Formulation, *in vitro* release and transdermal diffusion of isoniazide and rifampicin for dermal tuberculosis

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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 appendix containing relevant experimental data. The article contained in this dissertation is to be published in the International Journal of Pharmaceutics of which the complete guide for authors is included in the appendix.

ABSTRACT

Extrapulmonary tuberculosis makes up 10% of all tuberculosis cases and cutaneous tuberculosis (CTB) only a fraction of this 10%. CTB is caused by mainly *Mycobacterium tuberculosis* and can lead to scarring and deformities. The disease presents in different forms, from superficial granulomas to deeper ulceration and necrosis. Tissue cultures, polymerase chain reactions or purified protein derivative staining is used for the diagnosis of CTB (Barbagallo *et al.*, 2002:320).

Since the current treatment for CTB is oral anti-tubercular regimens and no topical treatment is available yet (Barbagallo *et al.*, 2002:320), this study aims to provide a topical preparation of isoniazide and rifampicin which will prevent the deformities and scarring caused by CTB and deliver quicker healing. This topical preparation is to be used in addition to oral treatment. Isoniazide and rifampicin are powerful first-line anti-tubercular drugs, active against both intra- and extracellular bacteria (SAMF, 2005:293).

Human skin is a resistant and protective barrier against the external environment and the stratum corneum is the main barrier against diffusion of compounds through the skin (Williams, 2003:9). The physicochemical characteristics (lipophilicity and molecular size) of neither isoniazide nor rifampicin are optimal for penetration of the stratum corneum and the skin-friendly Pheroid™ delivery system was incorporated in two of the formulations to investigate the possibility of improving drug delivery.

In this study the transdermal delivery of isoniazide and rifampicin was studied after formulation into four different topical preparations. The stability of these formulations were determined over a six month period under three different conditions of temperature and humidity (25°C/60% RH (relative humidity), 30°C/60% RH and 40°C/75% RH). Isoniazide and rifampicin were formulated into two Pheroid™ and two non-Pheroid™ spray formulations: lotion, Pheroid™ lotion, emulgel and Pheroid™ emulgel. Micrographs were taken with a confocal laser scanning microscope and it was seen that the formulations were homogenous and oil droplets were smaller than 10 µm, allowing permeation through skin.

Vertical Franz diffusion cells were used for *in vitro* permeation studies, with cellulose acetate membranes, for 12 h periods at pH 7.4, to determine drug release. The donor phase was the formulation, with 5 mg/ml of isoniazide and 10 mg/ml of rifampicin. The actives were released from the formulations and small concentrations penetrated the membranes. Release for isoniazide was best from the Pheroid™ emulgel and for rifampicin from the Pheroid™ lotion. Thus it can be concluded that the Pheroid™ improved drug release.

The diffusion study was repeated, substituting the membranes with female abdominal skin in order to investigate transdermal delivery. Isoniazide and rifampicin failed to permeate the skin from any of the formulations and no isoniazide or rifampicin could be found in the skin by means of tape stripping after 12 h.

Stability tests performed at 4, 8, 12 and 24 weeks was the determination of drug concentrations, pH, weight loss, viscosity, particle size, physical appearance and colour change tests. In these emulsion-type formulations, rifampicin proved to be more stable than isoniazide and after 24 weeks minimal concentrations of isoniazide (20.2 µg/ml) was left. The Pheroid™ formulations were proven to be more stable than the non-Pheroid™ formulations.

Keywords: Cutaneous tuberculosis, Transdermal delivery, Pheroid™, Vertical Franz cells, Diffusion study, Stability testing

UITTREKSEL

Van al die tuberkulose siektetoestande bestaan 10% daarvan uit ekstrapulmonêre siekte en slegs 'n klein persentasie van hierdie gevalle sal dermale tuberkulose (DTB) toon. DTB word hoofsaaklik veroorsaak deur *Mycobacterium tuberculosis* en kan lei tot erge littekenvorming. DTB kom in verskillende vorme voor; van oppervlakkige granulomas tot dieper ulserasie en nekrose. Die diagnose van 'n velletsel as DTB sluit in: weefsel kulture, polimerase-kettingreaksie of gesuiwerde proteienderivaatsmere (Barbagallo *et al.*, 2002:320).

Die huidige behandeling vir DTB, is die orale anti-tuberkulêre behandelings protokol wat ook vir pulmonêre tuberkulose gebruik word (Barbagallo *et al.*, 2002:320). Daar is nog geen topikale behandeling beskikbaar nie en daarom is die doel van hierdie studie om 'n formulering te verskaf wat die littekenvorming sal voorkom en vinniger herstel tot gevolg sal hê. 'n Topikale formulering van rifampisien en isoniasied behoort gebruik te word as bykomende behandeling tydens orale behandeling. Isoniasied en rifampisien is twee van die kragtigste eerste-linie anti-tuberkulose geneesmiddels en is aktief teen beide intra- en ekstrasellulêre bakterieë (SAMF, 2005:293).

Die vel bied weerstand en beskerming teen die eksterne omgewing en die stratum corneum is hoofsaaklik verantwoordelik vir die ondeurlaatbaarheid van die vel vir meeste geneesmiddels (Williams, 2003:9). Die fisies-chemiese eienskappe van isoniasied en rifampisien (lipofiliteit en molekulêre massa) bemoeilik penetrasie deur die stratum corneum en daarom word die Pheroid™ afleweringsisteem ingesluit in twee van die formulerings met die doel om moontlik geneesmiddelvrystelling te verbeter.

Die omvang van hierdie studie is om die transdermale aflewering van isoniasied en rifampisien deur middel van vier verskillende topikale formulerings te bestudeer en hulle stabiliteit te toets oor 'n tydperk van ses maande. Isoniasied en rifampisien is geformuleer in twee Pheroid™ en twee non-Pheroid™ sproeiformulerings: lotion, Pheroid™ lotion, emulgel en Pheroid™ emulgel. Mikrograwe was geneem met 'n konfokale laserskanderingsmikroskoop en dit het getoon dat die formulerings homogeen was en olie druppels kleiner as 10 µm, wat diffusie deur die vel moontlik maak.

Vertikale Franz selle is gebruik vir *in vitro* diffusiestudies deur gebruik te maak van sellulose asetaatmembrane, vir 12 h by pH 7.4 om geneesmiddel vrystelling vanuit die formulerings te bepaal. Die afsonderlike formulerings met 5 mg/ml isoniazide en 10 mg/ml rifampicin is gebruik as donor fase. Isoniasied en rifampisien is vrygestel uit die formulerings en klein

konsentrasies het deur die membraan gediffundeer. Vrystelling was die beste vir isonisied vanuit die Pheroid™ emulgel formulering en vir rifampisien vanuit die Pheroid™ lotion formulering.

Die diffusiestudie is herhaal deur die membrane te vervang met abdominale vel van 'n vroulike pasiënt om transdermale aflewering te ondersoek. Nie isoniasied of rifampisien kon deur die vel diffundeer vanuit enige van die formulerings nie en daar is geen geneesmiddel in die vel gevind met behulp van die "tape stripping" metode na 12 h nie.

Stabiliteitstoetse wat uitgevoer is op 4, 8, 12 en 24 weke behels geneesmiddelkonsentrasie bepaling, pH, massaverlies, viskositeit, deeltjiegrootte bepaling, fisiese voorkoms en kleurveranderinge. In hierdie emulsie-tipe formulerings was rifampisien meer stabiel as isoniasied en na 24 weke was daar minimale konsentrasies van isoniasied oor (20.2 µg/ml). Die Pheroid™ het die stabiliteit van die formulerings verbeter en die Pheroid™ formulerings was meer stabiel as hulle non-Pheroid™ ekwivalente.

Sleutelwoorde: Dermale tuberkulose, Topikale formulering, Pheroid™, Vertikale Franz selle, Diffusiestudie, Stabiliteitstoetse

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List of Abbreviations

AFB	-	Acid-fast bacilli
BCG	-	Bacilli Calmette-Guérin vaccine
BHT		Butylaled hydroxytoluene
CLSM		Confocal light scanning microscopy
CTB	-	Cutaneous Tuberculosis
EIB	-	Erythema induratum of Bazin
h	-	Hours
HIV	-	Human immunodeficiency virus
HPLC	-	High pressure liquid chromatography
INH	-	Isoniazide
MAC	-	Mycobacterium Avium Complex
MDR-TB	-	Multidrug resistant tuberculosis
MIC	-	Minimum inhibitory concentration
o/w	-	Oil in water emulsion
w/o	-	Water in oil emulsion
PBS		Phosphate buffer solution
pKa	-	Ionisation constant
PPD	-	Purified protein derivative
PCR	-	Polymerase chain reaction
RIF	-	Rifampicin
TB	-	Tuberculosis
TBVC		Tuberculosis verrucosa cutis

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

Tuberculosis (TB) is one of the most dangerous common infectious diseases of modern time. The World Health Organisation (WHO) estimated 9.27 million cases of TB and 1.76 million deaths in 2007, 456000 deaths was among HIV (human immunodeficiency virus)-positive patients and 1.3 million among HIV-negative patients (WHO, 2009:2). Of all these cases approximately 10 % present with extrapulmonary TB and only a small percentage of these cases present with cutaneous TB (CTB). CTB is caused by *Mycobacterium tuberculosis, Mycobacterium bovis* and the Bacilli Calmette-Guérin (BCG) vaccine (Barbagallo *et al.*, 2002:320) and the disease can cause permanent deformities especially when there are ulcerations on the face.

