate the SC faster than RMP (MW = 822.95).

2.6.2.4 SOLUBILITY / MELTING POINT

There exist a clear relationship between the solubility and melting point of a substance. Under normal conditions for temperature and pressure i.e. that typically prevail under conditions for transdermal drug delivery, substances with high melting points have relatively low aqueous solubility (Williams, 2003:37). RMP and INH have melting points of 183 °C and 171.4 °C, respectively. RMP being the more lipophilic of the two substances has the higher melting point (Drugbank, 2007).

2.6.2.5 DRUG CONCENTRATION

The flux of a substance across the skin is dependent on the drug concentration. If the drug concentration in the outermost layers of the SC is high, the concentration gradient will be steep (Behl *et al.*, 1993:241). The majority of vehicles used today are not inert and interact with the SC in some way or other (occlusion, lipid extraction etc.). If a vehicle does not act as a penetration enhancer, the flux of a permeant across the skin is directly proportional to the activity of the drug in the vehicle (Wiechers, 1989:194). Therefore a saturated solution of the permeant should be used to ensure that a maximum drug concentration is attained on the SC surface. Consequently the thermodynamic activity (leaving potential) for such a solution is maximal so that the molecules undergo the least amount of vehicle bonding

interactions and are most likely to leave the formulation by partitioning across the vehicle-membrane interface (Smith & Surber, 2000:32).

With regards to this study, the Pheroid™ system will act as a penetration enhancer itself. Pheroids™ cause a temporary disruption in the packed structure of the SC, thereby increasing the drug partitioning into the skin and delivering the entrapped drugs (Saunders *et al*, 1999:106).

2.6.2.6 IONISATION, pH AND pKa

Although the pH-partition hypothesis dictates that only unionised molecules are able to diffuse across the SC, studies have shown that electrolytes also cross the skin barrier, albeit with much difficulty (Smith, 1990:27; Wiechers, 1989:190). In the case of weak acids/bases the pH of the vehicle in combination with the permeant's pKa (ionisation constant) will determine the actual concentration of ionised or unionised species present in the formulation (Wiechers, 1989:190).

Diffusion experiments are going to be conducted at pH = 5.5 since it is the pH of maximum stability for both INH and RMP whilst simultaneously simulating the pH conditions (pH = 4 – 6.5) prevalent in normal skin (Yosipovitch & Hu, 2003:88). Both INH and RMP are amphoteric (zwitterions) substances. They contain both positive and negative charges depending on their functional groups as well as the surrounding pH. INH possesses three ionisation constants (pKa), 1.8 (hydrazine nitrogen); 3.5 (pyridine nitrogen) and 10.8 (acidic group) with an overall isoelectric point* (pI) of 1.82. Since the pH of the vehicle is higher than that of the pI, the INH molecule will therefore carry a net negative charge (Drugbank, 2007). This will greatly decrease INH's partitioning into the lipophilic SC.

RMP on the other hand has two pKa values at 1.7 (hydroxyl group at C8) and 7.9 (piperazine moiety at position N4) respectively, with an isoelectric point of 4.8. Therefore RMP is expected to be largely unionized at pH = 5.5 (Malassiney *et al.*, 1996:1128). Other factors excluded, we assume that RMP will partition across the SC with less difficulty than INH.

*Isoelectric point (pI): it is the pH at which a particular molecule carries no net electrical charge. A molecule's pI can affect its solubility at a certain pH. At a pH below the pI the molecule is positively charged; at a pH above its pI it is negatively charged and when the pH = pI the molecule is neutral (Wikipedia, 2007).

2.6.2.7 VEHICLE FORMULATION

The term “vehicle” refers to the liquid or semisolid in which the drug is dispersed for delivery to the SC (Smith, 1990:29). The physical properties of the vehicle may have effects on either the drug release rate or on the SC of the skin itself.

Drug release is affected by:

- a) The physical properties of the vehicle itself e.g. viscosity - a highly viscous formulation tends to retard the rate of drug diffusion through the vehicle.
- b) The interactions between the drug molecules and vehicle - the degree of solubility of the penetrant in the vehicle affects its partitioning out of the formulation and into the upper layers of the SC. This determines the drug concentration available for absorption (Wiechers, 1989:192).

2.6.2.8 OTHER FACTORS

Drug binding due to interactions between the penetrant and skin structure should also be taken into account. Interactions range from weak Van der Waals forces to strong hydrogen bonding, which in turn affect the flux of a compound across the SC. Drug binding also affects the lag time, delaying the time between that of drug application on the SC and its appearance in the systemic circulation. For patch therapy this phenomenon is not important, but for finite dosing for a topical action, it may result in poor availability of the applied active agent (Williams, 2003:40).

2.6.3 DOSING CONDITIONS

2.6.3.1 TEMPERATURE

Thermal analysis studies have shown that an increase in skin temperature led to higher diffusion coefficients and increased percutaneous absorption. This is due to changes in the lipid order of the SC. Furthermore there seems to be an increase in cutaneous blood flow at higher temperatures which permits the skin to act more as a sink. Factors that induce rises in skin temperature include: increased environmental temperature (weather/ clothing) and physiological responses (strenuous physical labour/ fever) (Wiechers, 1989:194).

As CTB is mostly associated with pulmonary TB and/or multiple organ involvement, fever is a common physical sign accompanying this disease (Jakubowiak *et al.*, 2001:21). In the clinical setting this effect is hardly measurable and the overall effect of temperature on drug permeation seems to be minimal as well as reversible at temperatures below 70 °C. Other factors such as occlusion or diseased skin generally have a much more prominent effect on skin permeation (Wiechers, 1989:194).

2.6.3.2 OCCLUSION

Occlusion prevents water-loss from the surface of the SC, thereby increasing its state of hydration as the endogenous water gets trapped within. This increases the SC's permeability especially with regards to lipophilic substances. Another important feature is that a larger quantity of the penetrant can be contained within the SC, thereby allowing for possible faster permeation to the viable epidermis (Wiechers, 1989:195).

2.7 MATHEMATICAL MODEL OF SKIN ABSORPTION

As drug permeation is in essence a passive diffusion process that takes place along a concentration gradient from a region of high drug concentration on the SC surface to a region with a low drug concentration within the skin. Fick's first law of diffusion can be applied to determine the rate of drug transport across the skin:

$$J = k_p \cdot \Delta C = \frac{K \times D}{l} \cdot \Delta C \quad \text{Equation 2.7.1}$$

Where

k_p is the permeability coefficient.

J is the steady state flux ($\text{mg} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ or $\mu\text{g} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) which is the total amount of drug that permeates a membrane per unit area per unit time.

K is the partition coefficient of the drug between the delivery vehicle and first layer of the membrane.

D ($\text{cm}^2 \cdot \text{h}^{-1}$) is the diffusivity of the drug in the membrane.

ΔC ($\text{mg} \cdot \text{cm}^{-3}$) is the concentration difference across the membrane and

l (cm) is the thickness of the membrane (Smith & Surber, 2000:24-26).

2.8 PENETRATION ENHANCERS

In order to improve transdermal drug delivery, scientists have been investigating a number of different approaches that will circumvent the barrier function of the SC. These include the use of penetration enhancers (chemical or physical) which reversibly alter the barrier resistance of the SC to allow for greater drug permeation through the skin (Barry & Williams, 2004:603).

2.8.1 CHEMICAL ENHANCERS

Table 8: Chemical penetration enhancers (Purdon *et al.*, 2004:100)

CLASS		EXAMPLES	MECHANISMS
Sulphoxides		Dimethylsulfoxide (DMSO)	Extraction of soluble components of the SC, disruption of the ordered structure of lipid chains, denaturing proteins
Alcohols		Ethanol, Octanol	Enhancing drug solubility, disruption of SC integrity through biochemical extraction
Polyols		Propylene glycol	Solubility modification by salivation of α -keratin in the SC
Fatty Alcohols		Lauryl & oleyl alcohol	Increase drug solubility & partitioning, disruption of skin barrier function, ion-pair formation
Fatty Acids		Oleic acid, lauric acid, linoleic acid, linolenic acid, capric acid	Perturbation of the intercellular lipid bilayers, ion-pair formation
Esters		Ethyl acetate, octyl acetate	Disruption of lipid packing, influence partitioning between vehicle and skin
Amines & amides	Urea	Dimethylacetamide, 1-alkyl-4-imidazolin-2-one	Disruption of lipid, facilitate hydration of the SC
	Pyrrolidones	1-dodecyl-azacycloheptan-2-one, 2-pyrrolidone-1-acetic acid dodecyl ester	Fluidising the lipids in the intercellular lipid domains, changing the SC lipid arrangement
Terpenes		D-limonene, menthol	Disruption of intercellular lipid, increase electrical conductivity of tissues
Surfactants	Anionic	Sodium laurate, sodium cholate	Alteration of the barrier function of the SC, removal of water-soluble agents, emulsifying sebum
	Cationic	Cetyltrimethyl ammonium bromide	
	Nonionic	Poloxamer, lecithin	
Cyclodextrins		α -, β - and γ -cyclodextrin	Inclusion of drugs into molecular cavity
Oxazolidinones		4-decyloxazolidin-2-one	Localising drugs in skin layers

Table 8: Chemical penetration enhancers (continued)

Chitosan	Poly[b-(1-4)-2-amino-2-deoxy-D-glucopyranose]	Ionic interactions with negatively charged sites on the cell surface
Lipid based delivery systems	Liposomes	Interacting with skin lipids
	Transfersomes	Squeezing through polar channels under a hydration gradient
	Ethosomes	Ethanol fluidises both ethosomal lipids and bilayers of the SC intercellular lipid
Drug-vehicle interactions	Supersaturated solution	Elevating thermodynamic activity of drug - maximize drug release from vehicle
	Microemulsions	Disruption of SC lipids, increasing the partitioning of the drug into the skin.

2.8.2 PHYSICAL ENHANCERS

Table 9: Physical penetration enhancers (Purdon *et al.*, 2004:100)

CLASS	EXAMPLES	MECHANISMS
Circumventing the SC	Micro-needle array	Solid silicone needles (coated with drug) or hollow metal needles (filled with drug) insert drug into SC without stimulating pain fibres
	SC ablation	Removing the SC e.g. skin stripping
Electrical techniques	Iontophoresis	Driving electrode repels oppositely charged species, electric current increases skin permeability, electro-osmosis moves uncharged molecules and large polar peptides
	Reverse iontophoresis	Systemic molecules (e.g. glucose) can be extracted at the skin surface using electro-osmosis (Barry, 2001:970)
	Sonophoresis (Ultrasound)	Cavitation disturbs lipid-packing in the SC and the increased free volume enhances drug permeation
	Electroporation	Creating transient pores through the SC by electrical pulse
Radio-wave energy	Radiofrequency thermal ablation	Creating an array of small microchannels across the SC by microablating skin cells using radiofrequency energy
Magnetic fields	Magnetophoresis	A method to move diamagnetic materials (e.g. benzoic acid) through biological barriers by means of a magnetic field. According to a study done by Murthy (1999:377) the influence of magnetic field strength on diffusion flux was found to increase with increasing applied field strength.
Photomechanical energy	Photomechanical waves	It entails a drug solution being placed on the skin, covered by a black polystyrene target and irradiated with a laser pulse. This photomechanical wave stresses the SC and thus enhances drug delivery (Barry, 2001:109).

Tables 8 and 9 give a brief overview of the best known chemical enhancers and physical or technological penetration enhancement techniques. A complete description of these enhancers falls outside the scope of this dissertation. Emphasis will, however, be given to the chemical enhancers oleic acid, linolenic acid and linoleic acid, because they have proven to increase diffusivity across the SC and they are also the primary constituents of all the Pheroid™ formulations (Purdon *et al.*, 2004:104; Grobler *et al.*, 2007). These chemical enhancers (oleic, linolenic and linoleic acid) will be discussed in sections 2.9.2 and 2.9.4.

2.9 USE OF PHEROID™ TECHNOLOGY AS A THERAPEUTIC DRUG DELIVERY SYSTEM FOR INH AND RMP

2.9.1 INTRODUCTION TO PHEROID™ TECHNOLOGY

The intellectual property on which Pheroid™ technology is based was purchased by the North-West University of South Africa in December 2003 from MeyerZall (Pty) Ltd. The novelty of Pheroid™ technology is underlined by patents registered in South Africa, China, Europe and the USA. The patents describe the use of Pheroid™ technology as a delivery or carrier system which is able to enhance the absorption and/or efficacy of various categories of actives resulting in improved control of size, charge and hydrophilic-lipophilic characteristics of therapies (Grobler *et al.*, 2007:4). The technology is trademarked, and will for ease of reading, be called Pheroid(s).

Pheroids comprise of a submicron emulsion type formulation. It can be considered to fall in the same general category as submicron emulsions, niosomes and liposomes - often being confused with the liposomes. However, Pheroids are regarded as a unique moiety as they differ remarkably in their constitution and preparation techniques (Saunders *et al.*, 1999:105). Various types of Pheroids can be manufactured, each with its own morphology and size, according to therapeutic need (Grobler *et al.*, 2007:1).

What makes the Pheroid carrier system stand out above its counterparts is the fact that its components can be manipulated in a manner that ensures high entrapment abilities, very fast transport rate, delivery and stability. Therefore the entrapment of

actives within Pheroids in general creates a safer, more effective formulation than one involving the said active alone (Grobler, 2004:4).

Currently all Pheroid-related products on the market are topical products. Ongoing research concerns itself with possible applications of Pheroid technology in oral as well as parenteral administration routes. Initial studies have indicated potential applicability in infectious disease treatment e.g. tuberculosis and HIV/AIDS (Grobler, 2004:3).

2.9.2 STRUCTURAL COMPONENTS AND MOLECULAR ORGANISATION OF PHEROIDS

The Pheroid delivery system is a colloidal system consisting of unique, stable lipid-based submicron- and micron-sized structures, called Pheroids, uniformly distributed in a dispersion medium. They are typically formulated to have a diameter of between 200nm - 2 μ m. Pheroids in essence consist of ethylated and pegylated poly unsaturated fatty acids, including the omega-3 and -6 fatty acids (excluding arachidonic acid) emulsified in water saturated with nitrous oxide (N₂O) (Grobler *et al.*, 2007:3).

The above mentioned essential fatty acids are vital to the maintenance of normal cell function, but cannot be manufactured by the human body. Therefore they have to be ingested (Grobler, 2004:4). The fatty acids contained in Pheroids are in the *cis*-conformation which is compatible with the orientation of fatty acids found in humans and are responsible for:

- Maintenance of membrane integrity of cells.
- Energy homeostasis.
- Modulation of the immune system through prostaglandins/ leukotriens and
- Some regulatory aspects of programmed cell death (Grobler *et al.*, 2007:6).

Pheroids generally contain a lipid bilayer, but unlike liposomes, it does not contain phospholipids or cholesterol. Pheroids form by a self-assembly process similar to that of low-energy emulsions and micro-emulsions with no lyophilization or hydration of the lipid components required. In contrast to regular emulsions, Pheroids do not

only consist of two liquid phases but also a dispersed gas (N_2O) phase which is associated with the fatty acid dispersed phase (Grobler *et al.*, 2007:9).

Nitrous oxide (N_2O) is known to exert at least three unique functions:

- It contributes to the miscibility of the fatty acids in the dispersal medium.
- It contributes to the self-assembly process of the Pheroids.
- It contributes to the stability of the formed Pheroids.

N_2O is a volatile anaesthetic compound that has both water and fat solubility - a characteristic which enables the gas to move freely through the epidermal and dermal layers. The resulting nitrous oxide essential fatty acid (NOEFA) matrix thus provides a functional model for the transport of hydrophobic and hydrophilic drugs (Grobler *et al.*, 2007:10).

Furthermore the specific ratio of pegylated to ethylated fatty acids used during manufacture of Pheroids adds some of the reservoir characteristics of the polymeric microspheres while the formulation of natural depots is reminiscent of the structure of macromolecular microspheres (Grobler *et al.*, 2007:9).

Pheroids can be manufactured as either one of the following:

- Lipid bilayer vesicles with nano- and micrometer diameters.
- Micro-sponges.
- Depots or reservoirs that contain pro-Pheroids (Grobler *et al.*, 2007:5).

2.9.3 UPTAKE OF PHEROIDS AND ENTRAPPED COMPOUNDS BY CELLS

The precise mechanism by which Pheroids are taken up by cells is still rather unclear and the relative importance of passive (diffusional) versus active (protein-mediated) mechanisms is yet to be determined. It is speculated that uptake is actively facilitated by the fatty acid membrane binding proteins generally present within lipid rafts in the cell membrane. A novel fatty acid-binding protein which is involved in essential fatty acid uptake as well as in recycling free fatty acids from the stratum compactum layer of the epidermis is present in keratinocytes (Grobler *et al.*, 2007:23).

Colocalization studies confirmed that once inside the cell, Pheroids are metabolised in either the mitochondria or peroxisomes, depending on their composition, with resulting release of the actives (Grobler *et al.*, 2007:23).

2.9.4 PENETRATION ENHANCEMENT MECHANISM OF PHEROIDS

Potential skin penetration enhancers should exert the following characteristics: they must be pharmacologically inert, non-toxic, non-irritating and be compatible with a wide range of ingredients (Saunders *et al.*, 1999:106). Studies have shown that Pheroids are able to penetrate keratinised tissue, skin, intestinal lining, vascular system, fungi, bacteria and even parasites (Grobler, 2004:4). The latter effect is exerted by the very components of which Pheroids are made up with.

The fatty acids contained in the Pheroid formulation cause a temporary disruption in the packed structure of the intercellular lipids, especially oleic acid due to its kinked structure (Saunders *et al.*, 1999:106). The presence of nitrous oxide in the formulation also contributes to increased dermal transport. It is proposed that N₂O causes an increase in fluidity of the membranes and intercellular lipids so that transport occurs in close association with the membranes (Saunders *et al.*, 1999:105).

A further advantage of the unique composition of the Pheroid system is that the use of N₂O together with the pliable pegylated tails of the fatty acids lends an extremely elastic structure to the Pheroid particles. Pegylation serves to sterically stabilise the Pheroids by electro-chemical interaction and to maintain their interior spaces (Grobler *et al.*, 2007:17). It follows then that Pheroid particles may be able to squeeze themselves through intercellular regions of the stratum corneum or vascular linings as intact structures, thereby delivering entrapped drugs. This characteristic distinguishes Pheroids from other lipid-based delivery systems in that most other systems contain cholesterol resulting in a rigid structure and rupture when exposed to pressure, e.g. extravasation from capillaries (Saunders *et al.*, 1999:105).

2.9.5 PHEROIDS VERSUS OTHER DELIVERY SYSTEMS

The following table lists the most important similarities and differences between Pheroids and other lipid-based delivery systems.

Table 10: List of the most important similarities and differences between Pheroids and other lipid-based delivery systems (Grobler, 2004:6).