In order for a lesion to be diagnosed as CTB, special staining, tissue culture or polymerase chain reaction (PCR) must be performed to indicate the presence of *Mycobacterium tuberculosis*. CTB presents in various forms, from the superficial dermal granulomas around sweat glands, to the ulceration of the lymph nodes, nose and mouth (Barbagallo *et al.*, 2002:320).

Current treatment for CTB is the same oral regimen as for pulmonary TB, since the majority of patients have systemic disease as well (Barbagallo *et al.*, 2002:320). No topical treatment is available yet and therefore the purpose of this study is to provide a topical preparation for the anti-tuberculids by use of isoniazide and rifampicin in order to deliver quicker healing and prevent the deformities caused by CTB. This topical preparation should be used in conjunction with oral treatment. Isoniazide and Rifampicin, two of the most powerful anti-tubercular drugs, are bactericidal for intra- and extracellular bacteria and are used as part of the first-line treatment regimen for TB. (SAMF, 2005:293).

Successfully formulating a transdermal product holds many challenges; the greatest being the skin's barrier function. The protein and lipid composition of the stratum corneum greatly contributes to this, making it very difficult to penetrate the skin (Williams, 2003:9). In order for a particle to cross the skin it has to be smaller than 10 µm in diameter (Barry, 2002:31) and have both hydrophilic and lipophilic characteristics (Schalla & Schaefer, 1981:42).

In this study, the Pheroid™ drug delivery system will be implemented to formulate isoniazide and rifampicin into a topical preparation. It was previously proven that Pheroid™ can penetrate skin and thus enhance the transdermal delivery of entrapped compounds (Grobler, 2004:4). *In vitro* Franz cell diffusion studies will be performed with human skin to investigate the ability of the actives to permeate the skin and with cellulose acetate membranes to determine drug release from the formulations. Tape stripping of the skin after the diffusion studies will be performed to determine the amount of actives in the different layers of the skin. The stability of the these formulations and their actives will be studied by use of six month stability tests, including concentration assays, determination of particle size, pH, weight loss, viscosity, physical appearance and colour changes.

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CHAPTER 2: TOPICAL DELIVERY OF ISONIAZIDE AND RIFAMPICIN

2 INTRODUCTION

Transdermal delivery and the advantages it holds over other routes of administration have been widely researched over the past few decades (Kumar & Philip, 2007:634). The barrier function of the stratum corneum is the limiting factor in transdermal delivery and a compound needs to possess the right physicochemical characteristics to be able to penetrate it.

In this chapter discussions will be given about the pathology, history classification, diagnosis and treatment of CTB. Skin structure and function will be reviewed and transdermal delivery of compounds and the role of the Pheroid™ delivery system will be described.

2.1 CUTANEOUS TUBERCULOSIS (CTB)

2.1.1 History of CTB

At the end of the 19th century cutaneous tuberculosis (CTB) became a major problem as the incidence of pulmonary tuberculosis (TB) was rising. Fortunately, due to improved hygiene and living standards, Bacilli Calmette-Guérin vaccine (BCG) immunisation and the implementation of anti-tubercular treatment, pulmonary TB started to decline. Thus CTB also declined as only a very small percentage of pulmonary TB patients presented with CTB. Patients with immunosuppression from immunosuppressive or chronic corticosteroid therapy, cancer or malnutrition are more likely to develop CTB. More recently CTB re-emerged due to multi-drug resistant pulmonary TB and the rapid increase of human immunodeficiency virus (HIV) cases (Barbagallo *et al.*, 2002:320).

2.1.2 Microbiology of CTB

Infection with *Mycobacterium tuberculosis, Mycobacterium bovis* and the BCG vaccine is responsible for CTB (Barbagallo *et al.*, 2002:320). They are all acid-fast bacilli (AFB) with low pathogenicity, implying that the patient's immune system has to be somewhat impaired in order for them to cause disease (Barbagallo *et al.*, 2002:320).

Mycobacterium tuberculosis is an aerobic organism that multiplies slower than other bacteria and thus TB evolutes slower than most bacterial infections (Varaine *et al.*, 2008:15).

2.1.3 Classification of CTB

CTB is classified as either multibacillary or paucibacillary (Table 2.1). Multibacillary forms of CTB present with multiple mycobacteria in the skin as demonstrated by Ziehl-Nielson stained material or cultured biopsy material of the skin. Paucibacillary forms present with very few mycobacteria in the skin and when fully developed are described as a tuberculid. True tuberculids are the following: Erythema induratum of Bazin (EIB); papulonecrotic tuberculid; lichen scrofulosorum; lupus milianis disseminatum fasciei and granulomatis mastitis (Bravo & Gotuzzo, 2007:174).

Table 2.1: *Classification of CTB (Bravo & Gotuzzo, 2007:174).*

Multibacillary:	Paucibacillary:
Direct inoculation ➢ Primary inoculation TB (tuberculous chancre)	**Direct inoculation (Re-exposure)** ➢ TB ➢ *Verrucosa cutis* ➢ Lupus vulgaris
Continuity ➢ Scrofuloderma ➢ TB ➢ *Periorificialis*	**Hematogenous spreading** ➢ Lupus vulgaris
Hematogenous spreading ➢ Acute miliary TB ➢ Gumma (cold abscess)	

2.1.5 Pathophysiology of acquired true CTB

CTB can be contracted exogenously or by the spread of a systemic TB infection. As stated in table 2.1 infection can be caused by direct inoculation, inoculation with re-exposure, (usually on broken skin), or by spreading of the already existing TB condition.

2.1.5.1 Pathophysiology of exogenously acquired true CTB

2.1.5.1.1 Primary inoculation TB (Tuberculous chancre)

In these cases the patient is usually a health care worker, care giver or family member of a pulmonary TB patient or a child that did not receive BCG immunisation. There are also some cases of tuberculous chancre after mouth-to-mouth resuscitation, circumcision, tattooing and piercing. These patients are thus non-sensitised (Barbagallo *et al.*, 2002:320).

A tuberculous chancre is acquired through accidental exposure to TB bacilli at entry points, caused by trauma to the skin. The chancre is usually situated on the face, hands or feet (Figure 2.1) and develops into a painless, reddish-brown nodule or papule which ulcerates 2-4 weeks after exposure. Similar to the Ghon complex in pulmonary TB, non-tender lymphadenopathy is present (Figure 2.1). Without anti-TB treatment these lesions leave scars and can take up to 6 months to heal (Bravo & Gotuzzo, 2007:174-175).

A diagnosis can be made by histology sections or smears from the ulcer showing AFB and it is confirmed by a positive culture for AFB. The purified protein derivative (PPD)-test is negative at first, but positive when repeated later on in the disease (Barbagallo *et al.*, 2002:321).

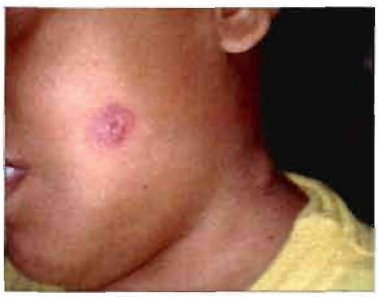

Figure 2.1: Illustration of primary inoculation TB with lymphadenopathy. (Bravo & Gotuzzo, 2007:175).

2.1.5.1.2 Tuberculosis verrucosa cutis (TBVC)

TBVC occurs where patients were previously infected and are re-infected with the mycobacterium. Trauma to the skin creates entry points for mycobacteria and it is acquired accidentally when handling autopsy material or infected patients (Barbagallo *et al.*, 2002:322).

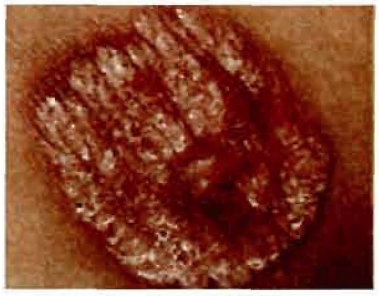

Figure 2.2: Illustration of TBVC (Barbagallo *et al.,* 2002:321).

TBVC presents as a single, slow-growing, painless verrucose plaque on the hand or foot (Figure 2.2) affecting both children and adults. When enlarged it resembles a common wart that is soft in the centre and firm on the outskirts with fissures and clefts on the surface, expressing pus and keratinous material. Lesions can also present on the face or around the anus, with the same verrucose plaque. *M. bovis* causes a similar infection, especially affecting ranch and cattle workers, resulting in a strong positive PPD test result.

A diagnosis can be made with a biopsy revealing non-caseating, ill-defined tuberculous granulomata in the dermis and hyperkeratosis and papillomatosis in the epidermis (Barbagallo *et al.*, 2002:321). Mycobacteria are not always present. Anti-TB treatment can also act as diagnosis, as the lesions, even after becoming chronic, stay sensitive to it. If left untreated the lesions can take years to heal, leaving atrophic scars.

2.1.5.2 Pathophysiology of endogenously acquired true CTB

2.1.5.2.1 Lupus vulgaris

In India, Pakistan and Tunisia this is the predominant form of CTB, more commonly affecting women. Facial lupus (Figure 2.3.a) is caused by haematogenous spread and lesions on the hands or feet by re-inoculation (Figure 2.3.b).

Lupus vulgaris presents as a soft, red-brown plaque with an "apple jelly" appearance, atrophy in the centre and an elevated verrucose border. Numerous coalescent micro-papules or "lupomes" enlarge to form a slowly growing plaque.

Facial lupus vulgaris causes severe scarring and deformation of facial features. Exophytic growth can be a complication if the lesion ulcerates at the centre, which happens especially on the nose.

Lesions on the earlobe can appear to be pseudo-tumoral. A biopsy would reveal numerous non-caseating granulomas with few AFB and PPD test would be positive (Bravo & Gotuzzo, 2007:177).

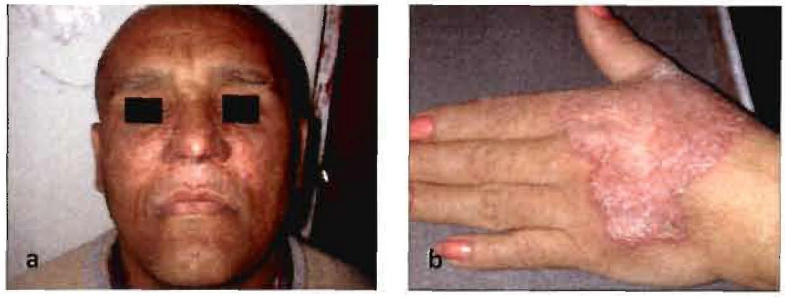

Figure 2.3: *Illustration of* **a)** Lupus vulgaris on the face and **b)** Lupus vulgaris on the hand (Bravo & Gotuzzo, 2007:177).

2.1.5.2.2 Acute disseminated miliary TB

This form of cutaneous TB is acquired when active pulmonary TB spreads from the internal focus, through the blood stream to the skin or it can be caused by viral exanthema infection. In HIV patients with TB, the immune suppression together with ineffective anti-TB treatment and multi-drug resistant organisms are the cause of haematogenous dissemination of TB to the organs and skin. Acute disseminated miliary TB presents as a rash of needlepoint papules with small vesicles on top that rupture and dry to form a crust. A diagnosis can be made with a biopsy confirming multiple AFB and micro-abscesses. Because of a lack of response to the antigen, the PPD test is usually negative (Barbagallo *et al.*, 2002:324).

2.1.5.2.3 Scrofuloderma

In developing countries scrofuloderma is a very common form of CTB amongst children and the elderly. Systemic symptoms and pleural or pulmonary disease may be present. Scrofuloderma is caused by spreading of the disease from a lymph node or bone structure that is infected; people can also become infected with *M. bovis* by consuming unpasteurised milk. An ulcer with keloid tissue forms on the skin of the underlying infected structure, resulting in an abscess and areas of wedge shaped necrosis. Scrofuloderma affects mostly the neck, groin, chest wall and axillae areas (Figure 2.4) (Bravo & Gotuzzo, 2007:175). Biopsy results reveal a strong positive AFB and the PPD test. Histology reveals granuloma in the lower dermis (Barbagallo *et al.*, 2002:323).

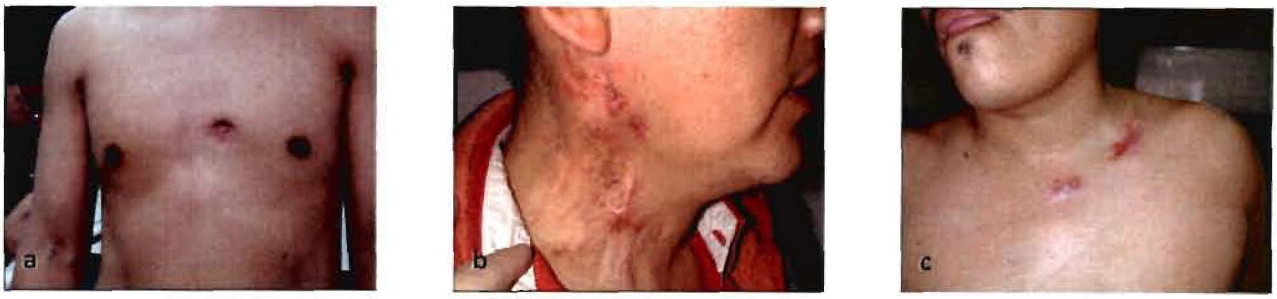

Figure 2.4: Illustration of scrofuloderma of the chest **(a)**, neck **(b)** and clavicular area **(c)** (Bravo & Gotuzzo, 2007:175).

2.1.5.2.4 Metastatic tuberculosis abscess (Tuberculous gumma)

In these cases, latent mycobacteria are aggravated by malnutrition or a compromised immune system. The mycobacteria are haematogenously spread and manifests as a cold abscess/gumma on the trunk, hands or feet (Figure 2.5.a)). Mycobacteria are also present in the infiltrate. If the patient is in poor general condition, the tuberculin test might be negative, though it is normally positive (Bravo & Gotuzzo, 2007:176).

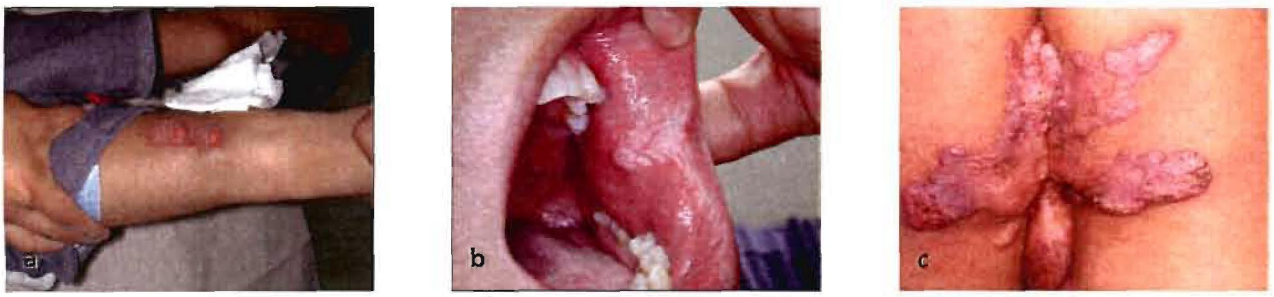

Figure 2.5: Illustration of **a)** gumma, **b)** oral secondary TB as expression of periorificial disease (Bravo & Gotuzzo, 2007:176) and **c)** periorificial TB around the anus (Bhutto, 2007:23).

2.1.5.2.5 Tuberculosis periorificialis

This rare form of cutaneous TB commences after the aggravation of an active focus in underlying tissue. It is secondary to pulmonary TB and middle-aged men are most commonly affected. The lesion presents as a painful ulcer or a lupus vulgaris-like plaque in the buccal mucosa (Figure 2.5.b)) and can also affect other regions, such as around the anus (Figure 2.5.c)), vulva or

penis. Biopsies reveal tuberculous granulomata and numerous AFB are present. PPD tests may be negative, but mycobacteria are normally present in tissue cultures.

2.1.5.3 Tuberculids

2.1.5.3.1 Papulonecrotic tuberculid

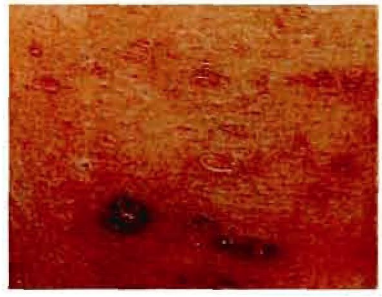

Figure 2.6: Illustration of papulonecrotic tuberculid (Barbagallo *et al.*, 2002:325).

This form of CTB is a paucibaccilary disseminated infection and it is haematogenously spread. It most commonly affects children and young adults with already existing active TB. The lesion presents as multiple papules on the hands or feet and sometimes on the lower abdomen, buttocks, trunk or earlobes (Figure 2.6). It is 1-5 mm in diameter, with central necrosis and results in variceliform scars. Biopsies reveal intradermal coagulative necrosis together with follicular necrosis. A combination with the lesions of EIB can sometimes be found. No bacilli can be found, but the tuberculin test is still positive (Bravo & Gotuzzo, 2007:177).