PHEROIDS	OTHER DELIVERY SYSTEMS
Consists primarily of essential fatty acids; a natural and essential ingredient of the body.	Generally contain substances foreign to the body e.g. artificial polymers, egg phosphatidylcholine and/or lysolecithin.
A variety of Pheroid types can be manufactured with variable sizes, charge, lipid composition and membrane packing. Desired types can be repeatably obtained.	Various liposomes have been described: single lamellar, multi-lamellar vesicles, multivesicular vesicles and nanosomes. Problems with repeatability are common. Filtration mechanisms limit size variations.
An affinity exists between the Pheroid and cell membranes due to the fatty acids. A specific protein family in cell membranes are responsible for the binding and uptake of essential fatty acids. This suggests that Pheroids follow the endosome sorting mechanism resulting in penetration and delivery. Rate of binding is high and rapid.	Specific binding and uptake mechanisms by mammalian cells have not been described for other delivery systems.
Fatty acids form part of the natural biochemical pathways, therefore it causes no cytotoxicity and assists with maintenance of cell membranes.	Cytotoxicity and impaired cell integrity are common problems. Liposomal systems may decrease or enhance cytotoxicity.
Drug resistance was reduced/ eliminated in all <i>in vitro</i> studies (decreased resistance observed for chloroquine-resistant malaria parasites) done. This is probably due to the intracellular release of drugs beyond the membrane zone, thus beyond the efflux pumps found in drug resistant organisms.	Some delivery systems are prone to drug resistance/ adverse immune responses. Their composition generally prohibits active compounds to be released beyond the drug efflux pumps developed by drug-resistant organisms.
Pheroids are polyphilic- drug with different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
Microsponges are ideal for combination therapies - one drug can be entrapped in the interior volume and the other in the sponge spaces. This separation into different interior spaces decreases drug-drug interactions.	Combination therapies are problematic.
Entrapment of drugs reduces their volume of distribution with resulting increase in drug concentration at target site. An enhanced but narrow therapeutic index is achieved, with decreased aspecific toxicity.	Similarly, liposomes encapsulating small molecule drugs have been shown to reduce the volume of distribution and enhance the drug concentration in tumours, with decreased aspecific toxicity.
The Pheroid protects the drugs from metabolism and inactivation in plasma/ body fluids. Little leakage of drug from the Pheroids is observed before it reaches its site of action.	Some liposomal systems protect drugs from metabolism and opsonization in the plasma after IV administration. Leaking of drugs into the plasma is frequently seen.
Pheroids are steirically stabilised without the disadvantages of increased size or decreased elasticity.	Delivery systems generally need to be stabilised steirically e.g. pegylation of liposomes in steirically stabilised liposomes. This leads to an increase in size and rigidity of the carrier.

Table 10: List of the most important similarities and differences between Pheroids and other lipid-based delivery systems (continued)

Pheroids contain no cholesterol but the interior volume is stably maintained. It shows a high degree of elasticity and fluidity, with a relatively high transition temperature. Pheroids are able to extravasate from capillaries intact with quick release of the actives in cells.	Most lipid-based delivery systems contain phospholipids and cholesterol. The latter is used for maintenance of the interior of the vesicles. Systems generally lose fluidity and elasticity - it cannot squeeze through intercellular spaces or extravasate.
Pheroids can be formulated as pro-Pheroids	Liposomes can be formulated as pro-liposomes.
Entrapment efficiency is high (between 85% and 100%).	Due to charge and steric limitations of delivery systems, entrapment efficiencies may be problematic.
Pheroids enhance bioavailability of oral, topical and buccal administration of drugs in all products tested so far. Absorption was similarly increased.	Some delivery systems have been shown to enhance absorption, whereas others decreased absorption.

2.10 SUMMARY

CTB refers to pathological lesions of the skin caused by either *Mycobacterium tuberculosis*, *Mycobacterium bovis* or the BCG vaccine. A confirmed diagnosis depends essentially on the demonstration of *M. tuberculosis* within the lesions by traditional acid-fast bacilli (AFB) staining, culture or polymerase chain reaction (PCR). Differential diagnosis is imperative to ensure timely initiation of chemotherapy.

Factors that govern the development of this disease include pathogenicity of the infecting organism, route of infection, the patient's prior sensitisation to TB and the patient's own cell-mediated immunity. After careful review of current literature, skin sites affected by the different CTB variants have been identified and is summarised in Table 11.

Table 11: Skin regions affected by the CTB variants

	ROUTE OF INFECTION	SKIN REGION(S) AFFECTED
TRUE CTB: EXOGENOUSLY ACQUIRED		
Tuberculous chancre	Direct inoculation	Any site where trauma occurs (i.e. affect all skin layers), may also involve mucous membranes.
Tuberculosis verrucosa cutis (TBVC)	Direct inoculation	Epidermis (hyperkeratosis and papillomatosis).
Lupus vulgaris (some cases)	Direct inoculation	Mainly the upper dermis.
TRUE CTB: ENDOGENOUSLY ACQUIRED		
Lupus vulgaris (most cases)	Hematogenous, lymphatic, contiguous	Mainly the upper dermis.
Miliary TB	Hematogenous	Superficial dermal layers.
Metastatic tuberculosis abscess (tuberculosis gumma)	Hematogenous	Lower dermis region and subcutaneous tissue.
Papulonecrotic tuberculid (most cases)	Hematogenous	Necrosis of the upper dermis extending into the epidermis.
Scrofuloderma	Contiguous	Sub-cutaneous nodules overlying an infected lymph node. Acid-fast bacilli are found in the purulent discharge, lower dermis and walls of the ulcer.
Orificial tuberculosis	Contiguous, autoinoculation	Upper dermis and layers of the lower dermis. Acid-fast bacilli are present in the dermis and ulcer walls.
TUBERCULIDS		
Lichen scrofulosorum	Hematogenous	Hair follicles and sweat ducts- surrounded by superficial tuberculoid granulomas.
Erythema induratum of Bazin	Hematogenous	Inflammation of subcutaneous fat lobules.
Nodular granulomatous phlebitis	Hematogenous	Epithelioid cell granulomas in the walls of cutaneous veins.

Current treatment of CTB comprises of the standard oral anti-TB three (smear-negative patients) or four (smear-positive patients) drug regimens. Lesions usually respond after 1 month of therapy with anti-TB drugs, but complete resolution is only attained after 4 - 6 months. To date no topical formulation exists to aid in the treatment of CTB lesions.

As indicated previously in section 2.5, transdermal drug delivery has advantages over conventional routes e.g. application of actives directly at the site of action together with elimination of first-pass metabolism.

However, dermal drug delivery is fraught with challenges and the formulation of a successful transdermal product depends upon the actual amount of drug that reaches its intended target site within the skin. The amount of drug that ultimately reaches its intended target site within the skin is determined by bio-medical factors, the physicochemical properties of the penetrant and vehicle, dosing conditions and a number of laws, such as Fick's laws which describe the role of each of the properties.

It is also evident that like the majority of drugs, INH and RMP do not display the characteristics of an ideal penetrant. Therefore it has become apparent that some reliable form of penetration augmentation must be used to improve their poor diffusivity. For this reason the novel Pheroid delivery system will be used to entrap INH and RMP as it holds some advantages over other lipid-based delivery systems including:

- A high entrapment efficiency.
- Rapid transport rate and delivery.
- Sufficient stability.
- Replenishing essential fatty acid deficiency in the skin essential for maintaining the integrity of the epidermal layer (Grobler, 2004:4).

Previous studies done by Grobler (2004:15) have demonstrated that the anti-tubercular drugs are compatible with and can be entrapped by the Pheroid system with ease. *In vitro* measurements have also shown that the Pheroids are capable of releasing the actives within the mycobacteria itself, thereby decreasing the minimum inhibitory concentration needed.

The *in vitro* transdermal permeation of the two principal anti-tubercular drugs, INH and RMP by means of the Pheroid therapeutic drug delivery system will be investigated in this study. Chapter 3 includes an article to be submitted to the European Journal of Pharmaceutical Sciences on our findings.

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**CHAPTER 3: ARTICLE FOR PUBLICATION IN THE
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**TRANSDERMAL DELIVERY OF ISONIAZID AND
RIFAMPICIN BY PHEROID™ TECHNOLOGY**

THE TRANSDERMAL DELIVERY OF ISONIAZID AND RIFAMPICIN BY PHEROID™ TECHNOLOGY

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Abstract

The scope of this *in vitro* study was to investigate the efficacy of the novel Pheroid™ technology system for the delivery of isoniazid (0.5% w/w) and rifampicin (1.0% w/w) across human skin. Particle size distribution for rifampicin and entrapment of actives within the Pheroid™ carrier system was determined by confocal laser scanning microscopy (CLSM) and revealed definite entrapment.

Vertical Franz diffusion studies were conducted using female abdominal skin as permeation membrane over a period of 12 hours. Concentrations of 5 mg/ml and 10 mg/ml for isoniazid (INH) and rifampicin (RMP) respectively were applied to the donor phase suspended in either phosphate buffered saline (PBS) or in Pheroid™.

Permeation studies were conducted at pH 5.5. *In vitro* penetration of INH and RMP were assayed directly by HPLC. Permeation profiles obtained for INH in Pheroids™ indicated a biphasic character, whilst that obtained for RMP in Pheroids™ showed a triphasic character. The Pheroid™ delivery system proved more efficacious for delivery of both anti-tubercular drugs and resulted in greater percentage yield as well as flux values than that for a PBS solution. Furthermore, the Pheroid™ formulation was able to deliver the entrapped INH and RMP in concentrations sufficient to exceed their respective minimum inhibitory concentrations (MIC).

Keywords: Isoniazid; Rifampicin; Pheroid™; Topical drug delivery; Cutaneous tuberculosis; Tuberculosis

1. Introduction

Cutaneous tuberculosis (CTB), also known as dermal tuberculosis or tuberculosis cutis, refers to pathological lesions of the skin caused by *Mycobacterium tuberculosis*, *Mycobacterium bovis* or the bacilli Calmette-Guérin (BCG) vaccine (Barbagallo et al., 2002). Though these cases are rare, it has been noted over the last two decades that their numbers seem to have increased proportionally with the rising incidence of pulmonary TB due to the AIDS pandemic and fatal synergy which exist between *M. tuberculosis* and HIV (human immuno-deficiency virus). Other causes include the emergence of multi-drug resistant TB strains, socio-economic problems (poverty, immigration) as well as non-compliance to the current anti-TB treatment regimens (Gopinathan et al., 2001; Maccari et al., 2005).

Confirmed diagnosis depends essentially on the demonstration of *M. tuberculosis* within the infected tissues by traditional acid-fast bacilli (AFB) staining, culture or polymerase chain reaction (PCR) (Barbagallo et al., 2002). In view of the fact that CTB is mostly associated with systemic involvement, current treatment comprises of the standard three/ four drug regimens. However, information regarding CTB is still needed regarding the response to anti-TB therapy. This is according to research done by Ramam et al. (2005) who concluded that cases of CTB showed improvement within 1 month of therapy with anti-TB drugs, but complete resolution was only attained after 4 - 6 months. The major drawback to current therapy is that patients not only remain a source of infection (viable organisms can still be demonstrated), but they also suffer from constant embarrassment due to the disfiguring nature of CTB, until these lesions have healed completely.

CTB is classified as either true CTB or tuberculids. The latter has a similar morphology to true CTB, but *M. tuberculosis* is not demonstrated in these lesions

(Barbagallo et al., 2002). True CTB lesions include tuberculous chancre, tuberculosis verrucosa cutis (TBVC), lupus vulgaris, metastatic tuberculosis abscess (tuberculosis gumma), scrofuloderma, miliary TB, orificial tuberculosis and papulonecrotic tuberculid (most cases). Tuberculids comprise of lichen scrofulosorum, erythema induratum of Bazin, nodular granulomatous phlebitis and papulonecrotic tuberculid (some cases). Both CTB and tuberculids show a wide spectrum of morphological presentations, consequently involving various sites within the skin such as the epidermis, upper dermis, lower dermis regions and subcutaneous tissue, hair follicles, sweat ducts and in some cases the subcutaneous nodules overlying an infected lymph node. These lesions are often associated with various degrees of necrosis (Barbagallo et al., 2002; Meltzer, 2006). The rationale behind the chosen drug combination and route of administration is to provide a possible means of treatment to patients with CTB in an effort to alleviate the psychological and physical suffering inherent to this disease. No evidence of an already existing topical formulation of this kind could be found.

Transdermal drug delivery holds some advantages over conventional routes, e.g. application of actives directly at the site of action together with elimination of first-pass metabolism. Formulation of a successful transdermal product, however, poses many challenges in its own right because of the resistance of the stratum corneum (SC) to penetration and the vast biological variability encountered in human skin (Sinha and Kaur, 2000).

Different measures are being investigated to circumvent the skin's barrier function in order to ensure that sufficient quantities of the active ingredients reach the intended site of action within the skin, whether it be surface effects (e.g. sunscreens) or systemic effects (e.g. nicotine patches) (Walters, 2002; Zatz, 1993). One such

measure is to select an active with the correct physicochemical properties (low molecular weight < 600 Da, low melting point, balanced partition coefficient, adequate oil-water solubility), to permeate across the SC at an acceptable rate.

Unfortunately like most drugs, INH and RMP do not display these ideal characteristics, meaning that some reliable form of penetration augmentation must be utilised to improve their poor diffusivity - thus their incorporation in the Pheroid™ drug delivery system.

Pheroid™, a stable skin-friendly carrier, comprises of a submicron (200 nm - 2 µm) emulsion type formulation. In essence it consists of ethylated and pegylated poly unsaturated fatty acids, including the omega-3 and -6 fatty acids (excluding arachidonic acid) emulsified in water saturated with nitrous oxide (N₂O) (Grobler et al., 2007). This technology is trademarked and will for ease of reading be called Pheroid(s) only.

Previous studies have shown that Pheroids are able to penetrate keratinised tissue, skin, intestinal lining, the vascular system, fungi, bacteria and even parasites (Grobler, 2004). This is due to their unique composition which provides the Pheroid particles with an extremely elastic character, enabling them to squeeze through intercellular regions of the stratum corneum or vascular linings as intact structures, thereby successfully delivering encapsulated drugs (Grobler et al., 2007). It is the latter characteristic that lends Pheroids its superiority above other cholesterol-containing lipid-based delivery systems which have a rigid structure that ultimately rupture when exposed to pressure, e.g. extravasating from capillaries (Saunders et al., 1999).

Pheroids can be manufactured as either one of the following:

- lipid bilayer vesicles with nano- and micrometer diameters
- micro-sponges
- depots or reservoirs that contain pro-Pheroids (Grobler et al., 2007).

Several authors (Sankar et al., 2003; Shishoo et al., 2001; Singh et al., 2000) have reported poor bio-availability for rifampicin (RMP) from fixed dose combinations containing isoniazid (INH) due to INH-catalysed degradation of RMP under acid conditions (i.e. during stomach residence time). In anticipation of this, a micro-sponge Pheroid formulation was used in this study as one active can be entrapped in the interior volume and the other in the sponge spaces according to the actives' hydro- or lipophilicity, thereby diminishing drug-drug interactions (Grobler, 2004). Furthermore, because the pH of the formulation to be applied is similar to that of normal skin (pH 5.5), which is also the pH of maximum stability for both INH and RMP, we assumed that degradation will be negligible (Lund, 1994; Yosipovitch and Hu, 2003).

If the Pheroid proves successful to deliver these drugs topically, it may result in a topical formulation to serve as an adjunctive to systemic therapy, which in turn might limit transmission of TB and lead to a more rapid cure for CTB lesions.

Materials and methods

2.1 Materials

Rifampicin ({2,7-(epoxypentadeca[1,11,13]trienimino)naphtho[2,1-b]furan-1,11}(2H)-dione, 5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20, {22-heptamethyl-8-[N-(4-methyl-1-piperazinyl)formimidoyl]-} 21-acetate), MW = 822.95 and isoniazid (pyridine-4-carbohydrazide), MW = 137.14, were obtained from Aventis, South Africa. Methanol (HiperSolv® for HPLC) obtained from VWR International Ltd., Poole, England, 1-Heptanesulphonic acid sodium salt ($C_7H_{15}O_3SNa$) and monobasic ammonium phosphate ($(NH_4)HPO_4$) which were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A) were utilised in the solvent systems during HPLC analyses. Absolute ethanol AR and methanol AR were purchased from Labchem, South Africa. Disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) as well as sodium chloride (NaCl), all of which were of analytical grade, was obtained from Merck Chemicals (Pty.) Ltd., South Africa. Pheroids were supplied by The Unit for Drug Research and Development, North-West University. All solutions were prepared in HPLC (deionised) water, purified by a Milli-Q Academic purification system (Millipore, Milford, USA).

2.2 Apparatus and instrumentation

Vertical amber Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) were used during diffusion studies. The high performance liquid chromatography (HPLC) system consisted of an Agilent series 1100 HPLC equipped with a pump, autosampler, UV detector and Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software. The column used was a Phenomenex®,

Synergi Max RP- 80A (250 x 4.6 mm, 4 μ m). The crystal size distribution of RMP and the entrapment ability of the actives within the Pheroid micro-sponges were done using a Nikon Eclipse TE 300 microscope equipped with a Nikon digital camera (DXM 1200), Nikon Plan Apo lens (60 x 1.40 oil, DIC H, ∞ /0.17, WD 0.21) and ACT-1 data acquisition software. Immersion oil for microscopy, type A (50 cc, nd = 1.515, 23 °C) Nikon, Japan was also utilised in conjunction with polarized light microscopy procedures.

2.3 Methods

2.3.1 Particle size distribution determination of RMP

The crystal size distribution of RMP was determined using the Nikon PCM 2000 confocal laser scanning microscope (CLSM) to ensure that entrapment would indeed take place within the micro-sponges. A sample quantity of RMP powder was spread out on a glass slide, covered with a glass cover-slip and subjected to polarized light microscopy (60x enlargement). Twenty two micrographs were taken at random and a calibration bar was added from which the relative sizes of the crystals could be determined.

2.3.2 Entrapment of INH and RMP in the Pheroid delivery system

INH (0.5%) was incorporated within the aqueous phase and RMP (1.0%) within the micro-sponge structure of the Pheroid formulation, due to their hydrophilic and lipophilic nature, respectively. Thereafter the two phases were combined, resulting in the formation of micro-sponge structures. The drug containing Pheroid system was shaken overnight before commencement of diffusion experiments to ensure complete

incorporation of the actives within the micro-sponge structures. The Pheroid micro-suspension was stored at 2-8 °C and used before the supplied expiry date.

Entrapment of both actives within the Pheroid formulation was monitored with the aid of a Nikon PCM 2000 confocal laser scanning microscope (CLSM).

According to protocol, Pheroids are usually labelled with the fluorophore Nile Red, which has an emission wavelength of between 640 and 650 nm. This wavelength was decided on as the fluorophore of choice as it predominantly accumulates in lipid-rich domains and therefore readily stains the fatty acid components of the Pheroid formulations (Saunders et al., 1999). Five microlitres of Nile Red was incubated with 100 µl of the formulation. The fluorescently labelled Pheroid formulation containing INH and RMP was layered on a glass slide and covered with a glass cover-slip. Micrographs were obtained after laser excitation at 505 nm, after which emission was captured at 568 nm, using a long pass filter. Simultaneous reflectance microscopy enabled the visualisation of crystals present in the formulation.

2.3.3 Skin preparation method

Excised skin from Caucasian female patients, who had undergone abdominal plastic surgery, was used. Ethical approval for the procurement and utilisation of the skin tissue was provided by the Research Ethics Committee of the North-West University (reference number 04D08). Immediately after procurement the full-thickness skin was frozen at -20°C for not longer than 24 hours. Prior to preparation of the epidermis layer for utilisation in the *in vitro* diffusion experiments, the skin was thawed at room temperature, remaining blood wiped off and all excess adipose tissue carefully removed with a scalpel. The skin was then immersed in water heated to 60°C for approximately one minute after which the epidermis layer was gently

separated from the underlying dermis using pincers (Bronaugh and Collier, 1993).

Extreme care was taken to ensure the epidermal layer remained intact.

The epidermal sections were floated on top of Whatman® filter paper whilst always ensuring that the stratum corneum (SC) side of the skin faced upwards. Epidermal skin sections were left to air dry, covered in aluminium foil and sealed in plastic bags. It was stored at - 20°C until used. Before starting a diffusion study, the frozen epidermal skin sections were thawed at room temperature and examined for defects. It was then cut into circles of about 10 mm in diameter before being mounted on vertical Franz cells.

2.3.4 Franz diffusion cells

Due to the photosensitivity of both drug substances (INH, RMP) and the Pheroid formulation, amber Franz diffusion cells were used during experiments (Lund, 1994). The epidermal skin disks were mounted on the lower half of vertical Franz diffusion cells (SC facing upwards), having a nominal surface area of 1.075 cm² and a receptor capacity of approximately 2 ml. The donor compartment was placed on the lower half with the skin acting as the membrane between the two halves, sealed with Dow Corning® high vacuum grease and fastened by means of a metal clamp.

A small magnetic stirrer bar was placed within each receptor compartment. Stirring was achieved by a Variomag® stirrer plate during the entire experiment at 750 rpm. Before the onset of each permeability experiment, tissue disks were equilibrated for one hour after filling both the donor and receptor compartments with phosphate buffered saline (PBS), pH 5.5, in a water bath held constant at 37°C.

This keeps the skin surface at approximately 32°C, simulating the temperature of human skin (El-Kattan et al., 2000). All bubbles were carefully removed from the interface between the underside of the skin and solution in the receiver compartment. For each diffusion study a single source of skin was used to diminish bio-variability between cells. After equilibration, the entire contents of the receptor compartments were withdrawn and replaced with a solution of 20% ethanol-PBS (pH 5.5), previously heated to 37°C. The inclusion of an organic solvent is to ensure the solubility of the lipophilic components namely rifampicin, Pheroids and skin tissue lipids. Likewise, the contents of the donor compartments were emptied and filled with 1000 µl (1 ml) of either a PBS or Pheroid solution containing 5 mg/ml INH and 10 mg/ml RMP, depending on the experiment. Cells were immediately covered with Parafilm® to prevent evaporation of the vehicle.

At predetermined intervals (15 min; 30 min; 45 min; 1; 1.5; 2; 3; 4; 6; 8; 10 and 12 hours), the entire content of the receptor cells were withdrawn and replaced with fresh receptor phase (20% ethanol-PBS solution) at 37°C. This was done to ensure that sink conditions prevailed during the entire experiment. Aliquots of 20 µl from each sample were directly assayed by HPLC to determine the drug concentration present within the receptor compartment.

2.3.5 Permeation experiments

Two sets of permeation studies were performed. During the first, a Pheroid formulation containing 5 mg/ml INH and 10 mg/ml RMP (pH 5.5) served as the donor phase. The second set involved the same drug combination at the above mentioned concentrations, but dispersed in a PBS solution (pH 5.5). This set also served as

positive control. The passive flux results for INH and RMP in each of these two sets of diffusion studies were compared and are described in section 3.

2.3.6 HPLC analysis

Samples collected from the receptor compartments during the permeation experiments were analysed using an Agilent series 1100 HPLC and a Phenomenex®, Synergi Max RP- 80A (250 x 4.6 mm, 4 µm) column. INH and RMP were separated by means of gradient elution. The mobile phase consisted of two solvents:

- A) A degassed mixture of 0.01 M monobasic ammonium phosphate and 0.005 M of 1-heptanesulphonic acid sodium salt in HPLC (deionised) water (pH 3.50).
- B) Methanol

Table 1 presents the gradient elution method for the mobile phase composition and adjustment. Aliquots of 20 µl from each sample were injected directly into the column. The flow rate was 1.0 ml/min and temperature was maintained at 25 °C. Both INH and RMP were detected at 263.4 nm and their retention times were 4.11 and 9.4 minutes respectively. Total run time was 13 minutes. Recording and integration of peaks were done by means of a Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software. The latter was used to determine the actual drug concentrations detected in the samples. Room temperature was maintained at 25 °C.

2.3.7 Statistical data analysis

The cumulative amount of drug that permeated the skin per unit time skin area was plotted against time for INH and RMP in both PBS and Pheroids. The slopes of the linear portions of the plots were estimated as the steady-state fluxes. The yield of each cell was depicted as a percentage of the applied concentration. Diffusion cells identified as exhibiting a compromised barrier function, % yield values $\geq 1.0\%$, were omitted from calculation of the mean values. Results are expressed as mean $\pm$ SD (standard deviation).

3. Results

3.1 Particle size distribution of RMP

The particle size distribution of RMP crystals are presented in Fig. 1. Data indicated that the majority of crystals (80%) ranged between 0-5 μm in size, whilst less than 9.0% of crystals were outside this ideal range. Therefore no additional size reduction procedure was necessary.

3.2 Entrapment of INH and RMP in Pheroids

Entrapment of RMP and INH within the Pheroid delivery system is shown in Fig. 2. Micrograph 2(a) and 2(b) represents a placebo Pheroid sample (no added actives) which served as the negative control. The Pheroids are clearly visible as red spheres. Micrograph 2(b) depicts the characteristic, spherical, sponge-like structure of the particular Pheroid formulation used during our permeation studies. Micrograph 2(c) shows the actual entrapment of RMP (yellow/green dots) within the Pheroid system (red sponge-like spheres). This proves that RMP molecules are indeed

entrapped within the sponge structure itself. INH could not be labelled with a fluorescent marker and is therefore not visible in the micrograph, but since CLSM indicated that the Pheroid micro-sponge formulation comprises of two clearly defined phases (aqueous and lipid) it can be assumed that INH is present within the watery phase due to its high water solubility (140 mg/ml) (Drugbank, 2007).

3.3 Permeation of INH and RMP with the aid of the Pheroid delivery system

Table 2 contains the mean flux values for INH (5000 µg/ml) incorporated in either PBS (pH 5.5) or Pheroids. Unlike a PBS solution, the Pheroid micro-suspension elicited a clear biphasic character, with phase one ranging from 0-2 hours and phase two from 2-12 hours (Fig. 3). The mean steady-state flux values for INH during the first phase were 0.29 ± 0.65 µg/cm²/h and 2.64 ± 2.31 µg/cm²/h for the PBS and Pheroid donors, respectively. Another important fact observed with regards to the Pheroid micro-suspension is that the % yield obtained was about 10 times higher than that of PBS within the first 2 hours after application (Table 3). Mean flux values yielded by PBS and Pheroids for INH during the second phase were 0.30 ± 0.15 µg/cm²/h and 1.14 ± 0.89 µg/cm²/h, respectively. In both instances the Pheroid micro-suspension proved superior to the PBS solution as is also evident from calculated enhancement ratio (ER) values. The ER values were 7.98 and 2.81 for the 0-2 and 2-12 hour intervals respectively (Table 2).

The permeation trends for RMP in either PBS or Pheroids (Fig. 4) differed remarkably from that of INH. Zero permeation was achieved for RMP dispersed in a PBS solution (0.00 ± 0.00 µg/cm²/h). It was observed that the RMP containing Pheroid micro-suspension elicited a triphasic character, with phase one being 0-1.5h, the second 1.5-4 h and the third 4-12 h. Mean steady-state flux values obtained were 1.87 ± 2.08 µg/cm²/h, 0.66 ± 0.09 µg/cm²/h and 0.23 ± 0.41 µg/cm²/h for each of the

three phases respectively (Table 4). Table 5 contains the % yield values obtained for RMP in either PBS or Pheroids for each of the three phases. It is evident that the RMP containing Pheroid formulation was superior to PBS. Finally, despite the fact that RMP's concentration was double that of INH, the overall mean flux values for INH in both PBS and Pheroid solutions were higher than those obtained for RMP.

4. Discussion

Transdermal delivery of therapeutic agents involves the permeation of adequate drug concentrations across the rate-limiting barrier, the SC, for either disease treatment, alleviation of symptoms accompanying underlying tissues or treatment of systemic disease conditions.

In view of Fig. 3 it was evident that the average flux attained for INH in Pheroids was about 4 times higher than that of PBS. The diminished penetration of INH in PBS can be related to the pH-partition hypothesis which dictates that the diffusion of unionised molecules is largely favoured above that of electrolytes (Smith, 1990; Wiechers, 1989). INH possesses three ionisation constants (pKa values) 1.8 (hydrazine nitrogen), 3.5 (pyridine nitrogen) and 10.8 (acidic group) with an overall isoelectric point (pI) of 1.82. Since the pH of PBS in the donor phase (pH 5.5) used during our study is above that of the pI, INH molecules exist predominantly in their ionised form (Drugbank, 2007). This diminishes INH's partitioning into the lipophilic SC. The fact that the same problem is not encountered when INH is entrapped within Pheroids under the same pH conditions is due to the unique ability of the Pheroids to undergo ion-pair formation with charged molecules which increases their partitioning and binding to fatty acid binding proteins in the cell membranes (Grobler et al., 2007).

Closer examination of the average flux achieved for INH in the Pheroid micro-suspension revealed a biphasic character where a quantity of 5.075 $\mu\text{g/ml}$ INH was delivered during time 0-2 h against 0.594 $\mu\text{g/ml}$ in PBS for the same interval.

Similarly a quantity of 11.532 $\mu\text{g/ml}$ was attained for INH in Pheroids over 3.077 $\mu\text{g/ml}$ in PBS for time 2-12 h. Thus, the flux obtained for Pheroids was approximately 10 and 4 times higher than for PBS during time 0-2 h and 2-12 h respectively. The decrease in flux during the second phase can be ascribed to depletion of INH in the donor medium, resulting in a decrease in concentration gradient across the membrane and consequently a decreased driving force.

According to Mandell, William (1996) the minimum inhibitory concentration (MIC) for INH is 0.025 to 0.05 $\mu\text{g/ml}$. The results of this study indicated that sufficient amounts of INH, in both PBS and Pheroids, permeated across intact epidermis over a period of 12 hours. The Pheroid micro-suspension, however proved more efficacious to such a degree that a reduction in the initial applied dosage might be considered.

From Fig. 4 it is evident that an actual flux was obtained for RMP in Pheroids whereas none could be achieved for RMP in PBS. RMP exhibits quite the opposite physicochemical characteristics to INH, being highly lipophilic ($\log P = 3.714$) and its molecular weight ($MW = 822.95$) is around six times that of INH. Because the intercellular route (movement of a permeant only through the intercellular lipid region) is favoured by lipophilic substances, RMP should therefore transverse the SC primarily *via* this laborious route (Zatz, 1993). This and the fact that RMP might have undergone extensive drug binding within the lipid domains of the SC due to its extreme lipophilic nature, might explain the absence of RMP (dissolved in PBS) in the receptor phase at the conclusion of the 12-hour permeation study (Fig. 4). Furthermore RMP has two pKa values at 1.7 (hydroxyl group at C8) and 7.9

(piperazine moiety at position N4) respectively, with a pI of 4.8. Thus RMP is expected to be largely unionized at pH 5.5; the latter also contributing to its lipophilicity (Malassiney et al., 1996).

Close examination of Fig. 4 revealed that a triphasic rather than biphasic nature could be identified for RMP in Pheroids, with the first phase ranging from 0-1.5 h, the second from 1.5- 4 h and the third from 4-12 h. Quantities of 2.796 µg/ml, 1.604 µg/ml and 1.780 µg/ml of RMP were obtained for each phase respectively. Again the decrease in flux during the second and third phases can be ascribed to gradual depletion of RMP in the donor medium, resulting in a decreased concentration gradient across the membrane and consequently a decreased driving force. When compared to the MIC values provided for RMP against *M. tuberculosis* (MIC = 0.005 to 0.2 µg/ml) *in vitro*, the above mentioned quantities of RMP delivered across human skin thus proved sufficient (Mandell, William, 1996).

The considerable improvements seen in the mean flux values obtained for INH and RMP in Pheroids can also be related to the Pheroids' ability to increase the permeability and fluidity of the SC caused by the individual fatty acids and N₂O, thereby reducing the diffusional resistance encountered by the actives (Grobler et al., 2007). This feature corresponds with literature findings which conclude that the SC lipid matrix is responsible for most of the barrier function as any extraction/fluidisation of intercellular lipid leads to enhanced solute penetration (Wiechers, 1989). In case of RMP in Pheroids this ability of the Pheroid to increase the permeability and fluidity of the SC is thought to be the single driving force behind RMP's transdermal delivery. Similar to results obtained previously by Grobler et al. (2007) the Pheroids delivery system led to an increase, albeit small, in the percentage yield attained for both INH and RMP.

5. Conclusion

We have demonstrated that both anti-tubercular drugs, INH and RMP, can be successfully entrapped within the microsphere-like structures of the Pheroid delivery system. The Pheroid drug delivery system was able to deliver INH and RMP simultaneously in quantities above their MIC values (an important factor to consider in the prevention of the emergence of drug resistant micro-organisms) over a period of 12 hours. Dosage reduction of the actives can also be considered, but that would require further investigation.

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Tables

Table 1: Mobile phase consisted of monobasic ammonium phosphate buffer (solvent A) and methanol (solvent B)

Mobile phase composition A : B		Duration (min) (Total runtime = 13 minutes)
80	20	3.50
20	80	4.50
20	80	9.00
80	20	9.20

Table 2: Average flux data for INH in both PBS solution and Pheroids

Donor phase (pH 5.5)	Av. Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (0-2 h)	Av. Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (2-12 h)	ER ^a (0-2 h)	ER (2-12 h)
INH in PBS (n = 13)	0.29 $\pm$ 0.65	0.30 $\pm$ 0.15		
INH in Pheroids (n = 13)	2.64 $\pm$ 2.31	1.14 $\pm$ 0.89	7.980	2.812

^a ER = enhancement ratio

Table 3: Average percentage (%) yield data for INH in both PBS solution and Pheroids

	PBS (n = 13)			Pheroids (n = 13)		
	% Yield (0-2 h)	% Yield (2-12 h)	% Yield (0-12 h)	% Yield (0-2 h)	% Yield (2-12 h)	% Yield (0-12 h)
Mean	0.01	0.06	0.07	0.10	0.23	0.33
SD^a	0.03	0.03	0.05	0.09	0.17	0.21
%RSD^b	215.47	49.09	66.64	85.51	73.03	63.91

^a ER = enhancement ratio

Table 4: Average flux data for RMP in both PBS solution and Pheroids

Donor Phase (pH 5.5)	Av. Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (0-1.5 h)	Av. Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (1.5-4 h)	Av. Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (4-12 h)	ER^a (0-1.5 h)	ER (1.5-4 h)	ER (4-12 h)
RIF in PBS (n = 13)	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00			
RIF in Pheroids (n = 13)	1.87 $\pm$ 2.08	0.66 $\pm$ 0.90	0.23 $\pm$ 0.41	1865.20	659.40	225.60

^a ER = enhancement ratio

Table 5: Average percentage (%) yield data for RMP in both PBS solution and Pheroids

	PBS (n = 13)				Pheroids (n = 13)			
	% Yield (0-1.5 h)	% Yield (1.5-4 h)	% Yield (4-12 h)	% Yield (0-12 h)	% Yield (0-1.5 h)	% Yield (1.5-4 h)	% Yield (4-12 h)	% Yield (0-12 h)
Mean	0.000	0.000	0.000	0.000	0.028	0.016	0.018	0.062
SD^a	0.000	0.000	0.000	0.000	0.030	0.022	0.033	0.063
% RSD^b	0.000	0.000	0.000	0.000	108.845	138.024	186.245	102.000

^aSD = standard deviation, ^b%RSD = percentage relative standard deviation

Figure legends and figures

Fig. 1: The size distribution for RMP crystals

Fig. 2: Confocal laser scanning micrographs of INH and RMP dispersed in the Pheroid delivery system. The Pheroids were labelled with Nile Red dye, which emits in the red spectrum.

Fig. 2(a): Placebo Pheroids (negative control).

Fig. 2(b): Sponge-like structure of the placebo Pheroids.

Fig. 2(c): Pheroids containing RMP and INH (though the latter is not visible).

Fig. 3: Average cumulative concentration per area ($\mu\text{g}/\text{cm}^2$) versus time (hours) after *in vitro* permeation of INH in (A) PBS solution and (B) Pheroids (pH 5.5) (n = 13).

▲ (A): INH dispersed in PBS solution (pH 5.5) (n = 13)

◆ (B): INH entrapped in Pheroids (pH 5.5) (n = 13).

Fig. 4: Average cumulative concentration per area ($\mu\text{g}/\text{cm}^2$) versus time (hours) after *in vitro* permeation of RMP in (A) PBS solution and (B) Pheroids (pH 5.5) (n = 13).

■ (A): RMP dispersed in PBS solution (pH 5.5) (n = 13)

● (B): RMP entrapped in Pheroids (pH 5.5) (n = 13).