2.1.5.3.2 Lichen scrofulosorum

This form of cutaneous TB is secondary to already existing TB of the lung, lymph nodes or bone. It most commonly affects children under the age of 16, presenting as clusters of small follicular or parafollicular papules on the trunk. Though no bacilli are present in the lesion, PPD test results are strongly positive. Up to 70 % of the patients have a history of BCG vaccination and lichen scrofulosorum can also present after *M. avium* infections. Lesions start to heal only after four weeks of anti-TB treatment and can take up to a year to fully recover (Bravo & Gotuzzo, 2007:178-179).

2.1.5.3.3 Erythema induratum of bazin (EIB)

According to Bravo & Gottuzo (2007:178) this is 'the most commonly reported form of tuberculid nowadays. It presents as ulcerated nodules on the back of the legs (Figure 2.7) and affects mostly female patients with active pulmonary disease. A PPD test is strongly positive and histology should show 3 of the following 4 elements: fat necrosis, vasculitis, septal panniculitis and granuloma formation (Bravo & Gotuzzo, 2007:178).

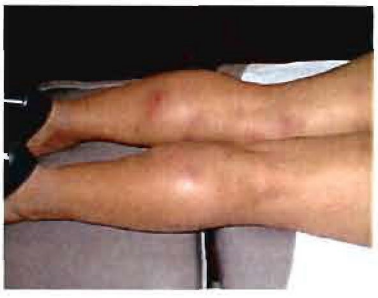

Figure 2.7: Illustration of EIB (Bravo & Gotuzzo, 2007:178).

2.1.5.3.4 Nodular granulomatous phlebitis

This uncommon tuberculid affects patients without active pulmonary disease, presenting as subcutaneous nodules on the front and insides of the legs along the veins. The lesion does not ulcerate as is the case with EIB. Histology shows epitheloid cell granulomas and Langerhans giant cells in the walls of the cutaneous veins (Barbagallo *et al.*, 2002:326).

2.1.5.3.5 Granulomatis mastitis

TB mastitis presents as nodules or ulcers on one breast of a female patient with a history of TB infection and can be seen as EIB of the breast (Figure 2.8.a)). Tissue Biopsies reveal tuberculous granulomas, fat necrosis and a positive PPD test, but no bacilli can be found. Breast carcinoma should be ruled out by means of biopsy (Bravo & Gotuzzo, 2007:179).

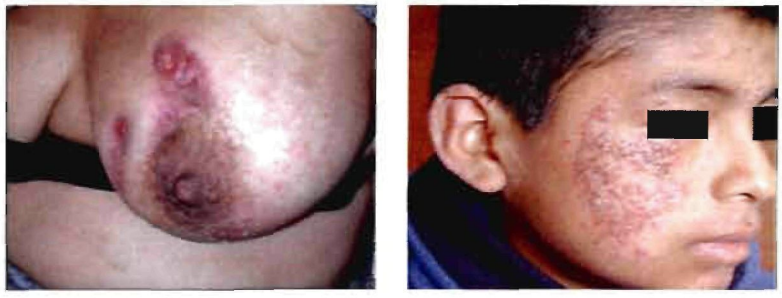

Figure 2.8: Illustration of **a)** tuberculous mastitis and **b)** acneiform lupus miliaris (Bravo & Gotuzzo, 2007:178).

2.1.5.3.6 Lupus miliaris disseminatus fasciei

This form of cutaneous TB presents as acne-like papulonecrotic or varicelliform lesions on the face of children or adults in areas of high TB prevalence (Figure 2.8.b)). PPD test results are positive and AFB can be found. Tissue biopsies reveal tuberculous granulomas and caseating necrosis (Bravo & Gotuzzo, 2007:179).

2.1.6 Diagnosis of CTB

The clinical picture should be considered, together with patient history, epidemiological data and PPD test results. Skin biopsies should be performed and the samples stained and cultured. A PPD test with a very positive result (>15 mm in diameter) is diagnostic. In lesions with a high bacillary load, such as scrofuloderma, tuberculous gumma and disseminated military TB, a smear for AFB is very helpful. A PCR test is very quick and valuable to identify *M. tuberculosis*, especially in tuberculid and granulomatous lesions (Barbagallo *et al.*, 2002:326-327).

2.1.7 Current Treatment Regimens for CTB

CTB should be treated with a multiple drug regimen for a period of time that would ensure the elimination of all viable organisms in order to prevent the re-emergence of disease and the developing of resistant strains. As the bacillary load of CTB is significantly smaller than for pulmonary disease, treatment with pulmonary anti-tubercular regimens is sufficient.

The Centre for Disease Control and Prevention suggests a two phase treatment: for the first 8 weeks - isoniazide, rifampicin, pirazinamide and either ethambutol or streptomycin daily, then for

16 weeks - isoniazide and rifampicin daily or 2-3 times a week if the patient can be directly observed. Treatment should not be stopped if the lesions have healed.

It might be necessary in some cases of scrofuloderma, TBVC or lupus vulgaris to have the lesion surgically removed together with the anti-tubercular treatment. In the case of lupus the nodules within the scars can be removed with cryotherapy (Barbagallo *et al.*, 2002:327).

2.2 TREATMENT WITH ANTI-TUBERCULAR ISONIAZIDE AND RIFAMPICIN

2.2.1 Introduction

The current treatment for CTB is the same oral regimen as for pulmonary TB and no topical treatment is available yet (Barbagallo *et al.*, 2002:327). A topical treatment would be ideal as complimentary to the oral treatment of patients with CTB to improve the topical effect and healing time.

Isoniazide and Rifampicin are two of the most active anti-tubercular agents and are used as first-line treatment in combination with pyrazinamide and ethambutol. Both actives are bactericidal for intra- and extracellular bacteria (SAMF, 2005:293).

2.2.2 Isoniazide

Isoniazide is the hydrazide of isonicotinic acid and was designed after chemical studies in 1945 found isonicotinic acid to have anti-tubercular properties. Isoniazide is tuberculostatic at a minimal concentration of 0.025-0.050 µg/ml (Petri, 2001:1274). It inhibits the synthesis of the mycobacterial cell wall selectively by inhibiting mycolic acids that are unique to mycobacteria (Petri, 2001:1275).

2.2.3 Rifampicin

Rifampicin is a complex macrocyclic antibiotic and a semi-synthetic derivative of the rifamycins produced by *Streptomyces mediterranei* (Petri, 2001:1277). Rifampicin is tuberculostatic at concentrations of 0.005-0.200 µg/ml and inhibits mycobacterial RNA polymerase which then inhibits RNA synthesis (Petri, 2001:1278).

2.3 HUMAN SKIN AND ITS APPENDAGES

2.3.1 Introduction

The human skin is the largest organ, protecting our bodies from harmful external stressors, critically maintaining homeostasis of the body and it is also a sensory organ (Tobin, 2006:52). It is a complex metabolically active organ, with immunological and histological responses and it has the ability to constantly regenerate itself (Aulton, 1996:384-385). The surface area of adult skin is about 2 m^2, it is 2.5 mm thick, has a density of 1.1 and weighs 6 % of our body mass (Tobin, 2006:52).

2.3.2 Functions

2.3.2.1 Mechanical function

The dermis is the main component contributing to the mechanical properties of the skin. The human skin is supple and certain factors influence its elasticity, including age and moisture levels (Aulton, 1996:384).

2.3.2.2 Barrier function

2.3.2.2.1 Microbiological barrier

The stratum corneum acts as a microbiological barrier as it is difficult to penetrate. Glandular excretions contain short chain fatty acids which inhibit bacterial and fungal growth (Aulton, 1996:384).

2.3.2.2.2 Chemical barrier

Chemicals penetrate the stratum corneum with great difficulty, making this the rate-limiting step in transdermal absorption. Only a small area for absorption is made up of the transappendageal route (about 0.1 %), and the stratum corneum's resistance to diffusion is a great obstruction for the penetration of actives through intact skin (Aulton, 1996:384).

2.3.2.2.3 Radiation barrier

The skin has an acute and chronic reaction against sunlight. The most damage is caused by ultraviolet light of 290-400 nm. Erythema, pigmentation and epidermal thickening are acute reactions and ageing, pre-malignancy and malignancy are chronic reactions following long-term exposure. When the skin is exposed to sunlight melanocytes are activated to produce melanin, which darkens the skin and protects it from further damage (Aulton, 1996:385).

2.3.2.2.4 Heat barrier and temperature regulation

Because the stratum corneum is so thin it does not effectively insulate the body and internal organs against extreme cold or heat, but the skin is responsible for regulating body temperature to keep it at 37°C. To keep warm, blood supply to the skin is minimized in order to prevent surface heat loss. Goosebumps form when the hair rises to entrap a layer of heat on the skin surface, warming the body. To lose heat the blood vessels in the skin will dilate and sweat is produced to cool the body. A dilute saline solution is secreted by the eccrine sweat glands and when water evaporates from the skin it creates a cooling effect (Aulton, 1996:385).

2.3.2.2.5 Mechanical barrier

Acute impact on the skin results in bruises or blisters. Friction causes blisters or epidermal thickening and chronic friction will result in corns or callosities (Aulton, 1996:385).

2.3.3 Structure

Human skin can be divided into multiple layers, with the main four being:

- Hypodermis (subcutaneous fatty layer)
- Dermis
- Viable epidermis
- Stratum corneum (non-viable epidermis)

2.3.3.1 Hypodermis (subcutaneous fatty layer)

This is the deepest layer of skin, closest to the internal body parts and underneath the dermis. In most parts of the body it is thick – up to several centimetres, but can also be absent as seen on the

eyelids, penis, scrotum, nipple and areola. The hypodermis protects the body against physical shock, serves as insulator and is a source of energy. It also supplies nerves and blood vessels to the skin and joins the skin to connective tissue and the deeper situated body structures (Williams, 2003:2).

2.3.3.2 Dermis

The dermis is 3-5 mm thick. Its ground substance is mucopolysaccharride and it contains collagen, elastin and reticulin (fibrous proteins), which make up the connective tissue of the skin. Collagen provides support to the skin and elastin provides its flexibility. Nutrients are carried to the skin by blood vessels and waste products are removed by the lymphatic vessels. Nerve endings, eccrine and apocrine sweat glands and hair follicles are imbedded in the dermis. Arterial blood supply lies within 0.2 mm of the skin surface, enabling percutaneous absorption (Aulton, 1996:383-384).

2.3.3.3 Epidermis

The thickness of the epidermis ranges from 0.006 mm on the eyelids to 0.8 mm on the palms and soles (Aulton, 1996:383). The epidermis consists mainly of keratinocytes, which differentiate towards the skin surface. The four layers of the epidermis are the stratum basale/stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum (McGrath *et al.*, 2004:7). The lipids of the stratum corneum attribute to the hydrophilic-lipophilic character of the epidermis, indicating that only compounds with hydrophilic and lipophylic characteristics can cross the skin (Schalla & Schaefer, 1981:42). Rifampicin is highly lipophylic (Sousa, 2008:873) and isoniazide highly hydrophilic (Stephan *et al.*, 2004:4167) and by incorporating the Pheroid™ drug delivery system the permeability of the skin might be improved.

2.3.3.3.1 Stratum basale/stratum germinativum

This is the deepest layer of the epidermis. It is only 2-3 cell layers thick and consists of 10-14 nm cuboidal cells with large nuclei and many ribosomes (McGrath *et al.*,2004:7).

2.3.3.3.2 Stratum spinosum

The stratum spinosum lies just above the stratum basale and is made up of spinous/prickle cells. Together the stratum basale and stratum spinosum is called the Malpighian layer (McGrath *et al.*, 2004:8).

2.3.3.3.3 Stratum granulosum

The stratum granulosum is the granular layer superior to the stratum spinosum. It contains intracellular keratohyalin granules and cytoplasmic lamellar granules. The latter is also present in the stratum spinosum and contains lipids which are important for intercellular cohesion and the barrier function of the skin (McGrath *et al.*, 2004:8).

2.3.3.3.4 Stratum corneum

The stratum corneum is the outermost layer of the epidermis and is approximately 10-15 µm thick (Kumar & Philip, 2007:634). Cells of this layer are flat corneocytes without nuclei or cytoplasmic organelles. It contains keratin and fillagrin, which enables keratin filament aggregation. Corneocytes have highly insoluble plasma membranes, making penetration more difficult and thus contributing to the barrier function of the skin (McGrath *et al.*, 2004:8).

The stratum corneum barrier is dependent on the balance of ceramides, cholesterol and fatty acids in the skin. UV-damaged and aged skin contains mostly cholesterol, skin with atopic dermatitis mostly ceremides and psoriatic skin mostly fatty acids, all imbalances which affect the skin's barrier function (Tobin, 2006:55).

2.3.3.4 Appendages

Human skin has three main appendages:

- Hair follicles over all skin, except palms, soles and lips.
- Sebaceous glands that secrete sebum for the follicles, moisturise the skin and maintain skin pH at approximately 5.
- Eccrine glands that secrete sweat and apocrine glands that secrete lipoidal and protein secretions (Williams, 2003:4).

2.3.4 Advantages and limitations of transdermal delivery

2.3.4.1 Advantages

- The first-pass metabolism is avoided.
- Fewer side effects, due to lower plasma levels.
- Plasma levels are more stable.

- Drugs with a short half-life and low therapeutic index can be delivered safely and effectively.
- Less toxic and drug delivery can easily be stopped.
- Fewer doses and improved patient compliance (Kumar & Philip, 2007:634).

2.3.4.2 Limitations:

- Drugs with a high molecular weight (>500 Da) cannot cross the stratum corneum.
- Systemic circulation cannot be reached by drugs with a very low or very high partition coefficient.
- Drugs with poor solubility and high melting point cannot cross the stratum corneum (Kumar & Philip, 2007:634).
- Drug release and absorption is affected by the type of base used in the formulation.
- The skin's permeability varies with anatomic site, gender, age and condition (Shargel *et al.*, 2005:372).

2.3.5 Routes of transdermal absorption

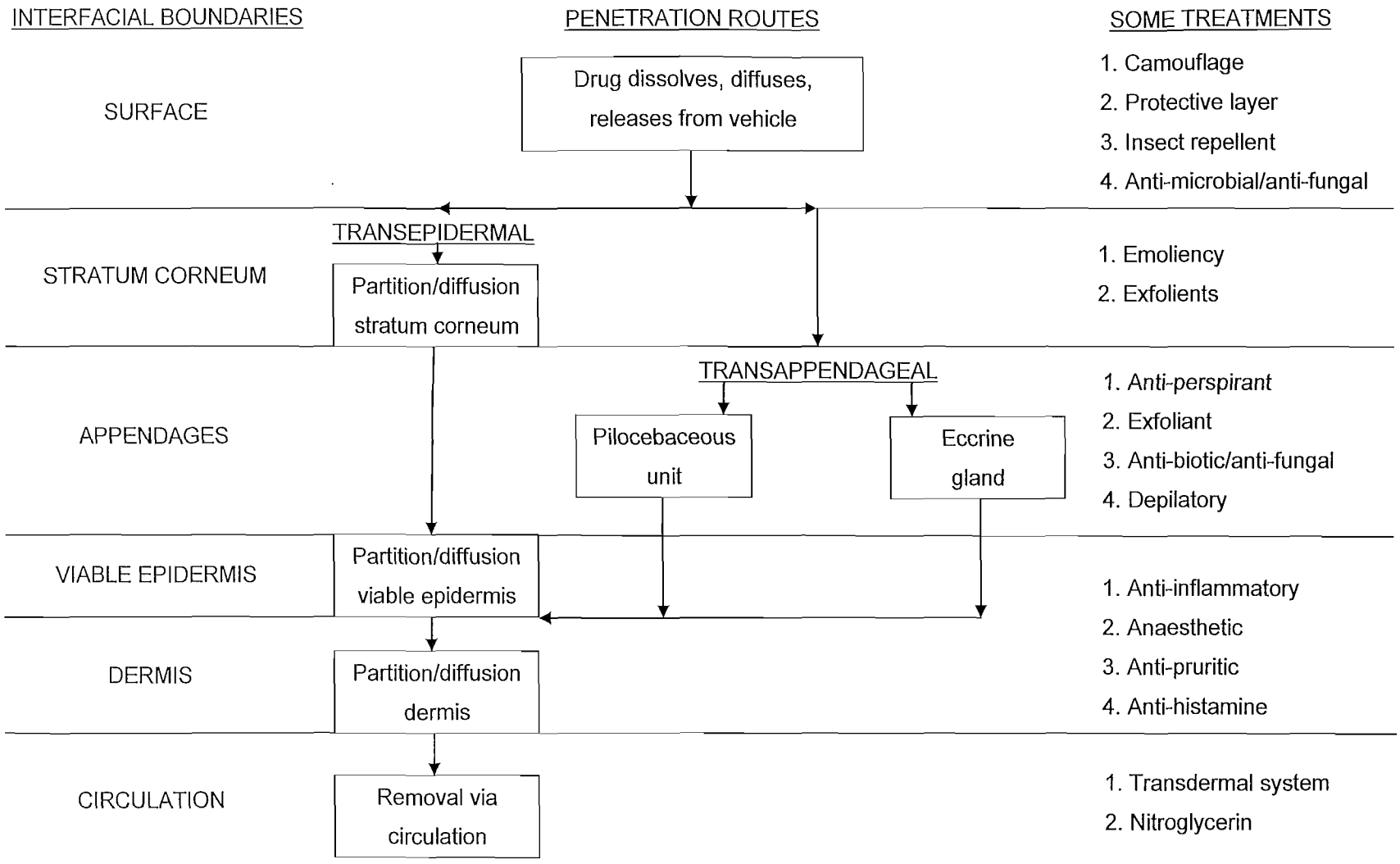

Figure 2.9: Schematic illustration of the transdermal delivery routes and treatment examples (Aulton, 1996:386)

2.3.6 Factors that influence transdermal absorption

In order to produce a clinical result the active ingredient has to be released from the dosage form, it has to cross the skin barriers and it has to activate a pharmacological response. This will be influenced by the skin, the drug and its vehicle and other biological and physicochemical factors (Aulton, 1996:391).