FIGURES

Figure 1

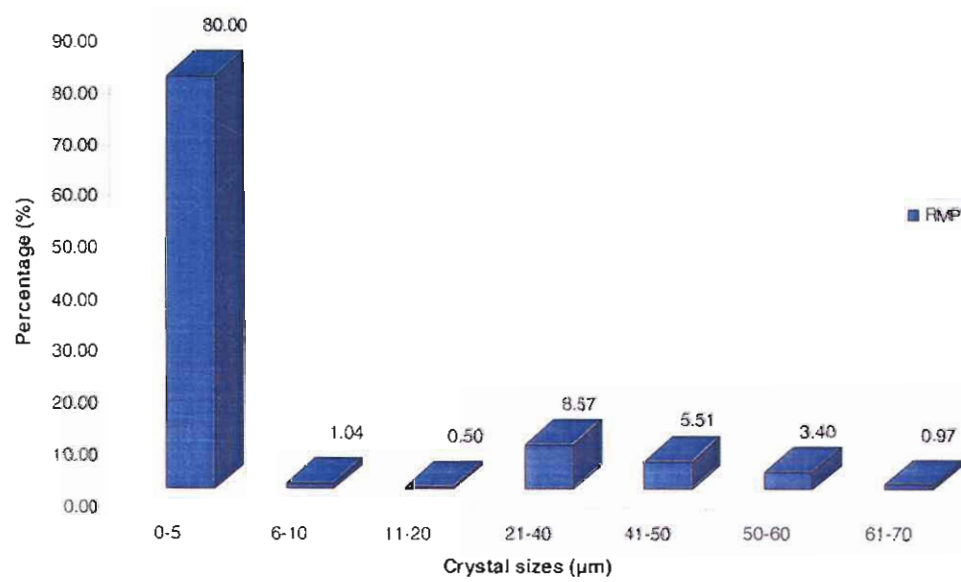


Figure 2

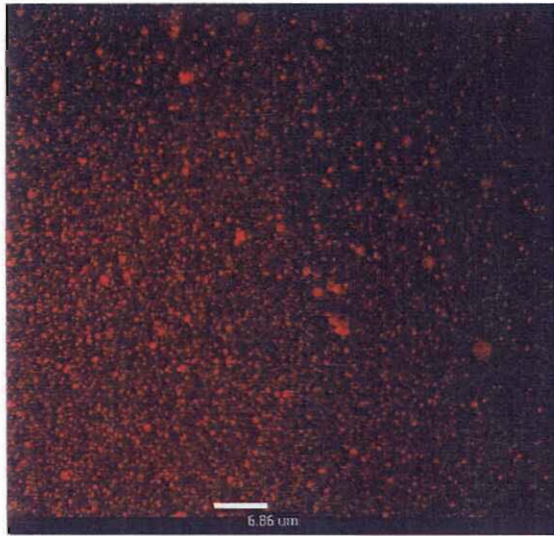


Fig. 2(a): Placebo Pheroids



Figure 2(b): Sponge-like structure of the placebo Pheroids

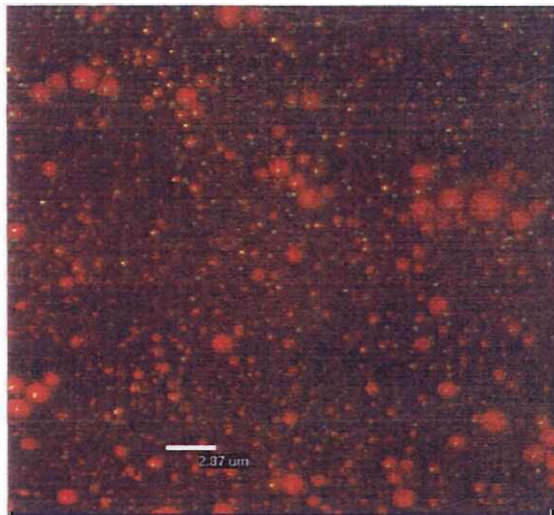


Figure 2(c): Pheroids with RMP and INH (though INH is not visible)

Figure 3

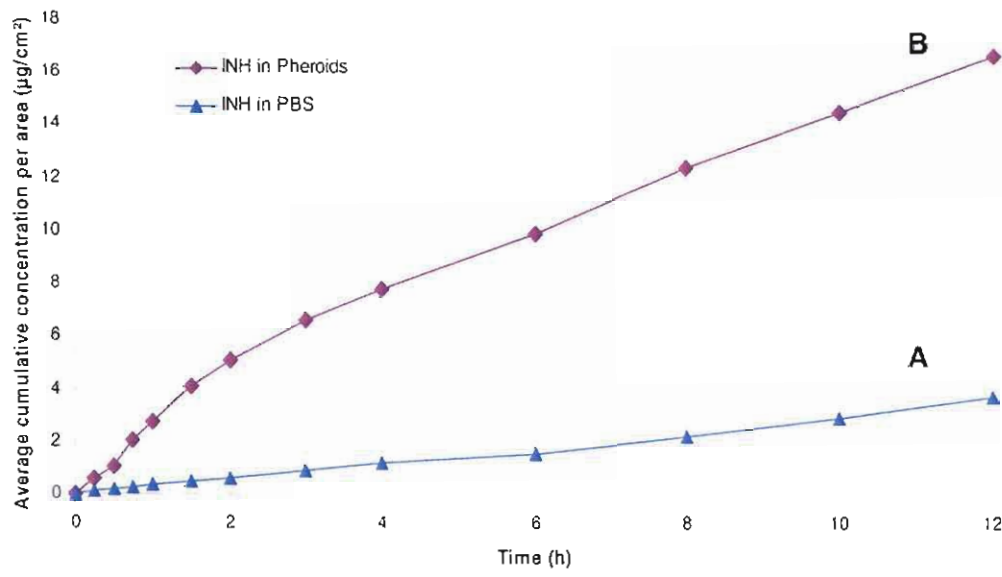
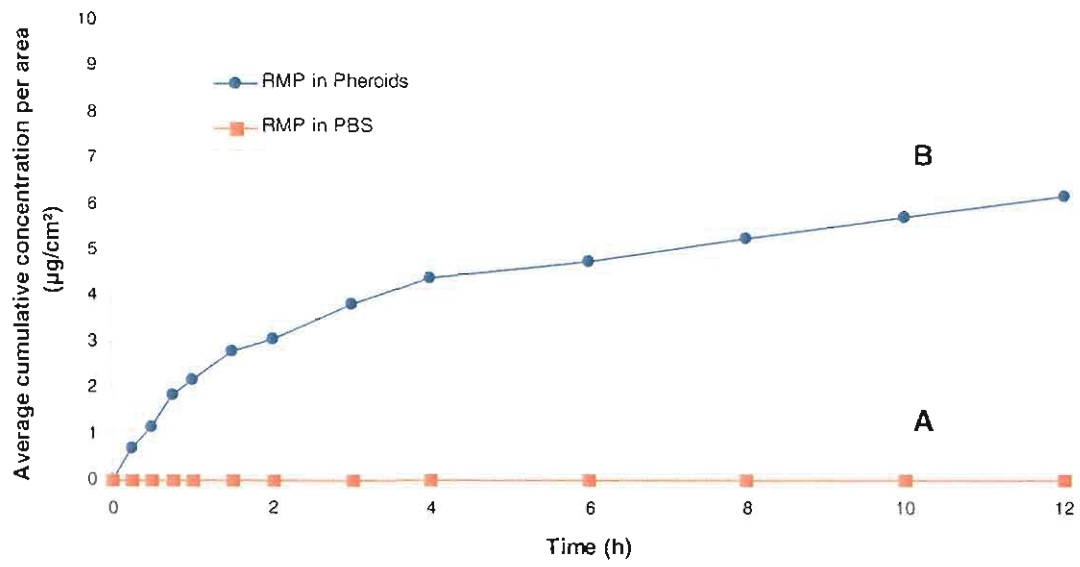


Figure 4



CHAPTER 4: FINAL CONCLUSIONS AND FUTURE PROSPECTS

The scope of this *in vitro* study was to investigate the transdermal delivery of isoniazid (INH) and rifampicin (RMP) by means of the novel Pheroid™ technology system. Since INH and RMP, like the majority of drugs, do not display the ideal physicochemical properties which will allow for easy permeation across the rate-limiting barrier of the skin, the stratum corneum (SC), some reliable form of penetration augmentation had to be utilised to improve their poor diffusivity. For this reason both actives were entrapped within the Pheroid™ drug delivery system.

Pheroid™, a stable skin-friendly carrier, comprises of a submicron (200 nm - 2 µm) emulsion type formulation for which previous studies have confirmed the ability to penetrate keratinised tissue, skin, intestinal linings, the vascular system, fungi, bacteria and even parasites.

Entrapment of INH and RMP within the Pheroid™ micro-sponge structures was monitored by confocal laser scanning microscopy (CLSM). *In vitro* permeation studies were conducted, using vertical Franz diffusion cells and female abdominal skin as permeation membranes over a period of 12 hours. Concentrations of 5 mg/ml and 10 mg/ml for isoniazid (INH) and rifampicin (RMP) respectively, were applied to the donor phase suspended in either phosphate buffered saline (PBS) or in Pheroid™. Permeation experiments conducted using PBS as the drug delivery vehicle served as positive control against which the efficacy of the Pheroid™ delivery system was measured. All permeation studies were conducted at pH 5.5.

The rationale behind the chosen drug combination and route of administration was to investigate the feasibility for the development of a topical product which could serve as an adjunctive to current systemic chemotherapy for cutaneous tuberculosis (tuberculosis involving the skin).

Cutaneous tuberculosis (CTB) refers to pathological lesions of the skin caused by any one of the following: *Mycobacterium tuberculosis*, *Mycobacterium bovis* or the BCG vaccine. It can develop either as disseminated pulmonary tuberculosis (TB) or after direct skin inoculation with *M. tuberculosis*. Demonstration of *M. tuberculosis*

within the infected tissues by traditional acid-fast bacilli (AFB) staining, culture or polymerase chain reaction (PCR) confirms the diagnosis.

CTB lesions are associated with various degrees of one or more of the following ulceration, plaque formation, hyperkeratosis or the presence of necrotic matter. All of these may influence the diffusion of INH and RMP in the Pheroid™ vehicle to their intended sites of action on the tubercle bacilli.

As CTB is mostly associated with systemic involvement, current treatment comprises of the standard three/four drug regimens used for pulmonary TB. Cases of CTB generally show improvement within 1 month of therapy with anti-TB drugs, but complete resolution is only attained after 4 - 6 months. The major drawback to current therapy is that patients not only remain a source of infection (viable organisms can still be demonstrated in the lesions), but they also suffer from constant embarrassment due to the disfiguring nature of CTB until these lesions have healed completely. A topical application might therefore induce a more speedy recovery and reduce the scarring and disfigurement associated with this disease.

The following observations were made during the course of this study:

- Confocal laser scanning microscopy revealed definite entrapment of both actives within the Pheroid™ micro-suspension.
- *In vitro* permeation profiles for INH in Pheroids™ showed a biphasic character (phase one ranging from 0-2 hours and phase two from 2-12 hours) with the absence of a lag-time.
- The percentage yield obtained for the INH containing Pheroid™ micro-suspension was about 10 times higher than that of PBS within the first 2 hours after application. For time 2-12 h, the percentage yield was 4 times higher than that obtained with a PBS solution.
- Zero permeation was achieved for RMP dispersed in a PBS solution.
- *In vitro* permeation profiles for RMP in Pheroids™ showed a triphasic, rather than biphasic, character with phase one ranging from 0-1.5 h, the second 1.5-4 h and the third 4-12 h. A lag-time was also absent.

- The Pheroid™ drug delivery system was able to deliver INH and RMP simultaneously in quantities above their MIC values (an important factor to consider in the prevention of the emergence of drug resistant micro-organisms) over a period of 12 hours.
- Despite the fact that RMP's concentration in the formulation was double that of INH, the overall mean flux values for INH in both PBS and Pheroid solutions were higher than those obtained for RMP.
- In the case of RMP in Pheroids™, the Pheroids™ is thought to be the single driving force behind RMP's transdermal delivery.
- Overall, the Pheroid™ micro-suspension drug delivery system proved to be superior over a PBS solution in that two actives with totally opposite physicochemical properties could be entrapped and delivered across intact epidermis in sufficient quantities.

Aspects identified which might render further investigation are listed below:

- The development of an INH and RMP containing Pheroid™ micro-suspension as a topical metered spray for easy application (preventing cross infection to other body sites) in CTB.
- Stability testing of the Pheroid™ micro-suspension containing INH and RMP.
- *In vitro* permeation studies to investigate the degree of dosage reduction of the entrapped actives that would still render drug quantities above that of their respective MIC values.
- *In vitro* efficacy testing of the proposed Pheroid™ micro-suspension, using reference strains of *Mycobacterium tuberculosis*.
- *In vivo* testing of the studied Pheroid™ formulation to determine actual blood and plasma levels for INH and RMP and safety of concomitant use with oral anti-tubercular drugs.
- Clinical trials in patients with CTB to determine the efficacy of the INH and RMP Pheroid™ formulation as well as duration of treatment necessary to achieve

resolution of CTB lesions, thus total eradication of *M. tuberculosis* mycobacteria.

- Development of similar topical Pheroid™ formulations with RMP in combination with other drugs e.g. dapsone, which might be considered for use in leprosy patients.

.....**END**.....

APPENDICES

APPENDIX 1: GUIDE FOR AUTHORS – EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES



ELSEVIER

EUROPEAN JOURNAL OF
PHARMACEUTICAL SCIENCES

Journal of Pharmaceutical Sciences and Technology

Official Journal of the European Federation for Pharmaceutical Sciences (EUFEPS).
<http://www.elsevier.com>

Guide for Authors

1.1. Manuscripts

Authors should submit their manuscript electronically via the homepage of this journal (<http://www.elsevier.com/journals>).

After registration, authors will be asked to upload their manuscript and associated artwork. Full instructions on how to use the online submission tool are available at the web address listed above.

Manuscripts submitted to the journal are accepted on the understanding that: (1) they are subject to editorial review, (2) they have not been and will not be published in whole or in part in any other journal and (3) the recommendations of the Declarations of Helsinki and Tokyo, for humans, and the European Community guidelines as accepted principles for the use of experimental animals, have been adhered to.

The European Journal of Pharmaceutical Sciences will, therefore, only consider manuscripts that describe experiments which have been carried out under approval of an institutional or local ethics committee. Only manuscripts written in English should be submitted.

1.2. Format

Manuscripts should be neatly typed, double-spaced throughout, including tables, with at least 2.5 cm margins on all sides. Use one font type and size throughout the manuscript. Author(s) should not break or hyphenate words. The manuscript should be submitted with a cover letter containing the declaration that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and that the protocol complies with the particular recommendation and that approval of their protocols was obtained.

Webster's New International Dictionary or the Oxford English Dictionary should be consulted for spelling. Latin plurals should not be used if the English equivalent has become the accepted form, e.g., formulas not formulae. Use of hyphens, capital letters, numbers written or spelled out (e.g., 8 or eight) should be consistent throughout the manuscript. Words at the end of a line should not be divided.

1.3. Electronic manuscripts

Ensure that the letter "l" and digit "1" (also letter "O" and digit "0") have been used properly, and format your article (tabs, indents, etc.) consistently. Characters not available on your word processor (Greek letters, mathematical symbols, etc.) should not be left open but indicated by a unique code (e.g., gralpha, @, #, etc., for the Greek letter α). Such codes should be used consistently throughout the entire text. Please make a list of such codes and provide a key. Do not allow your word processor to introduce word splits and do not use a 'justified' layout. Please adhere strictly to the general instructions on style/arrangement and, in particular, the reference style of the journal. If your word processor features the option to save files "in flat ASCII", please do **not** use it.

LaTeX documents

If the LaTeX file is suitable, proofs will be produced without rekeying the text. The article should preferably be written using Elsevier's document class "elsart" or, alternatively, the standard document class "article".

The Elsevier LaTeX package (including detailed instructions for LaTeX preparation) can be obtained from the Quickguide: <http://www.elsevier.com/latex>. It consists of the files: elsart.cls, guidelines for users of elsart, a template file for quick start, and the instruction booklet "Preparing articles with LaTeX".

Additional instructions on how to prepare your manuscript can be found at Elsevier's Quickguide: <http://www.elsevier.com>. For more detailed instructions please visit our artwork instruction pages at <http://www.elsevier.com/artworkinstructions>.

1.4. Abbreviations

Abbreviations are a hindrance for the reader. Use as few abbreviations as possible and write out names of compounds, receptors, etc., in full throughout the text of the manuscript, with the exceptions given below. Unnecessary and nonsense abbreviations are not allowed. Generic names should not be abbreviated. As an example, AMP, HAL, HIST, RAMH, TAM, SST, for amphetamine, haloperidol, histamine, (R)- α -methylhistamine, tamoxifen, somatostatin, are not accepted. Abbreviations which have come to replace the full term (e.g., GABA, DOPA, PDGF, 5-HT, for γ -aminobutyric acid, 3,4-dihydroxyphenylalanine, PDGF, 5-hydroxytryptamine) may be used, provided the term is spelled out in the abstract and in the body of the manuscript the first time the abbreviation is used. Unwieldy chemical names may be abbreviated. As an example, 8-OH-DPAT, DOI, DTG, BAPTA, for 8-hydroxy-2-(di-*n*-propylamino)tetralin, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, 1,3-di(2-tolyl)-guanidine, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, are acceptable; however, the full chemical name should be given once in the body of the manuscript and in the abstract, followed in both cases by the abbreviation. Code names may be used, but the full chemical name should be given in the text and in the abstract. *Authors not conforming to these demands may have*

their manuscripts returned for correction with delayed publication as a result.

Some abbreviations may be used without definition:

ADP, CDP, GDP, IDP	5'-pyrophosphates of adenosine
UDP	cytidine, guanosine, inosine, uridine
AMP etc.	adenosine 5'-monophosphate etc.
ADP etc.	adenosine 5'-diphosphate etc.
ATP etc.	adenosine 5'-triphosphate etc.
CM-cellulose	carboxymethylcellulose
CoA and acetyl-CoA	coenzyme A and its acyl derivatives
DEAE-cellulose	O-(diethylaminoethyl)-cellulose
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether) <i>N,N,N',N'</i> -tetraacetic acid
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione, reduced and oxidized
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
Pi, PPi	orthophosphate, pyrophosphate
RNA	ribonucleic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

Two alternative conventions are currently in use in some cases. For example, for the phosphoinositides there are both the abbreviations recommended by the IUPAC-IUB and those of the Chilton Convention (e.g., PtdIns(4,5)P₂ vs. PIP₂ for phosphatidylinositol 4,5-bisphosphate). The journal will accept either of these forms but not their combination.

Abbreviations of units of measurements and other terms are as follows:

Units of mass

kilogram	kg
gram	g
milligram	mg
microgram	µg
nanogram	ng
mole (gram-molecule)	mol
millimole	mmol
micromole	µmol
nanomole	nmol
picomole	pmol
femtomole	fmol
equivalent	eq

Units of time

hour	h
minute	min
second	s
millisecond	ms
microsecond	µs

Units of volume

litre	l
millilitre	ml
microlitre	µl

Units of length

metre	m
centimetre	cm
millimetre	mm
micrometre	μm
nanometre	nm

Units of concentration

molar (mol/l)	M
millimolar	mM
micromolar	μM
nanomolar	nM
picomolar	pM

Units of heat, energy, electricity

joule	J
degree Celsius (centigrade)	$^{\circ}\text{C}$
coulomb	C
ampere	A
volt	V
ohm	Ω
siemens	S

Units of radiation

curie	Ci
counts per minute	cpm
disintegrations per minute	dpm
becquerel	Bq

Miscellaneous

gravity	g
dissociation constant	K_d
median doses	LD ₅₀ , ED ₅₀
probability	P
routes of drug administration	i.v., i.p., s.c., i.m.
square centimetre	cm ²
standard deviation	S.D.
standard error of the mean	S.E.M.
Svedberg unit of sedimentation coefficient	S
Hill coefficient	n_H

The isotope mass number should appear before the atomic symbol, e.g., [³H]noradrenaline, [¹⁴C]choline. Ions should be written: Fe³⁺, Ca²⁺, Mg²⁺. The term absorbance (A) is preferred to extinction or optical density. For abbreviations not included in this list consult: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Authors and Editors*, 1994 (The Royal Society of Medicine, London), ISBN 0-905958-78-0, or *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 6th edn. (Cambridge University Press, Cambridge), ISBN 0-521-47154-0.