2.3.6.1 Biological factors

2.3.6.1.1 Skin age

Adults have less permeable skin than babies, children or the elderly. Because the body surface of children are much bigger than that of adults, medicines and chemicals can become toxic at much lower levels (Aulton, 1996:392).

2.3.6.1.2 Skin condition

Damage to the skin influences its barrier function. Acids and alkalis promote penetration, as do cuts, abrasions and dermatitis. Permeation enhancers such as dimethylsulphoxide improve transdermal absorption. Inflammation, altered keratinisation and loss of stratum corneum due to disease increase permeability. Penetration is reduced with frequent contact with irritants, thickening with corns and calluses or ichthyosis (Aulton, 1996:392).

2.3.6.1.3 Regional skin sites

Permeability varies from person to person, depending on the stratum corneum thickness and density of skin appendages. Diffusivity for the different skin regions is as follows: plantar > palmar and dorsum of hand > scrotal and post auricular > axillary and scalp > arm > leg > trunk (Aulton, 1996:393).

2.3.6.1.4 Skin metabolism

Metabolism of drugs in the skin influences the therapeutic effect of topical formulations, especially pro-drugs, and carcinogenic effects (Aulton, 1996:393).

2.3.6.1.5 Circulatory effects

Increased blood flow to the skin removes the drug from the dermis quicker and thus raises the concentration gradient across the skin (Aulton, 1996:393).

2.3.6.2 Physicochemical factors

2.3.6.2.1 Partition coefficient

The partition coefficient is an indication of the distribution of particles between the water- and oil phase (usually octanol). It is also known as the octanol-water partition coefficient or log P. In transdermal studies the log P of a substance determines the route of permeation through the skin (Williams, 2003:36).

Compounds with a low log P value are not well distributed into the skin lipids and therefore poorly permeates the skin. Compounds with a very high log P also have low permeability, because they distribute well into the skin lipids in the stratum corneum and do not easily diffuse out of it (Thomas & Finnin, 2004:699).

Lipophilic compounds with a log P greater than 3, such as rifampicin (log P = 3.714), permeate skin by the intercellular route and hydrophilic compounds with a log P smaller than 1, like isoniazide (log P = -0.640), permeate skin by the intercellular and transappendageal routes (Drugbank, 2009).

2.3.6.2.2 Molecular size

Smaller particles have higher diffusivity through skin and the ideal molecular weight for skin permeation is 100-500 g/mol. Particles with a molecular weight up to 5000 g/mol can still permeate skin (Wiechers, 1989:190). Therefore isoniazide (MW = 137.14 g/mol) will cross the skin much faster than rifampicin (MW = 822.95 g/mol) (Drugbank, 2009).

2.3.6.2.3 Drug/skin interactions

- Skin hydration
  When skin is saturated with water, its permeability increases, because it swells, softens and wrinkles. Thus, hydrated skin allows for efficient transdermal penetration.
- Drug/skin binding
  This is important for topically active preparations, sunscreens and controlled release preparations (Aulton, 1996:394).

2.3.6.2.4 Vehicle/skin interactions

- Vehicle effects on skin hydration

  Drug penetration is increased by occlusion, which minimizes water loss and increases the moisture content of the skin. Vehicles in order of decreasing hydration: lipids > water in oil emulsions > oil in water emulsions > powders (dry the skin) (Aulton, 1996:394).

Table 2.2: Expected effects of common vehicles on skin hydration and skin permeability – in approximate order of decreasing hydration (Aulton, 1996:395)

Vehicle	Examples	Effect on skin hydration	Effect on skin permeability
Occlusive dressing	Plastic film, imperforated waterproof plaster	Prevents water loss, full hydration	Definite increase
Lipophilic	Paraffins, oils, fats, waxes, fatty acids, alcohols, esters, silicones	Prevents water loss, may produce full hydration	Definite increase
Absorption base	Anhydrous lipid material plus water/oil emulsifiers	Prevents water loss, marked hydration	Definite increase
Emulsifying base	Anhydrous lipid material plus oil/water emulsifiers	Prevents water loss, marked hydration	Definite increase
Water/oil emulsion	Oily creams	Retards water loss, *raised hydration*	Increase
Oil/water emulsion	Aqueous creams	May donate water; slight hydration increase	Slight increase
Humectants	Water soluble bases, glycerol, glycols	May withdraw water; decreased hydration	Decrease enhances penetration
Powder	Clays, organics, inorganics, 'shake' lotions	Aid water evaporation; decreased excess	Little effect on stratum corneum

- Temperature

  Temperature greatly influences drug penetration. Occlusion raises temperature somewhat. Wearing adequate clothing controls changes in temperature and the tempo of penetration (Aulton, 1996:394).

- Penetration enhancers

  Penetration enhancers are compounds that minimize the barrier function of the skin, increasing its permeability and the level of drug penetration. The ideal penetration enhancer should be pharmacologically inert, non-toxic, non-allergenic, non-irritating, compatible with other ingredients in the formulation. It should also be a good solvent, cosmetically acceptable, odourless, colourless, tasteless and inexpensive. The ideal penetration enhancer should have an immediate action and a predictable effect, it should not cause loss of body fluids or electrolytes, it should allow immediate recovery of the skin's barrier function when removed and it should be able to be formulated into all topical products. Aprotic solvents such as dimethylsulphoxide, dimethylformamide and dimethylacetamode are the most effective penetration enhancers. Other penetration enhancers are the pyrrolidones like azone, surface active ingredients like decylmethylsulphoxide and combinations of oleyl alcohol/oleic acid with propylene glycol. The safest and most effective penetration enhancer of all is water (Aulton, 1996:394-396).

2.3.7 Mathematical model for skin absorption

Fick's law of diffusion can be accepted to describe transdermal delivery of a drug. Drug permeation is a passive diffusion process: from an area of high concentration of drug on the surface of the stratum corneum to an area of low concentration of drug within the skin.

$$J = k_p.\Delta C = (K.D/\ell).\Delta C \qquad \textbf{Equation 2.1}$$

J = steady state flux ($mg.cm^{-2}.h^{-1}$)

k_p = permeability coefficient

ΔC = difference in concentration across the membrane ($mg.cm^{-3}$)

K = partition coefficient between the donor and the outer layer of the membrane

D = diffusivity of the drug in the membrane ($cm^2.h^{-1}$)

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

2.4 PHEROID™ DELIVERY SYSTEM

2.4.1 Introduction

The Pheroid™ drug delivery system is a system designed to enhance the absorption of a number of drugs and it is currently being tested for oral as well as transdermal formulations. It

is based on its predecessor, the Emzaloid™ drug delivery system, containing mainly plant oils and essential fatty acids (Grobler, 2004:3-4).

2.4.2 Structural characteristics and molecular organisation

In order for a particle to cross the stratum corneum and follicles, it has to be smaller than 3 µm. Particles in the range of 3-10 µm enter the follicles, but cannot cross them and particles larger than 10 µm stay on the surface of the skin (Barry, 2002:31).

The Pheroid™ drug delivery system is a stable system of dispersed micron-size lipids. The basic Pheroid™ structures are 200-440 nm sized vesicles made up of polyunsaturated omega-3 and omega-6 fatty acids. There are also other types of Pheroid™ namely micro-sponges and depots or reservoirs with pro-Pheroid™ (Grobler *et al.,* 2007:5).

Penetration and delivery are improved by the affinity of human cell membranes for the Pheroid™ and the compatibility of the fatty acids of Pheroid™ and human fatty acids. The main ingredients in Pheroid™, linoleic acid and linolenic acid, are normally used by the skin to form the skin's barrier properties and thus they ensure for a skin-friendly carrier. They are also responsible for cell membrane integrity, energy homeostasis, immune system modulation and programmed cell death (Grobler *et al.*, 2007:6).

Nitrous oxide (N_2O)-gas is dispersed into the oil and water phases. It ensures the miscibility of the fatty acids in the medium, the formation of the Pheroid™ and the stability thereof (Grobler *et al.*, 2007:10). All topical Pheroid™ formulations contain tocopherol or tocopherol-derivatives as anti-oxidants and emulsion stabilizers.