1.5. Nomenclature

Only generic and chemical names of drugs should be used, although a proprietary equivalent may be indicated once, in parentheses. *Pharmacological and Chemical Synonyms*, E.E.J. Marler, 9th edn. (Elsevier, Amsterdam, 1990) may be consulted.

The nomenclature of chemical substances should be consistent, clear and unambiguous, and should conform to the usage of the American Chemical Society and the convention recommended by the International Union of Pure and Applied Chemistry (IUPAC).

When in doubt, writers should consult the indexes of *Chemical Abstracts*; the various reports and pamphlets of the American Chemical Society Committee on Nomenclature, Spelling and Pronunciation; and from the International Union of Biochemistry and Molecular Biology (IUBMB): *Biochemical Nomenclature and Related Documents* (Portland Press, London).

When drugs, which are mixtures of stereoisomers are used, the fact that they have a composite nature and the implication of this for interpretation of the data and drawing of conclusions should be made clear. The use of the appropriate prefix is essential. Use of the generic name alone without prefix would be taken to refer to agents with no stereoisomers. The nomenclature of the various isomers and isomeric mixtures can be found in: (i) *IUPAC, Nomenclature of Organic Chemistry*, eds. J. Rigaudy and S.P. Klesney (Pergamon Press, London), 1979, p. 481; (ii) *Signs of the times: the need for a stereochemically informative generic name system*, Simonyi, M., J. Gal and B. Testa, 1989, *Trends Pharmacol. Sci.* 10, 349. For nomenclature of peptides, see *Neuropeptides*, Vol. 1, 1981, p. 231.

The nomenclature of receptors and their subtypes should conform to the *TIPS 1995 Receptor & Ion Channel Nomenclature Supplement* (*Trends Pharmacol. Sci.* Receptor Nomenclature Supplement 1995). Copies of this supplement are available from the publisher (Elsevier Trends Journals, Oxford Fulfilment Centre, P.O. Box 800, Kidlington, Oxford OX5 1DX, UK. Tel.: (44-1865) 843-699; Fax: (44-1865) 843-911).

The trivial name of the enzyme may be used in the text, but the systematic name and classification number according to *Enzyme Nomenclature*, rev. edn. (Academic Press, New York, NY, 1984) should be quoted the first time the enzyme is mentioned.

1.6. Editorial review

All manuscripts are generally submitted to 2-3 referees who are chosen for their ability to evaluate the work. Supplementary material may be included to facilitate the review process. Authors may request that certain referees should not be chosen. Members of the editorial board will usually be called upon for advice when there is

disagreement among the referees or between referees and authors, or when the editors believe that the manuscript has not received adequate consideration by the referees.

All referees' comments must be responded to, and suggested changes be made. The author should detail the changes made in response to the referees' comments and suggestions in an accompanying letter. If the author disagrees with some changes, the reason, supported by data, should be given. The editors may refuse to publish manuscripts from authors who persistently ignore referees' comments. Handwritten additions or corrections will not be accepted. Only complete retyping of the pages affected by revision is acceptable. A revised manuscript should be received by the editorial office no later than 4 months after the editorial decision was sent to the author(s); otherwise it will be processed as a new manuscript.

2. Organization and style of manuscripts

Authors should consult a current issue of the journal for the general manner of presentation. Manuscripts should be written in clear, concise English (see section 1.1), bearing in mind that English is not the native language of many of the readers. Terms that are not generally understood should be avoided; however if it is absolutely necessary to use such terms, they must be defined.

2.1. Research articles

2.1.1. General

The manuscript of a research article should be arranged as follows.

First page: title, surname(s) and full first name(s) of each author; name and address of the establishment where the work was done; name, full postal address, telephone and telefax numbers and e-mail of author to whom proofs and other correspondence should be sent. Next page: abstract and keywords (indexing terms, normally 3-6 items). Pages 3 to end: 1. Introduction; 2. Materials and methods; 3. Results; 4. Discussion; Acknowledgements; References; Tables; Figure legends and Figures. Parts 3 & 4 may be combined into one item: Results & Discussion. Subdivisions of a section should also be numbered within that section: 2.1., 2.2., 2.3., etc. All pages should be numbered consecutively, the title page being p. 1. See section 2.7 for

further information.

Supplementary material for electronic publication can be published on the journal website alongside the article. In the print version, a URL reference will be made to point readers to the location of the article and supplementary material.

2.1.2. Abstract and keywords

The abstract with keywords should be typed on a separate sheet. The abstract should include: the reason why the experiments were done, a very brief description of the experiments (including species, tissue, etc.), followed by the main results, and finally, a conclusion giving the relevance of the results to the question asked. The abstract must be completely self-explanatory. The abstract should not exceed approximately 200 words. No footnotes may be used and a reference, if cited, must be given in full. Standard terms and scientific nomenclature should be used. Abbreviations and contractions, except those for weights and measures and those explained, should not be used. Below the abstract, type 3-6 keywords or short phrases suitable for indexing. These terms will be printed at the end of the abstract. If possible, keywords should be selected from *Index Medicus* or *Excerpta Medica Index*.

2.1.3. Introduction, Materials and Methods, Results, Discussion

The introduction should not be an extensive review of the literature but should refer only to previous work which has a direct bearing on the topic to be discussed.

Materials and methods should be written clearly and in such detail that the work can be repeated by others. Procedural detail that has been published previously should be referred to by citation. When a modified procedure is used, only the author's modifications of the previously published method need to be given in detail.

Results should be described concisely. Text, tables and figures must be internally consistent.

The discussion should involve the significant findings presented. Wide digressions are unacceptable because of the limitations of space.

2.2. References

Authors are responsible for the accuracy and completeness of their references as these will not be checked by the editorial office.

References should be listed alphabetically (see sample references) according to the "Harvard" system. Articles written by the same first author with different second authors should be listed according to the second author's surname. Articles written by the same first author with more than one co-author should be listed alphabetically according to the first author's surname and then according to the year of publication. Two or more references to the same first author with the same publication year should have a, b, c, etc., suffixed to the year indicating the alphabetical order of the second or third author, etc.

References to journals should contain the names and initials of the author(s), the year, the full title, the abbreviation of the name of the periodical according to those in the Bibliographic Guide for Editors and Authors (American Chemical Society, Washington, DC.) followed by the volume and page numbers.

References to books should include the title and name and city of the publisher.

References in the text should be cited by the author's name and the year of publication. For 3 or more authors the name of the first author followed by et al. should be used, e.g., Davis, Robinson (1990) or (Davis, Illum, 1984; de Ber et al., 1988, 1989; Borchardt et al., 1990, 1991a,b,c).

Journals:

Fagerholm, U., Lennernas, H., 1995. Experimental estimation of the effective unstirred water layer thickness in the human jejunum and its importance in oral drug absorption. Eur. J. Pharm. Sci. 3, 247-253.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug. Deliv. Rev.* 23, 3-25.

Books:

Alderborn, G., Nystrom, C., 1998. *Pharmaceutical Powder Compaction Technology*. Marcel Dekker, New York.

Kissel, T., Koneberg, R., 1996. Injectable biodegradable microspheres for vaccine delivery. In: Cohen, S. and Bernstein, H. (Eds.), *Microparticulate systems for the delivery of proteins and vaccines*. Marcel Dekker, New York, pp. 51-87.

Unpublished observations, personal communications and manuscripts in preparation or submitted for publication may be referred to in the text but should not appear in the list of references. Manuscripts in press (i.e., accepted for publication) may be included in the references citing the DOI article identifier, which enables the citation of a paper before volume, issue and page numbers are allocated. The name of the journal in which they are to appear must be given.

Articles in Special Issues: Please ensure that the words 'this issue' are added (in the list and text) to any references to other articles in this Special Issue.

2.3. Illustrations

The number of illustrations should be limited to the essential.

(a) It is important to allow for reduction to fit a single column, 8.4 cm wide or at most a double column, maximally 17.6 cm wide. Of preference, illustrations, especially photomicrographs, should be submitted in their final size (single or double column). When possible, all key symbols should be explained in the figures. All letters and numerals appearing in a particular illustration should be of the same size (approximately 1.4-2.0 mm height when reduced to 8.4 cm width). Comparable illustrations should carry letters, figures and numerals of the same size when reduced to 8.4 cm width.

(b) Graphs should be prepared by a skilled photographer so that the dark, cross-hatched background is eliminated, the faint portions of the graphs are intensified, and a sharp print is obtained. This process may be avoided by using blue-ruled instead of black-ruled recording paper for the originals.

(c) Drawings of chemical structures should as far as possible be produced with the use of a drawing program such as ChemDraw. Authors using the current versions of ChemDraw, ChemIntosh and ChemWindows should use the JOC format.

(d) A calibration bar should be drawn on the micrographs instead of giving a magnification factor in the figure legend.

(e) All illustrations should be referred to as figures and numbered in Arabic numerals (Fig. 1, 2, etc.).

(f) Legends to figures should make the figures comprehensible without reference to the text.

(g) If, together with your accepted article, you submit usable colour figures then Elsevier will ensure, at no additional charge, that these figures will appear in colour on the web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in colour in the printed version. For colour reproduction in print, you will receive information regarding the total cost from Elsevier after receipt of your accepted article. The 2006 colour prices are EUR 285.00 for the first page and EUR 191.00 for subsequent pages. In some cases, colour costs may be waived at the discretion of the Editor-in-Chief. For more detailed instructions please visit our artwork instruction pages at <http://www.elsevier.com/artworkinstructions>.

Please note: Because of technical complications which can arise by converting colour figures to 'grey scale' (for the printed version should you not opt for colour in print) please submit in addition usable black and white prints corresponding to all the colour illustrations.

2.4. Tables

Tables should be prepared for use in a single column (8.4 cm wide) or be of page width (17.6 cm).

- (a) Each table should have a brief explanatory heading and sufficient experimental detail (following the table body as a footnote) so as to be intelligible without reference to the text.
- (b) Tables should not duplicate material in text or illustrations.
- (c) Short or abbreviated column headings should be used and, if necessary, explained in footnotes, and indicated as ^a, ^b, ^c, etc.
- (d) Statistical measures of variation, S.D., S.E., etc. should be identified.
- (e) Tables should be numbered separately in Arabic numerals (Table 1, 2, etc.).

2.5. Formulas and equations

Structural chemical formulas, process flow diagrams and complicated mathematical expressions should be very clearly presented. All subscripts, superscripts, Greek letters and unusual characters must be identified. Structural chemical formulas and process flow diagrams should be prepared in the same way as graphs.

2.6. GenBank accession numbers

Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should reference this information in the following manner:

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Example 1: "GenBank accession nos. **AI631510**, **AI631511**, **AI632198**, and **BF223228**), a B-cell tumour from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**)".

Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.**

In the final version of the **printed article**, the accession number text will not appear bold or underlined (see Example 2 below).

Example 2: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

In the final version of the **electronic copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article (see Example 3 below).

Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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The journal is looking for a stimulating and provoking essays, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to preferably recent peer-reviewed material. The word count should be approximately 2,000 words maximum.

The commentary should have a short abstract summary of 150 to 200 words and 4-5 key words should be included, The text should be broken down into 4-5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes

2.9.2 Mini-review (Guidance)

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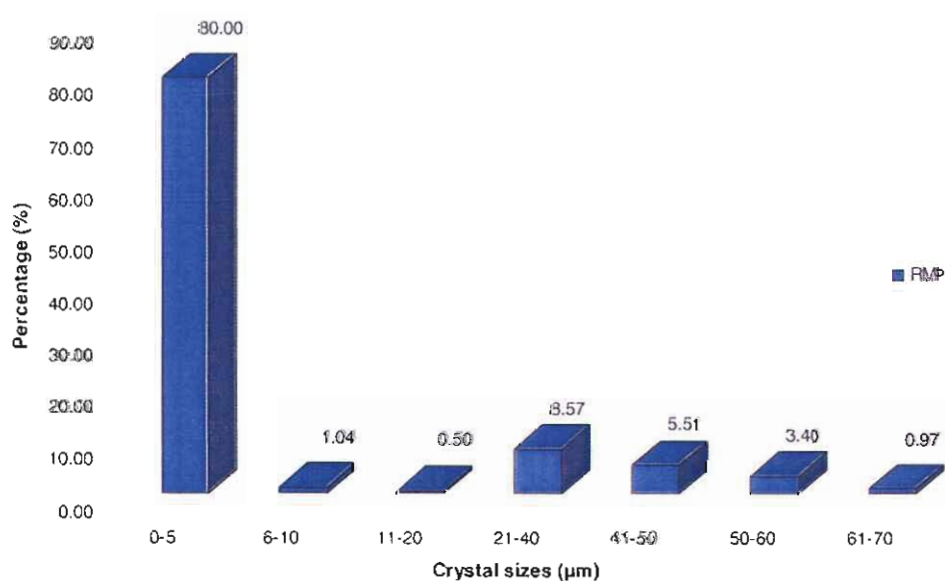
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APPENDIX 2: PARTICLE SIZE DISTRIBUTION OF RIFAMPICIN (RMP)

The following table and figure expresses the size distribution of RMP crystals as determined by confocal laser scanning microscopy (CLSM).

CRYSTAL SIZE RANGES (μm)	NUMBER OF PARICLES (n = 4411)	PERCENTAGE (%)
0-5	3529	80.00
6-10	46	1.04
11-20	22	0.50
21-40	378	8.57
41-50	243	5.51
50-60	150	3.40
61-70	43	0.97

Graph of the percentage of particles (%) vs Crystal sizes (μm):
Rifampicin (RMP)



APPENDIX 3: DATA OF FRANZ CELL DIFFUSION STUDIES

ISONIAZID (IN COMBINATION WITH RIFAMPICIN) IN PBS SOLUTION (pH 5.5)

Presented below is the permeation data obtained for 13 cells containing INH (5 mg/ml) dissolved in PBS solution (pH 5.5) after a 12-hour period. This study served as positive control against which the efficacy of the Pheroid™ delivery system was measured.

Table 1: Permeation data for INH (5 mg/ml) in PBS (pH 5.5)

Cell#	Cum.conc. ^a (µg/ml) (0-2 h)	Cum.conc. (µg/ml) (2-12 h)	Cum.conc (µg/ml) (0-12 h)	% Yield (0-2 h)	% Yield (2-12 h)	% Yield (0-12 h)
1	0.00	1.93	1.93	0.00	0.04	0.04
2	0.00	4.21	4.21	0.00	0.08	0.08
3	0.00	3.00	3.00	0.00	0.06	0.06
4	3.22	5.70	8.92	0.06	0.11	0.18
5	0.00	3.07	3.07	0.00	0.06	0.06
6	0.61	4.44	5.05	0.01	0.09	0.10
7	0.00	1.88	1.88	0.00	0.04	0.04
8	3.89	4.14	8.03	0.08	0.08	0.16
9	0.00	2.64	2.64	0.00	0.05	0.05
10	0.00	5.13	5.13	0.00	0.10	0.10
11	0.00	2.34	2.34	0.00	0.05	0.05
12	0.00	0.75	0.75	0.00	0.02	0.02
13	0.00	0.76	0.76	0.00	0.02	0.02
Mean				0.01	0.06	0.07
SD ^b				0.03	0.03	0.05
% RSD ^c				215.47	49.09	66.64

^a Cum. conc. = cumulative concentration, ^b SD = standard deviation

^c % RSD = percentage relative standard deviation

Table 2: Flux values for INH (5 mg/ml) in PBS (pH 5.5)

Cell #	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (0-2 h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (2-12 h)
1	0.000	0.195
2	0.000	0.404
3	0.000	0.297
4	1.630	0.560
5	0.000	0.323
6	0.234	0.425
7	0.000	0.190
8	1.957	0.399
9	0.000	0.262
10	0.000	0.493
11	0.000	0.232
12	0.000	0.050
13	0.000	0.050
Mean	0.294	0.298
SD	0.646	0.151
% RSD	219.650	50.481

Permeation profiles for 13 cells containing INH (5 mg/ml) dissolved in PBS solution (pH 5.5).

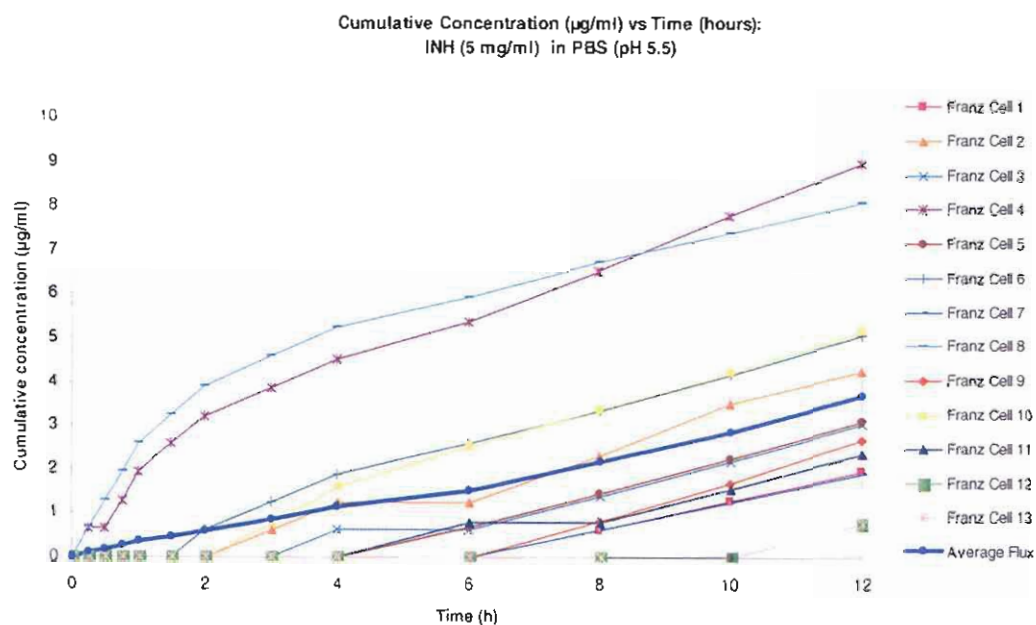


Figure 1

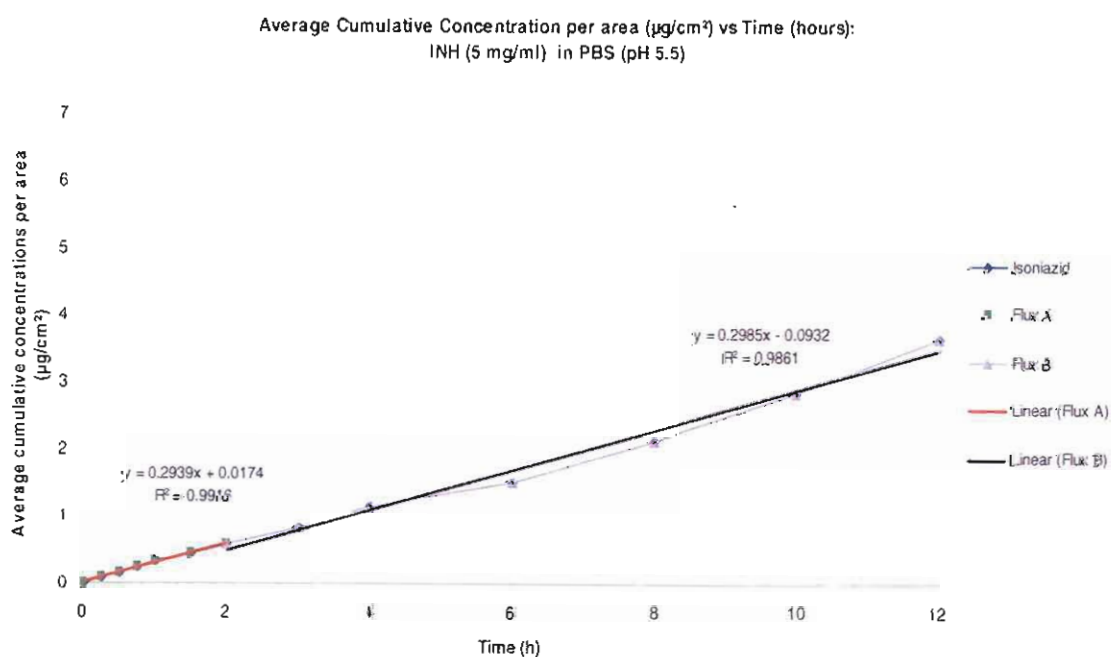


Figure 2

ISONIAZID (IN COMBINATION WITH RIFAMPICIN) IN THE PHEROID™ DELIVERY SYSTEM

Presented below is the permeation data obtained for 13 cells containing INH (5 mg/ml) entrapped within the Pheroid™ delivery system (pH 5.5) after a 12-hour period. This data was compared to those obtained for PBS to investigate the influence of the Pheroid™ on the transdermal delivery of INH.

Table 3: Permeation data for INH (5 mg/ml) in Pheroid™ (pH 5.5)

Cell #	Cum.conc. (µg/ml) (0-2 h)	Cum.conc. (µg/ml) (2-12 h)	Cum.conc (µg/ml) (0-12 h)	% Yield (0-2 h)	% Yield (2-12 h)	% Yield (0-12 h)
1	6.16	5.77	11.92	0.12	0.12	0.24
2	7.33	6.97	14.30	0.15	0.14	0.29
3	0.63	3.64	4.28	0.01	0.07	0.09
4	0.64	6.42	7.06	0.01	0.13	0.14
5	3.61	6.64	10.25	0.07	0.13	0.21
6	8.31	9.72	18.03	0.17	0.19	0.36
7	0.00	5.76	5.76	0.00	0.12	0.12
8	0.00	6.27	6.27	0.00	0.13	0.13
9	14.56	13.13	27.69	0.29	0.26	0.55
10	10.48	17.35	27.84	0.21	0.35	0.56
11	3.41	9.43	12.84	0.07	0.19	0.26
12	7.94	26.99	34.93	0.16	0.54	0.70
13	2.89	31.83	34.72	0.06	0.64	0.69
Mean				0.10	0.23	0.33
SD				0.09	0.17	0.21
% RSD				85.51	73.03	63.91

Table 4: Flux values for INH (5 mg/ml) in Pheroid™ (pH 5.5)

Cell #	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (0-2 h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (2-12 h)
1	3.166	0.547
2	3.576	0.680
3	0.243	0.380
4	0.246	0.655
5	2.041	0.646
6	4.896	0.863
7	0.000	0.588
8	0.000	0.620
9	7.671	1.072
10	5.340	1.698
11	1.891	0.928
12	4.034	2.696
13	1.214	3.397
Mean	2.640	1.136
SD	2.307	0.883
% RSD	87.375	77.765

Permeation profiles obtained for 13 cells containing INH (5 mg/ml) entrapped within the Pheroid™ delivery system (pH 5.5). The average flux curve shows a distinct biphasic character, with the first phase from 0-2 hours (Flux A) and the second from 2-12 hours (Flux B).

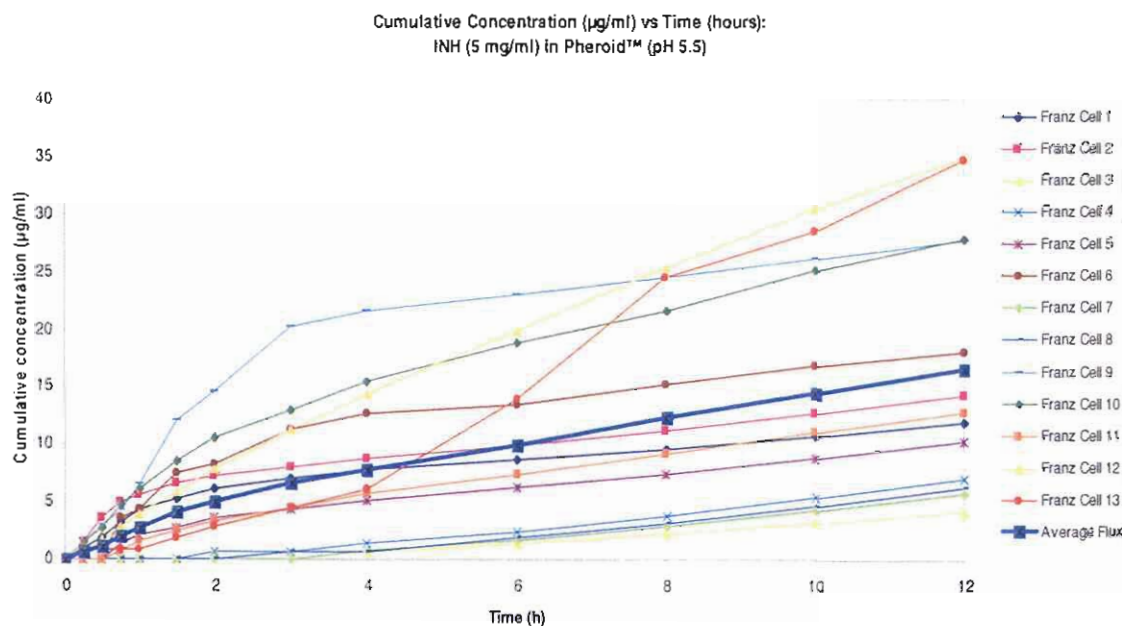


Figure 3

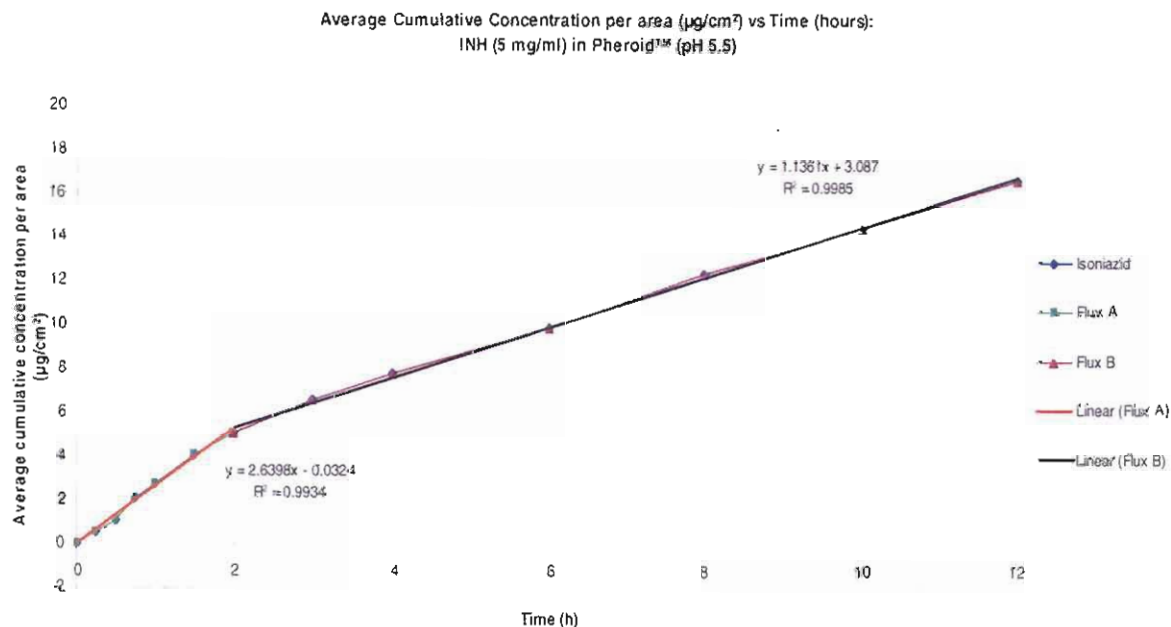


Figure 4

RIFAMPICIN (IN COMBINATION WITH ISONIAZID) IN PBS SOLUTION (pH 5.5)

Permeation data obtained for 13 cells containing RMP (10 mg/ml) dispersed in a PBS solution (pH 5.5) after a 12-hour period. This study served as positive control against which the efficacy of the Pheroid™ delivery system was measured.

Table 5: Permeation data for RMP (10 mg/ml) in PBS (pH 5.5)

Cell #	Cum.conc. ^a (µg/ml) (0-1.5 h)	Cum.conc. (µg/ml) (1.5-4 h)	Cum.conc (µg/ml) (4-12 h)	Cum.conc (µg/ml) (0-12 h)	% Yield (0-1.5 h)	% Yield (1.5-4 h)	% Yield (4-12 h)	% Yield (0-12 h)
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mean					0.00	0.00	0.00	0.00
SD ^b					0.00	0.00	0.00	0.00
%RSD ^c					0.00	0.00	0.00	0.00

^a Cum. conc. = cumulative concentration, ^b SD = standard deviation

^c % RSD = percentage relative standard deviation

Table 6: Flux values for RMP (10 mg/ml) in PBS (pH 5.5)

Cell #	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (0-1.5 h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (1.5-4 h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (4-12 h)
1	0.000	0.000	0.000
2	0.000	0.000	0.000
3	0.000	0.000	0.000
4	0.000	0.000	0.000
5	0.000	0.000	0.000
6	0.000	0.000	0.000
7	0.000	0.000	0.000
8	0.000	0.000	0.000
9	0.000	0.000	0.000
10	0.000	0.000	0.000
11	0.000	0.000	0.000
12	0.000	0.000	0.000
13	0.000	0.000	0.000
Mean	0.000	0.000	0.000
SD	0.000	0.000	0.000
% RSD	0.000	0.000	0.000

Below follows the permeation profiles obtained for 13 cells containing RMP (10 mg/ml) dissolved in PBS solution (pH 5.5).

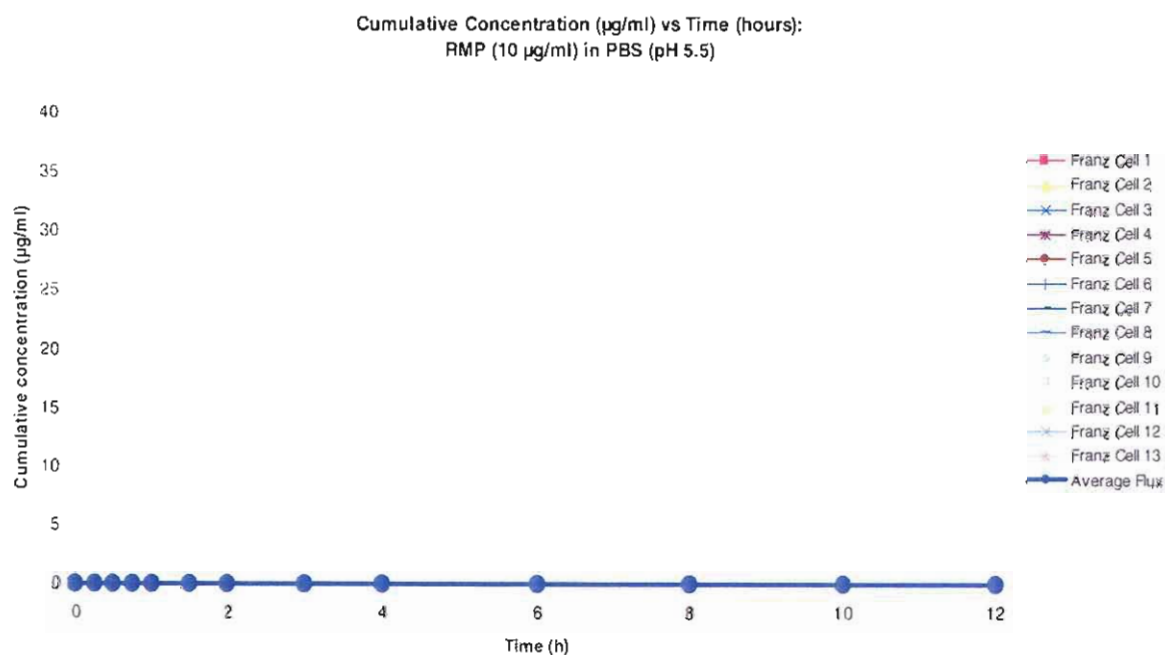


Figure 5

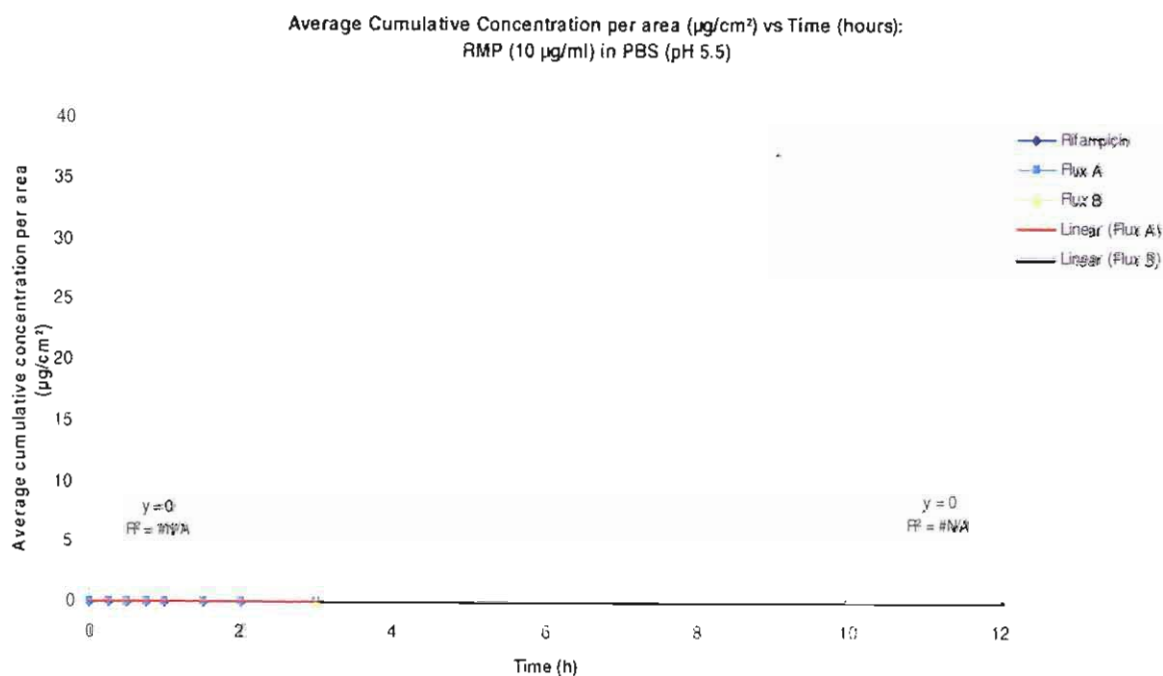


Figure 6

RIFAMPICIN (IN COMBINATION WITH ISONIAZID) IN THE PHEROID™ DELIVERY SYSTEM

Presented below is the permeation data and permeation profiles obtained for 13 cells containing RMP (10 mg/ml) entrapped within the Pheroid™ delivery system (pH 5.5) over a 12-hour period. This data was compared to those obtained for PBS to investigate the influence of the Pheroid™ on the transdermal delivery of RMP.

Table 7: Permeation data for RMP (10 mg/ml) in Pheroid™ (pH 5.5)

Cell #	Cum.conc. (µg/ml) (0-1.5 h)	Cum.conc. (µg/ml) (1.5-4 h)	Cum.conc (µg/ml) (4-12 h)	Cum.conc (µg/ml) (0-12 h)	% Yield (0-1.5 h)	% Yield (1.5-4 h)	% Yield (4-12 h)	% Yield (0-12 h)
1	4.83	1.06	0.00	5.88	0.05	0.01	0.00	0.06
2	5.38	1.11	0.00	6.49	0.05	0.01	0.00	0.06
3	1.08	0.00	0.00	1.08	0.01	0.00	0.00	0.01
4	1.09	0.00	0.00	1.09	0.01	0.00	0.00	0.01
5	1.33	0.00	0.00	1.33	0.01	0.00	0.00	0.01
6	4.36	3.94	2.42	10.71	0.04	0.04	0.02	0.11
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	9.29	6.71	0.00	16.00	0.09	0.07	0.00	0.16
10	7.65	5.02	6.45	19.12	0.08	0.05	0.06	0.19
11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	3.03	2.96	5.99	0.00	0.03	0.03	0.06
13	1.35	0.00	11.30	12.65	0.01	0.00	0.11	0.13
Mean					0.03	0.02	0.02	0.06
SD					0.03	0.02	0.03	0.06
% RSD					108.84	138.02	186.24	102.00

Table 8: Flux values for RMP (10 mg/ml) in Pheroid™ (pH 5.5)

Cell #	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (0-1.5 h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (1.5-4 h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$) (4-12 h)
1	3.466	0.502	0.000
2	3.583	0.525	0.000
3	0.492	0.000	0.000
4	0.497	0.000	0.000
5	1.138	0.000	0.000
6	3.133	1.735	0.363
7	0.000	0.000	0.000
8	0.000	0.000	0.000
9	6.152	2.739	0.000
10	5.170	1.943	0.793
11	0.000	0.000	0.000
12	0.000	1.129	0.372
13	0.616	0.000	1.405
Mean	1.865	0.659	0.226
SD	2.079	0.896	0.412
% RSD	111.453	135.870	182.847

Permeation profiles obtained for 13 cells containing RMP (10 mg/ml) entrapped within the Pheroid™ delivery system (pH 5.5). The average cumulative curve shows an interesting triphasic character, with the first phase from 0-1.5 hours (Flux A), the second from 1.5-4 hours (Flux B) and the third from 4-12 hours (Flux C).