The rate of delivery, route of administration and the size of the active ingredient determine the size and type of Pheroid™ to be formed (Grobler *et al.*, 2008:283-293).

2.4.3 Uptake of Pheroid™ and entrapped compounds

Though the exact process is still uncertain, it is thought that fatty acid membrane proteins aid the cell-uptake of Pheroid™ structures. These proteins are found in epidermal keratinocytes. Pheroid™ metabolism takes place in the mitochondria or peroxisomes and the actives are then released (Grobler *et al.*, 2007:23).

Permeation of Pheroid™ through the skin are influenced by the size of the Pheroid™, amount of fatty acid and active component, hydration of the formulation, pH, characteristics of the active component and the presence of electrostatic molecules (Grobler *et al.*, 2008:299).

2.4.4 Penetration enhancement

Pheroid™ can penetrate skin, keratinized tissue, intestine, vasculature, bacteria, fungi and certain parasites (Grobler, 2004:4).

N_2O gas and the pegylated tails of the fatty acids in Pheroid™ give it a very pliable structure (Grobler *et al.*, 2008:294). Fatty acids in Pheroid™ disrupt intercellular lipids in the skin, allowing intercellular, transdermal transport of the Pheroid™. The kinked structure of oleic acid contributes to this even further (Saunders *et al.*, 1999:106).

2.5 SUMMARY

CTB is a somewhat complicated disease. Although it is quite rare, it can be serious and can cause deformities if left untreated. Because of the slow recovery with current oral treatment regimens, a topical formulation might provide a more specific treatment with faster recovery and better patient compliance.

The anti-tubercular actives isoniazide and rifampicin were chosen due to their powerful bactericidal action and proven oral efficiency. In this study the actives will be formulated into topical preparations by means of the Pheroid™ drug delivery system in order to provide a formulation to be used for the treatment of CTB.

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CHAPTER 3: ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

PHEROID™ TECHNOLOGY FOR THE TOPICAL FORMULATION OF ISONIAZIDE AND RIFAMPICIN

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Abstract

Cutaneous tuberculosis is an infection caused mainly by *Mycobacterium tuberculosis* and can be acquired by direct inoculation or the spreading of pulmonary disease. Treatment consists of the anti-tubercular treatment used for pulmonary tuberculosis and no topical products are available yet. In this study the anti-tubercular drugs, isoniazide and rifampicin, were formulated into four topical formulations (two with and two without the Pheroid™ technology). Six month stability testing consisting of concentration assay, pH, viscosity, weight loss, particle size, appearance and colour proved the Pheroid™ formulations to be more stable. The concentration assay proved isoniazide more stable in the non-Pheroid™ emulgel and rifampicin more stable in the Pheroid™ emulgel. Diffusion studies demonstrated that the Pheroid™ improved drug release and permeation through cellulose acetate membranes after 12 h. In none of the formulations the actives were able to penetrate into or across human skin.

Keywords: Isoniazide, Rifampicin, Pheroid™, Cutaneous tuberculosis, Franz cell diffusion

1 Introduction

Tuberculosis (TB) is one of the most dangerous common infectious diseases of modern time. According to estimates of the World Health Organisation (WHO) there were 9.27 million cases of TB and 1.76 million deaths caused by TB in 2007 (WHO, 2009). Approximately 10 % of these cases comprise of extrapulmonary TB and cutaneous TB makes up a small percentage of them. Cutaneous TB is caused by *Mycobacterium tuberculosis, Mycobacterium bovis* and the Bacilli Calmette-Guérin (BCG) vaccine and can lead to permanent deformities when necrosis is present. To confirm diagnosis of a lesion as cutaneous TB, *Mycobacterium tuberculosis* needs to be identified by purified protein derivative staining, polymerase chain reaction or culture of the affected tissue. The various forms of cutaneous TB range from superficial dermal granulomas to ulceration and necrosis of the nose, mouth and lymph nodes. Since most patients with cutaneous TB also have pulmonary TB; treatment currently is the same anti-tubercular chemotherapy regimen (Barbagallo et al., 2002). The aim of this study is to improve recovery time and prevent scarring of cutaneous TB lesions and the deformities it can cause, by developing a topical preparation containing the anti-tuberculids, isoniazide and rifampicin. The greatest challenge of successfully formulating a transdermal product is the barrier function of the skin and the significant role of the stratum corneum's protein and lipid composition, in preventing skin penetration (Williams, 2003). A particle or drug has to be smaller than 10 µm in diameter (Barry, 2002) and must be both hydrophilic and lipophilic (Schalla and Schaefer, 1981). In this study the Pheroid™ delivery system will be implemented to formulate isoniazide and rifampicin into a topical spray preparation. The ability of these actives to be released from Pheroid™ and non-Pheroid™ formulations and permeate the skin will be studied by an *in vitro* method using Franz cells. The stability of the these formulations and the actives in them will be tested by six month stability tests, including assay, determination of particle size, pH, weight loss, viscosity, physical appearance and colour changes.

2 Methods and materials

2.1 Materials

Isoniazide (pyridine-4-carbohydrazide), MW = 137.14 and rifampicin ({2,7-(epoxypentadeca [1,11,13]-trienimino)naphtha[2,1-b]furan-1,11](2H)-dione,5,6,17,19,21-hexahydroxy-23-methoxy -2,4,12,16,18,20,{22-heptamethyl-8-[N-(4-methyl-1-piperazinyl)formidoyl]-}21-acetate), MW = 822,95 were obtained from Aventis (South-Africa). Water was purified by a Milli-Q Academic purification system (Millipore, Milford, USA). Vitamin F-ethyl ester, cremophor RH 40 and *dl*-α-tocopherol (vitamin E) were supplied by The Unit for Drug Research and Development, North-West University. Oleic acid, cetylalcohol, span 60, tween 80, methylparaben, propylparaben, butylated hydroxytoluene (BHT), metolose and glycerol were obtained from Merck Chemicals (Pty.) Ltd., South-Africa. Nitrous oxide (N_2O)-gas was dispersed into distilled water by The Unit for Drug Research and Development, North-West University. Dow-corning® vacuum grease was used to seal Franz cells.

2.2 Methods

2.2.1 Preparation of topical formulations for the donor phase and stability testing

2.2.1.1 Lotion

100 g of the lotion formulation was produced by this method. 1 g rifampicin and 14 g oleic acid were heated together to 70°C, stirring continuously. 2.5 g cetylalcohol, 1.5 g span 60, 5 g tween 80, 0.4 g methylparaben, 0.08 g propylparaben and 0.2 g BHT were added. 0.5 g isoniazide and 74.8 g purified water were heated to 70°C and added to the oil phase while homogenising at 13500 rpm and room temperature. Homogenising was continued until the preparation cooled to 40°C. The preparation was then shaken until cooled to 25°C.

2.2.1.2 Pheroid™ lotion

100 g of the Pheroid™ lotion formulation was produced by this method. 1 g rifampicin and 14 g oleic acid were heated together to 70°C, stirring continuously. 2.5 g cetylalcohol, 1.5 g span 60, 5 g tween 80, 0.4 g methylparaben, 0.08 g propylparaben and 0.2 g BHT, 2.8 g vitamin F-ethyl ester and 1 g cremophor RH 40 were added, cooled to 55°C and 0.2 g *dl*-α-tocopherol was

added. 0.5 g isoniazide and 70.8 g N_2O-water were heated to 70°C and added to the oil phase while homogenising at 13500 rpm and room temperature. Homogenising was continued until the preparation cooled to 40°C. The preparation was then shaken until cooled to 25°C.

2.2.1.3 Emulgel

100 g of the lotion formulation was produced by this method. 1 g rifampicin and 14 g oleic acid were heated together to 70°C, stirring continuously. 2.5 g span 60, 6.5 g tween 80, 0.4 g methylparaben, 0.08 g propylparaben and 0.2 g BHT were added and the mixture was cooled to 55°C. 0.35 g metolose and 2.5 g glycerol were mixed together and added to the oil phase. 0.5 g isoniazide and 71.9 g purified water were heated to 70°C and added to the oil phase while homogenising at 13500 rpm and room temperature. Homogenising was continued until the preparation cooled to 40°C. The preparation was then shaken until cooled to 25°C.

2.2.1.4 Pheroid™ emulgel

100 g of the lotion formulation was produced by this method. 1 g rifampicin and 14 g oleic acid were heated together to 70°C, stirring continuously. 1.5 g span 60, 5 g tween 80, 0.4 g methylparaben, 0.08 g propylparaben, 0.2 g BHT, 2.8 g vitamin F-ethyl ester and 1 g cremophor RH 40 were added, the mixture cooled to 55°C and 0.2 g *dl*-α-tocopherol was added. 0.35 g metolose and 2.5 g glycerol were mixed together and added to the oil phase. 0.5 g isoniazide and 70.5 g N_2O-water were heated to 70°C and added to the oil phase while homogenising at 13500 rpm and room temperature. Homogenising was continued until the preparation cooled to 40°C. The preparation was then shaken until cooled to 25°C.