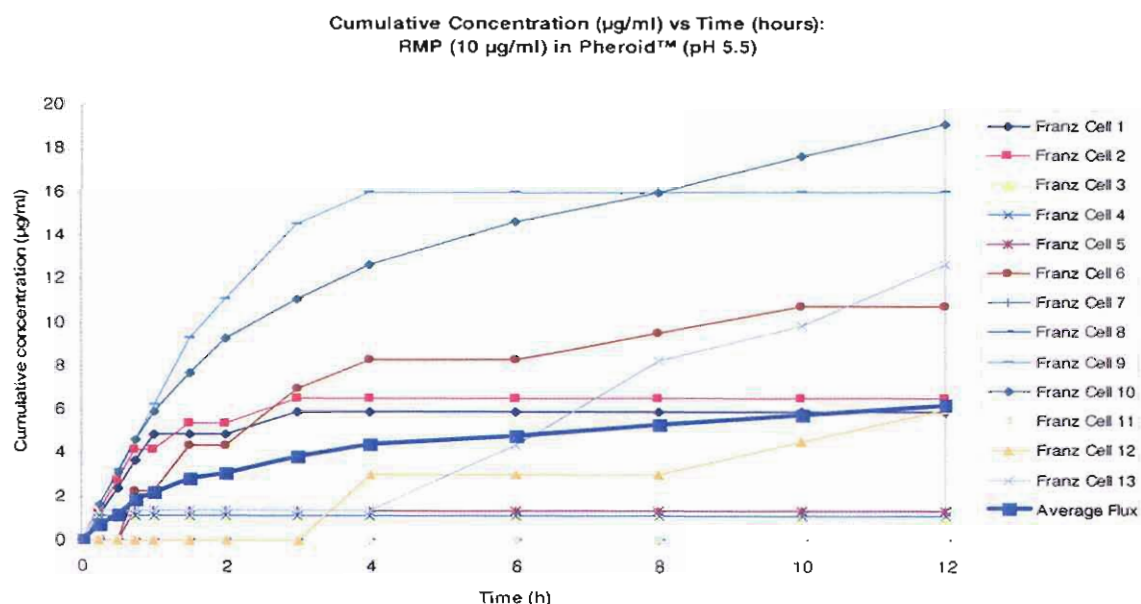


Figure 7

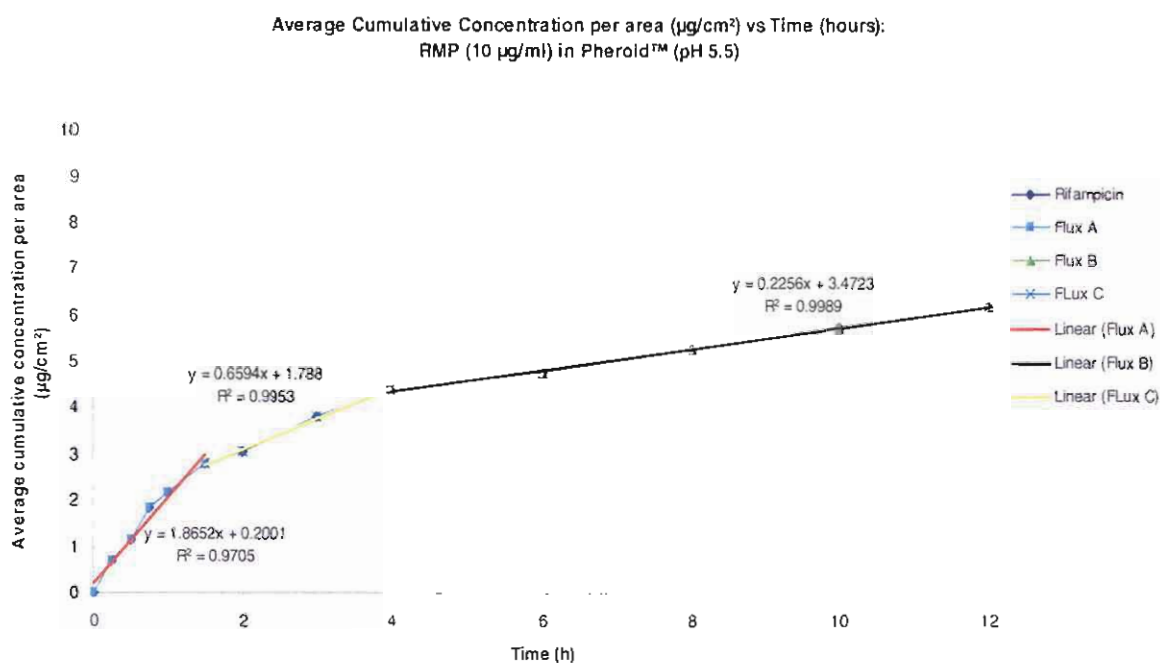


Figure 8

APPENDIX 4: VALIDATION OF EXPERIMENTAL METHODS

VALIDATION OF THE HPLC GRADIENT ELUTION METHOD FOR SIMULTANEOUS DETERMINATION OF RIFAMPICIN (1.0%) AND ISONIAZID (0.5%)

SUMMARY:

Table 1: Summary of the validation results for isoniazid (INH) and rifampicin (RMP)

Test	Results	
	Isoniazid	Rifampicin
Specificity	Complies	Complies
Range	1.25- 500 µg/ml	2.5- 1000 µg/ml
Linearity	$R^2 = 0.9992$	$R^2 = 0.9956$
Precision	RSD ^a = 1.05 %	RSD = 0.39 %
Robustness/ Stability	Complies	Complies

^a RSD: relative standard deviation

PURPOSE OF THE VALIDATION

To ensure that the method was suitable, sensitive and accurate enough for the reliable determination of amounts of drug substances that permeated the skin.

CHROMATOGRAPHIC CONDITIONS

Analytical instrument: Agilent series 1100 HPLC equipped with a pump, autosampler, UV detector and Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software were used.

Column: Phenomenex®, Synergi Max RP- 80A, 250 x 4.6 mm, 4 µm column.

Mobile phase: Isoniazid (INH) and rifampicin (RMP) were separated by means of gradient elution. The mobile phase consisted of two solvents:

- A. A degassed mixture of 0.01 M monobasic ammonium phosphate $[(\text{NH}_4)\text{H}_2\text{PO}_4]$ and 0.005 M 1-heptanesulphonic acid sodium salt $[\text{C}_7\text{H}_{15}\text{NaO}_3\text{S}]$ in HPLC (deionised) water. 10% Phosphoric acid (H_3PO_4) solution was used to adjust the pH of the mobile phase to 3.50.
- B. Methanol - HPLC grade (100%).

The following table describes the mobile phase composition:

Table 2: Mobile phase consisted of monobasic ammonium phosphate buffer (solvent A) and methanol (solvent B)

Mobile phase composition A : B		Duration (min) (Total runtime = 13 minutes)
80	20	3.50
20	80	4.50
20	80	9.00
80	20	9.20

Flow rate:	1.0 ml/min
Injection volume:	20 µl
Detection:	UV at 263.4 nm
Retention time:	Isoniazid and rifampicin eluted at 4.11 and 9.4 minutes respectively.
Solvent:	A 20:80 mixture of methanol and phosphate buffered saline (PBS) was used during the entire validation process. Methanol, however, can be substituted with ethanol without causing any disturbance. The inclusion of an organic solvent is to ensure the solubility of the lipophilic components namely rifampicin, Pheroid™ and skin tissue lipids.

STANDARD PREPARATION

1. Weighed approximately 10 mg rifampicin and 5 mg isoniazid accurately in a 100 ml volumetric flask which was then diluted to volume with PBS and methanol (80:20) to obtain a concentration of 100 µg/ ml. Rifampicin was dissolved in methanol prior to adding the isoniazid and PBS. The mixture was sonicated for a few minutes.
2. Transferred a quantity to an amber HPLC vial which was analysed.

SAMPLE PREPARATION

1. Weighed approximately 1g of the Pheroid™ preparation accurately and diluted it with methanol R (100%) to obtain a concentration equivalent to 100 µg of rifampicin and 50 µg of isoniazid per millilitre.
2. Transferred a quantity to an amber HPLC vial which was analysed.

VALIDATION PARAMETERS

CALIBRATION CURVE

A calibration curve for isoniazid and rifampicin was established by using standard solutions with the following concentrations: 2.5, 5, 10, 20, 50, 100, 200, 500, 800 and 1000 µg/ml for rifampicin and 1.25, 2.5, 5, 10, 25, 50, 100, 250, 400 and 500 µg/ml for isoniazid. The solutions were prepared as follows:

1. Weighed approximately 1 g rifampicin and 0.5 g isoniazid accurately in a 100 ml volumetric flask and diluted to volume with 50:50 methanol and PBS to obtain a standard stock solution (RMP 1.0% & INH 0.5%). Rifampicin was dissolved in methanol prior to adding isoniazid and PBS to ensure complete dissolution. The mixture was sonicated for 10 minutes.
2. Diluted 10 ml of the standard stock solution to 100 ml with solvent (1000 µg/ml)*.
3. Diluted 40 ml of the 1000 µg/ml solution to 50 ml with solvent (800 µg/ml).
4. Diluted 12.5 ml of the 800 µg/ml solution to 20 ml with solvent (500 µg/ml).
5. Diluted 8 ml of the 500 µg/ml solution to 20 ml with solvent (200 µg/ml).
6. Diluted 10 ml of the 200 µg/ml solution to 20 ml with solvent (100 µg/ml).
7. Diluted 10 ml of the 100 µg/ml solution to 20 ml with solvent (50 µg/ml).
8. Diluted 8 ml of the 50 µg/ml solution to 20 ml with solvent (20 µg/ml).
9. Diluted 10 ml of the 20 µg/ml solution to 20 ml with solvent (10 µg/ml).
10. Diluted 10 ml of the 10 µg/ml solution to 20 ml with solvent (5 µg/ml).
11. Diluted 10 ml of the 5 µg/ml solution to 20 ml with solvent (2.5 µg/ml).
12. 20 µl of each dilution was injected in duplicate into the chromatograph.

*Values in parenthesis refer to the rifampicin concentration in the mixture.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Standard solutions were prepared as described above. Linearity of isoniazid and rifampicin were established from a plot of the mean peak area ratios versus concentration ($\mu\text{g/ml}$) from which the regression lines were calculated by method of least squares. The data is best described by a linear equation $y = mx + c$ where:

y = peak area ratio of isoniazid/ rifampicin

m = slope

x = concentration of isoniazid/ rifampicin ($\mu\text{g/ml}$)

c = y- intercept

Table 3: Peak area ratio values for isoniazid standards

Isoniazid				
Spike	$\mu\text{g/ml}$	Area 1 (mAU)	Area 2 (mAU)	Mean
1	1.25	42.98	39.61	41.30
2	2.50	71.77	71.57	71.67
3	5.00	138.77	141.74	140.26
4	10.00	278.56	273.89	276.23
5	25.00	602.44	603.22	602.83
6	50.00	1199.12	1200.74	1199.93

Table 4: Regression statistics for isoniazid

R squared	0.9992	Lower 95%	Upper 95%
Intercept	15.2679	-1.2693	31.8051
Slope	23.7359	22.9694	24.5024

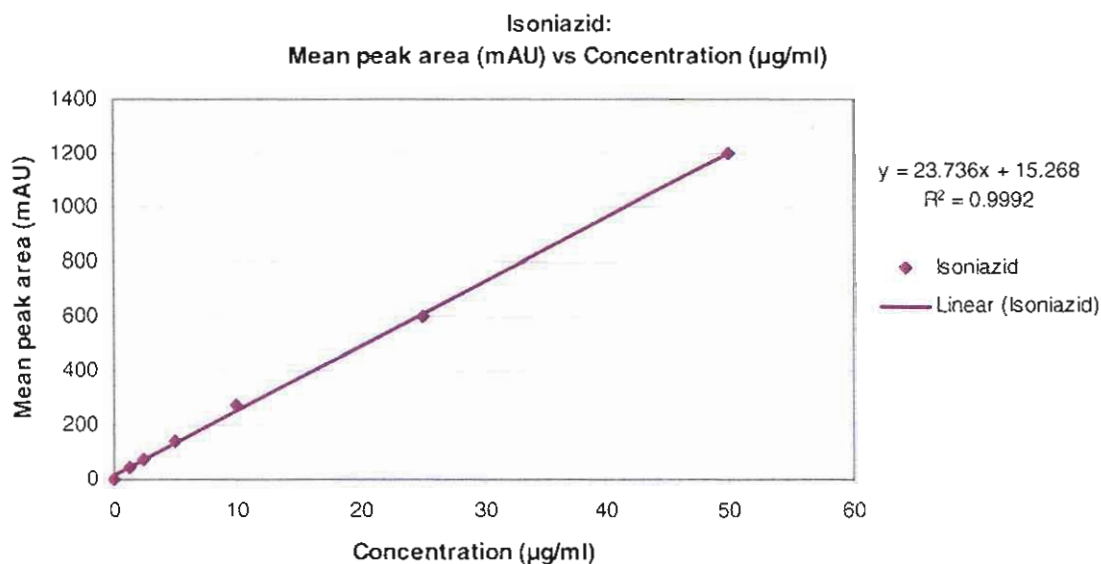


Figure 1: Linear regression curve for isoniazid standards (n = 6)

The R squared value (R^2) obtained for isoniazid indicates a high degree of linearity and therefore demonstrates the stability of the analytical system.

Table 5: Peak area ratio values for rifampicin standards

Rifampicin				
Concentration Spiked	µg/ml	Area 1 (mAU)	Area 2 (mAU)	Mean
1	2.50	99.37	99.72	99.55
2	5.00	191.55	192.77	192.16
3	10.00	386.67	387.19	386.93
4	20.00	754.56	754.12	754.34
5	50.00	1627.24	1633.00	1630.12
6	100.00	2924.97	2950.40	2937.69

Table 6: Regression statistics for rifampicin

R squared	0.9956	Lower 95%	Upper 95%
Intercept	72.2874	-24.5634	169.1383
Slope	29.3045	27.0599	31.5492

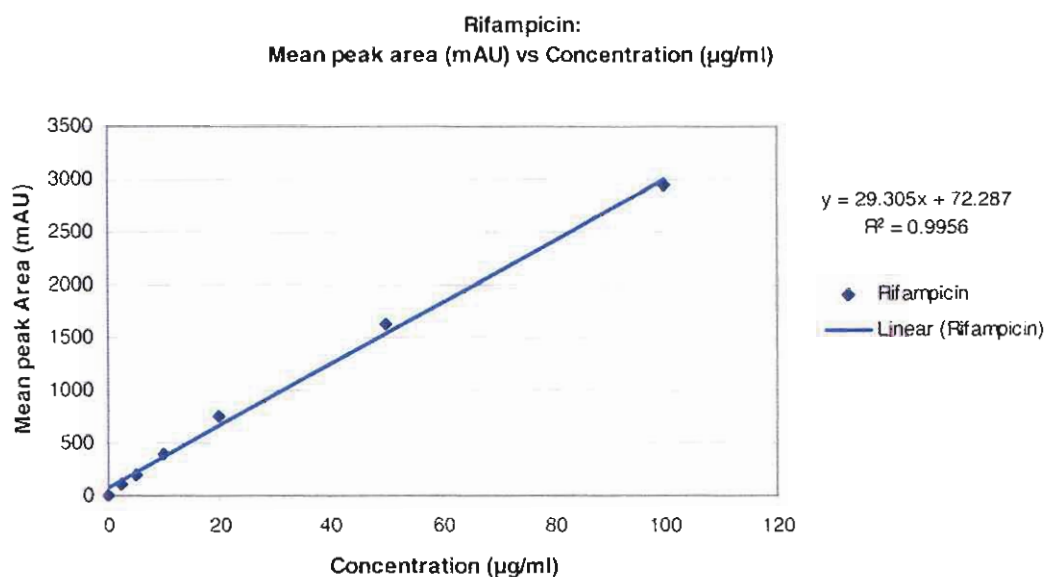


Figure 2: Linear regression curve for rifampicin standards (n = 6)

The R squared value (R^2) obtained for rifampicin indicates a high degree of linearity and therefore demonstrates the stability of the analytical system.

SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components such as impurities, degradation products or matrix which might be expected to be present.

The method is specific when samples do not contain any peaks that interfere with those of the drug substances under investigation.

None of the components of the Pheroid™ formulation or of the solvents used, interfered with the analyte peaks. Extra peaks formed during forced degradation did not interfere with the remainder of the analyte peaks. Figures 3 to 8 depict the HPLC chromatograms which illustrate above findings.

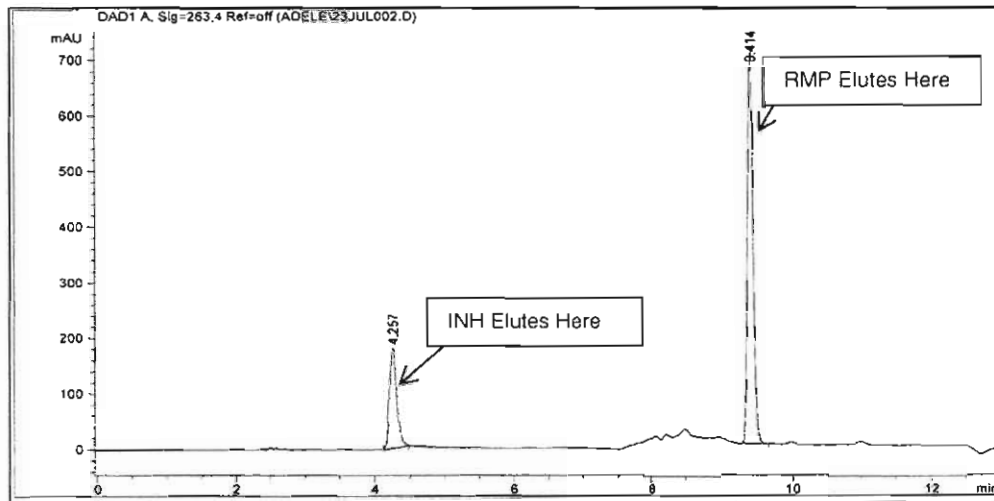


Figure 3: Chromatogram of a standard solution

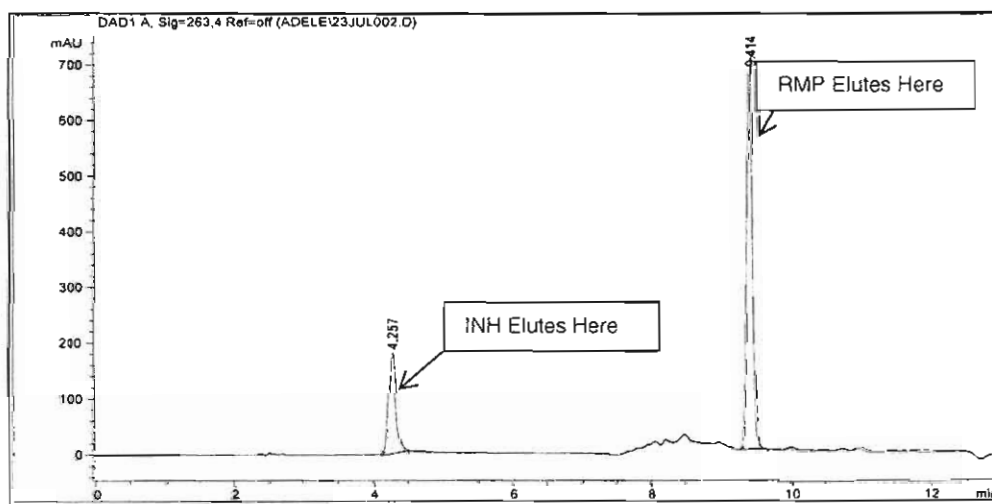


Figure 4: Chromatogram of a sample solution

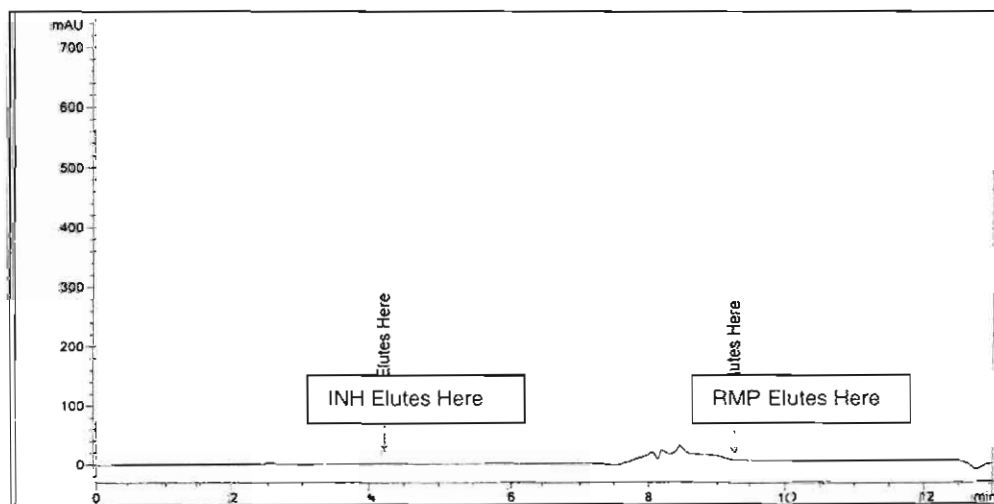


Figure 5: Chromatogram of a placebo solution

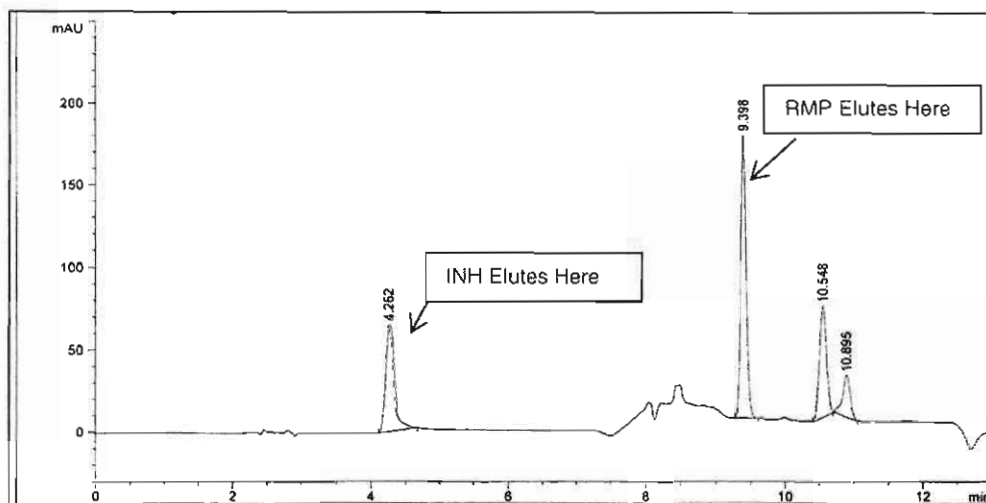


Figure 6: Chromatogram of a sample solution stressed in 0.1M hydrochloric acid at 40°C for 24 hours

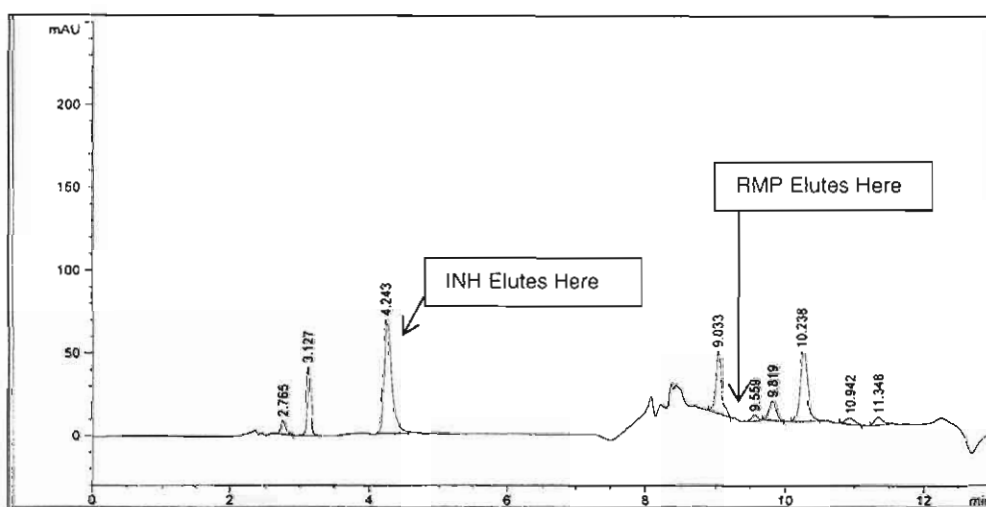


Figure 7: Chromatogram of a sample solution stressed in 0.1M sodium hydroxide at 40°C for 24 hours

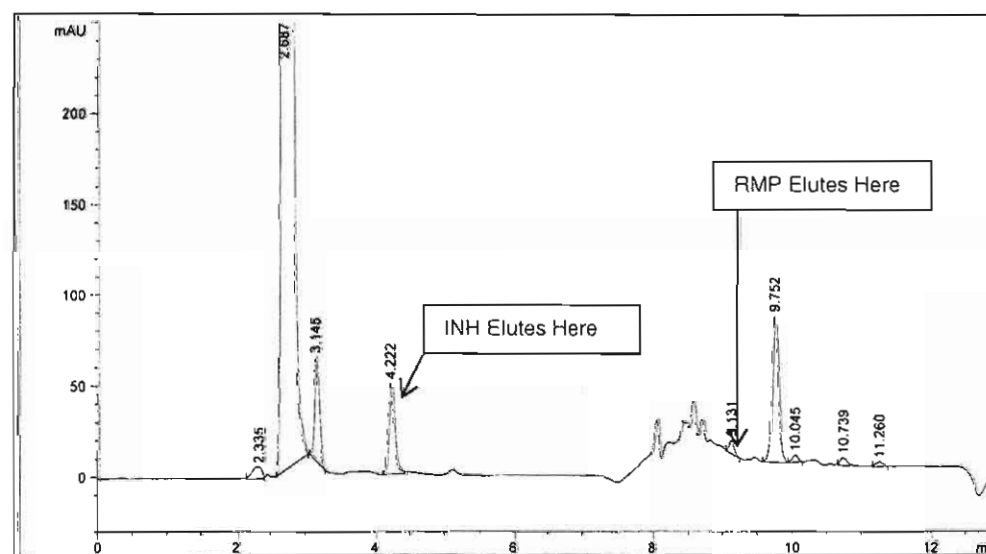


Figure 8: Chromatogram of a sample solution stressed in 10% hydrogen peroxide at 40°C for 24 hours

ACCURACY AND PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the same prescribed conditions.

Precision was investigated in terms of intra-day (repeatability) variation as well as inter-day (reproducibility) precision.

Intra-day precision (Repeatability)

Repeatability expresses the precision under the same operating conditions over a short period of time.

The intra-day precision was determined by performing HPLC analyses on three different samples ($n = 3$) with known concentrations: 5, 25, 50 $\mu\text{g/ml}$ and 10, 50, 100 $\mu\text{g/ml}$ for isoniazid and rifampicin respectively. Each sample was injected in duplicate and the procedure was repeated three times on that same day.

Table 7: Intra-day precision values for isoniazid

Isoniazid					
Concentration Spiked ($\mu\text{g/ml}$)	Area 1 (mAU)	Area 2 (mAU)	Mean	Conc. ^a ($\mu\text{g/ml}$)	%
5.02	112.70	112.89	112.79	4.86	96.94
5.02	112.43	111.51	111.97	4.83	96.23
5.02	111.72	112.83	112.28	4.84	96.49
25.08	560.14	563.58	561.86	24.22	96.57
25.08	559.44	564.73	562.09	24.23	96.61
25.08	563.86	560.97	562.41	24.24	96.67
50.15	1145.16	1153.92	1149.54	49.55	98.79
50.15	1144.39	1145.07	1144.73	49.34	98.38
50.15	1145.33	1157.03	1151.18	49.62	98.93
Mean					97.29
SD ^b					1.02
% RSD ^c					1.05

^a Conc. = concentration, ^b SD = standard deviation

^c % RSD = percentage relative standard deviation

Precision for isoniazid was satisfactory with a RSD of 1.05%.

Table 8: Intra-day precision values for rifampicin

Rifampicin					
Concentration Spiked ($\mu\text{g/ml}$)	Area 1 (mAU)	Area 2 (mAU)	Mean	Conc. ($\mu\text{g/ml}$)	%
10.01	389.58	388.10	388.84	10.00	99.96
10.01	387.58	386.27	386.92	9.95	99.47
10.01	384.01	382.35	383.18	9.86	98.50
50.03	1940.50	1941.49	1941.00	49.92	99.79
50.03	1938.49	1935.58	1937.03	49.82	99.59
50.03	1933.01	1932.18	1932.60	49.71	99.36
100.05	3882.88	3869.96	3876.42	99.70	99.65
100.05	3868.67	3860.99	3864.83	99.40	99.35
100.05	3866.35	3858.23	3862.29	99.34	99.29
Mean					99.44
SD					0.39
% RSD					0.39

Precision for rifampicin was satisfactory with a RSD of 0.39%.

Inter-day precision (Reproducibility)

The inter-day precision was determined by the HPLC analyses of three different samples ($n = 3$) each with a known concentration (50 $\mu\text{g/ml}$) isoniazid and (100 $\mu\text{g/ml}$) rifampicin. Samples were injected in duplicate into the HPLC and the procedure was repeated on three consecutive days.

Table 9: Inter-day precision values for isoniazid

Isoniazid	Day 1	Day 2	Day 3	Between days
	112.27	102.23	105.43	
	108.93	103.08	105.95	
	110.37	105.22	105.59	
Mean	110.52	103.51	105.66	106.56
SD	1.37	1.26	0.21	0.52
% RSD	1.24	1.21	0.20	0.49

Table 10: Inter-day precision Anova single factor statistics for isoniazid

SUMMARY				
Groups	Count	Sum	Average	Variance
Day 1	3	331.5714	110.5238	2.8025
Day 2	3	310.5261	103.5087	2.3672
Day 3	3	316.9674	105.6558	0.0692

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Days	77.5185	2	38.7593	22.1950	0.0017	5.1432
Within Days	10.4778	6	1.7463			
Total	87.9964	8				

Where SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Table 11: Inter-day precision values for rifampicin

Rifampicin	Day 1	Day 2	Day 3	Between days
	93.74	104.49	99.76	
	94.35	104.29	99.91	
	94.54	104.08	99.95	
Mean	94.21	104.29	99.87	99.46
SD	0.34	0.17	0.08	0.11
% RSD	0.36	0.16	0.08	0.11

Table 12: Inter-day precision Anova single factor statistics for rifampicin

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Day 1	3	282.6305	94.2102	0.1754
Day 2	3	312.8590	104.2863	0.0415
Day 3	3	299.6055	99.8685	0.0101

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Days	153.0629	2	76.5315	1011.5398	2.59E-08	5.1432
Within Days	0.4540	6	0.0757			
Total	153.5169	8				

The intra-day and inter-day variance was not significantly different. The accuracy and repeatability was within acceptable limits ($RSD \leq 2\%$).

STABILITY

A standard sample was prepared as described under standard preparation. The sample was left in the auto-sampler tray and reanalysed at hourly intervals up to 12 hours to determine the stability.

Table 13: Stability values for isoniazid

Isoniazid		
Time (hours)	Peak Area (mAU)	%
0	1179.18	100.00
1	1175.54	99.69
2	1178.42	99.94
3	1176.57	99.78
4	1177.09	99.82
5	1180.65	100.13
6	1179.37	100.02
7	1178.25	99.92
8	1178.58	99.95
9	1177.71	99.88
10	1176.88	99.81
11	1177.49	99.86
12	1172.76	99.46
Mean	1177.58	99.86
SD	1.89	0.16
% RSD	0.16	0.16

Table 14: Stability values for rifampicin

Rifampicin		
Time (hours)	Peak Area (mAU)	%
0	38580.30	100.00
1	38474.50	99.73
2	38435.50	99.62
3	38329.90	99.35
4	38382.40	99.49
5	38329.10	99.35
6	38143.50	98.87
7	38014.30	98.53
8	37968.20	98.41
9	37919.20	98.29
10	37775.60	97.91
11	37802.70	97.98
12	37674.00	97.65
Mean	38140.71	98.86
SD	288.42	0.75
% RSD	0.76	0.76

Both isoniazid and rifampicin proved to be stable over a period of 12 hours.

SYSTEM REPEATABILITY

System repeatability was determined by injecting a standard solution of (50 µg/ml) isoniazid and (100 µg/ml) rifampicin six times ($n = 6$) consecutively into the HPLC. The standard solution was prepared as described under standard preparation.

Table 15: Repeatability values for isoniazid

Isoniazid		
Sample	Peak area (mAU)	Retention Time (minutes)
1	1223.27	4.11
2	1221.73	4.10
3	1213.89	4.10
4	1216.45	4.11
5	1217.94	4.12
6	1217.64	4.11
Mean	1218.49	4.11
SD	3.15	0.01
% RSD	0.26	0.17

Table 16: Repeatability values for rifampicin

Rifampicin		
Sample	Peak area (mAU)	Retention Time (minutes)
1	3125.51	9.14
2	3116.92	9.15
3	3122.49	9.42
4	3126.55	9.42
5	3136.84	9.42
6	3141.82	9.43
Mean	3128.36	9.33
SD	8.46	0.13
% RSD	0.27	1.40

The retention times for isoniazid as well as rifampicin were within acceptable limits ($RSD \leq 2\%$).

CHROMATOGRAPHIC PERFORMANCE PARAMETERS

The chromatographic performance parameters were obtained by duplicate injection of a standard solution of (50 µg/ml) isoniazid and (100 µg/ml) rifampicin from which the relative standard deviation (RSD) was calculated. The number of theoretical plates and resolution between isoniazid and rifampicin peaks were calculated using the tangent method.

Table 17: Peak areas (n = 2) for isoniazid and rifampicin

	Isoniazid	Rifampicin
Peak area 1 (mAU)	1216.98	3155.93
Peak area 2 (mAU)	1219.84	3152.94
Mean	1218.41	3154.43
SD	1.43	1.49
% RSD	0.12	0.05

Table 18: Chromatographic performance parameters for isoniazid and rifampicin

	Isoniazid	Rifampicin
Retention time (minutes)	4.123	9.428
Relative standard deviation (n = 2)	0.12 %	0.05 %
Number of theoretical plates (N)/ column (tangent method)	9424	66876
USP tailing factor (T)	1.467	1.098
Capacity factor (k')	0.459	2.337
Resolution between peaks (R)	33.611	33.611

SYSTEM SUITABILITY

System suitability parameters were determined from the chromatographic performance parameters of the analyte peaks. The chromatographic procedure should be subjected to system suitability testing to verify the effectiveness of the final operating system.

The system is suitable to perform the analysis if the following criteria are met:

- Relative standard deviation (RSD) of two injections ≤ 2.0 %
- The column has more than 5000 and 30000 theoretical plates for isoniazid and rifampicin respectively
- The capacity factor (k') for rifampicin is ≥ 2.0

LIMIT OF QUANTIFICATION (LOQ)

The limit of quantification of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (RSD < 15%). The limit of quantification for isoniazid and rifampicin were determined as 0.125 and 0.25 $\mu\text{g/ml}$ respectively.

CONCLUSION

The method performed well and proved to be reliable and sensitive enough for the determination of the concentrations of isoniazid and rifampicin.

APPENDIX 5: LIST OF ABBREVIATIONS

AFB	-	Acid fast bacilli
BCG	-	Bacilli Calmette-Guèrin vaccine
CLSM	-	Confocal laser scanning microscopy
CTB	-	Cutaneous tuberculosis
DMSO	-	Dimethylsulfoxide
E	-	Ethambutol
h	-	Hours
HIV	-	Human immunodeficiency virus
HPLC	-	High pressure liquid chromatography
INH	-	Isoniazid
MAC	-	<i>M. avium</i> complex
MDR-TB	-	Multidrug resistant tuberculosis
MIC	-	Minimum inhibitory concentration
PCR	-	Polymerase chain reaction
pI	-	Isoelectric point
pKa	-	Ionisation constants
PZ	-	Pyrazinamide
RMP	-	Rifampicin
S	-	Streptomycin
SC	-	Stratum corneum
STD	-	Sexually transmitted disease
TB	-	Tuberculosis
TBVC	-	Tuberculosis verrucosa cutis
TG	-	Tuberculous gumma

APPENDIX 6: PHOTOS OF INSTRUMENTATION USED DURING DIFFUSION EXPERIMENTS AND ANALYSES



Photo 1: Amber Franz cell (donor & receptor compartments)



Photo 2: Franz cell stand, metal clamps, parafilm®, magnetic stirrer bars & Dow Corning® vacuum grease



Photo 3: Assembled Franz cells



Photo 4: Syringes used to withdraw the receptor compartment fluid



Photo 5: Grant water bath



Photo 6: Variomag® magnetic stirrer plate



Photo 7: Separations® amber HPLC vials
used to collect samples



Photo 8: Phenomenex®, Synergi Max RP-80A, 250 x 4.6 mm, 4 µm column



Photo 9: Agilent® series 1100 HPLC



Photo 10: Satorius® BP-211D
balance