2.2.2 Stability testing

700 ml of each formulation was prepared and placed in 250 ml glass containers for viscosity testing and a small, white plastic container for every set of stability tests. The containers were then placed in stability chambers at 25°C/60 % RH (relative humidity), 30°C/60 % RH and 40°C/75 % RH. The glass containers were placed in the 25°C stability chamber. Stability tests were performed at 4, 8, 12 and 24 weeks and included pH, weight loss, viscosity, determination of particle size, physical appearance and colour changes, as well as concentration assay. pH

was measured with a Mettler Toledo SevenMulti pH meter (Switzerland) after calibration with Mettler Toledo buffer solutions at pH 4.01, 7.00 and 10.01 with a slope at 25°C. Sealed containers were cooled to room temperature and weighed with a Mettler Toledo AB204 balance. A Brookfield LVDVII+ viscometer on a helipath stand D (Brookfield engineering labs, U.S.A) was used for viscosity measurements and a Brookfield temperature controller TC202 to maintain temperature at 25°C. The LV1 61 spindle was used for the lotion and the Pheroid™ lotion and the small sample adaptor for the emulgel and Pheroid™ emulgel. Viscosity measures were taken at 30 and 60 rpm respectively, with torque kept above 10 %. 32 data points were collected at intervals of 10 sec. Particle size was measured by confocal laser scanning microscopy (CLSM) micrographs after a tab was added to indicate size in µm. CLSM micrographs were taken with a CLSM Nikon PCM2000, digital camera DMX1200 with a He/Ne laser-(543 nm), an Argon ion laser (457-517 nm) and a CLSM Nikon D-eclipse C1 si with a violet diode laser (400-405 nm), a He/Ne laser (543 nm) and a Argon ion laser (457 – 514 nm). Type A microscopy immersion oil (Nikon, Japan) was used for polarized light microscopy. Colour changes were measured by comparing photos taken with a Kodak® EasyShare C503 camera. The physical appearance of the formulation was assessed by comparison of photos and CLSM micrographs with the initial colour and appearance of the formulation.

2.2.2.1 High pressure liquid chromatography method for stability testing

The analytical instrument used was an Agilent® series 1100 high performance liquid chromatography (HPLC) with a pump, auto sampler, UV detector and Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software. The column used was a Phenomenex®, Jupiter 5u, C18, 300A, 250 x 4.6 mm, 5 µm column. Isoniazide and rifampicin were separated by means of gradient elution. The solvents of the mobile phase were a degassed mixture of octane-sulphonic acid in HPLC (deionised) water with pH adjusted to 3.5 with 10 % phosphoric acid (A) and methanol (HiperSolv for HPLC) (B). The mobile phase was composed as follows (A:B): 50:50 for 8 min, 0:100 for 16 min and 50:50 again for the remaining minute. The flow rate was set at 1.0 ml/min, with the injection volume 10 µl and the stop time

25 min. Both isoniazide and rifampicin was detected with UV at 254.4 nm and their retention times were at 3.4 and 9.8 min, respectively. The mobile phase was used as solvent.

2.2.3 Skin preparation

The skin used was Caucasian female abdominal skin removed by abdominoplastic surgery. Ethical approval for the procurement and use of the skin was provided by the Research Ethics Committee of the North-West University under reference number 04D08. Patient consent was given and the patients were kept anonymous. For this study full thickness skin was used, containing all the layers – stratum corneum, epidermis and dermis. Skin was frozen within 24 h after surgery. When completely frozen the subcutaneous fatty layer was carefully cut away with a scalpel and the skin punched into 15 mm discs, which were frozen again until use within 4 months. The discs were thawed at room temperature and mounted onto the Franz diffusion cell prior to the diffusion study.

2.2.4 Franz cell diffusion

Because both isoniazide and rifampicin are light-sensitive (WHO, 2009) 10 amber Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) were used. The diffusion area is 1.075 cm^2 and the capacity of the receptor compartment approximately 2 ml. The cellulose acetate membranes were mounted between the receptor and donor compartments; sealed with vacuum grease and clamped in place with metal horseshoe clamps. Small magnetic stirrer bars were placed in the receptor compartments and it was filled with a mixture of 20 % ethanol and 80 % phosphate buffer solution (PBS) at pH 7.4 and 37°C, taking care to avoid air bubbles. The diffusion cells were placed on a Variomag stirrer plate to ensure stirring of the receptor phase and in a Grant water bath at 32°C, human skin temperature (Azarmi et al., 2007). The donor phase was 1 ml of the formulation and it was covered with Parafilm® to prevent evaporation. Withdrawals were made at 20 and 40 min, as well as at 1, 2, 4, 6, 8, 10 and 12 h and directly assayed by HPLC.

Skin diffusion studies were performed with this same method, substituting the membranes with thawed skin discs (stratum corneum facing upwards) to determine transdermal penetration.

2.2.4.1 High pressure liquid chromatography method for Franz cell diffusion studies

The same analytical instrument and column was used as for stability testing and isoniazide and rifampicin were also separated by means of gradient elution. The solvents of the mobile phase were a degassed mixture of 0.01 M monobasic ammonium phosphate and 0.005 M 1-heptanesulphonic acid sodium salt in HPLC (deionised) water at pH 3.5 (A) and methanol(B). The mobile phase was composed as follows (A:B): 80:20 for 3.5 min, 20:80 for 1 min and the flow rate was set at 1.0 ml/min, with the injection volume 20 µl and the stop time 13 min. Both isoniazide and rifampicin was detected with UV at 263.4 nm and their retention times were at 4.0 and 8.5 min, respectively. The mobile phase was used as solvent.

2.2.5 Tape stripping

Tape stripping was done on the skin discs immediately after the 12 h diffusion study, to determine the drug concentrations in the stratum corneum and epidermis. The skin discs were carefully dismounted from the Franz cells, placed on Parafilm and stapled to the surface. Excess formulation was wiped away with tissue paper and the diffusion area (≈ 11.70 mm diameter) visibly indented. Pieces of tape were used to carefully strip away the epidermis of the diffusion area. The first tape was discarded and the next fifteen placed in 5 ml of 20 % ethanol-PBS solution overnight at 4°C. A sample was then analysed by HPLC. The skin around the diffusion area was cut away and the diffusion area into small pieces. It was placed in 2 ml of 20 % ethanol-PBS solution and centrifuged with an Eppendorf centrifuge 5804 R at 10°C and 14000 rpm for 10 min. A sample was then analysed by HPLC.

2.2.6 Diffusion and statistical analysis

The average cumulative concentration for isoniazide and rifampicin for each formulation was determined by calculating the cumulative amount per area (µg/cm^2). For statistical analysis of the membrane diffusion study the median (centre of data) cumulative concentration was determined. The median is a more accurate method if there is a big variation in data (Gerber et al., 2008). The data obtained was statistically analysed by the nonparametric, two tailed Kruskal-Wallis test of comparison for 3 or more groups of data. The p-values (measure of the

statistical correctness of data) were calculated and compared to the level of significance (0.05) (Lang and Secic, 1997). If there is a definite difference between two groups the p-value will be smaller than the level of significance and can be seen as statistically significant. When there is no difference between two groups the p-value is the same as the level of significance. If there is a very small difference between two groups the p-value will be greater than the level of significance and can be seen as statistically insignificant (Lang and Secic, 1997).

3 Results and discussion

3.1 Stability testing

After formulation CLSM micrographs proved all four formulations were homogenous and no crystals could be seen. Thus it can be concluded that the actives were fully dissolved and homogenising of the formulations were satisfactory. According to Barry (2002) particles of 3 µm and smaller than can cross the stratum corneum, paticles between 3 and 10 µm can enter follicles and particles larger than 10µm stay on the skin surface. The oil droplets were smaller than 3 µm for all except for the lotion formulation, but in the lotion it was smaller than 10 µm, thus transdermal penetration of the lotion can be expected via the transfollicular route and the other formulations via the across the stratum corneum.

According to the ICH (2003) a 5 % difference in the assay concentration is already accepted as "significant" change". Concentration assay showed significant breakdown of isoniazide after only 4 weeks to below 50 % of the initial concentration for the Pheroid™ formulations. The breakdown of rifampicin was much slower and concentrations decreased to below 50 % only after 12 weeks at 25°C. Hence, all four formulations were unstable. It was noted that isoniazide was most stable in the non-Pheroid™ emulgel formulation and rifampicin in the Pheroid™ emulgel formulation. This can be ascribed to the hydrophilic character of isoniazide (Stephan et al., 2004) and the lipophilic character of rifampicin (Sousa et al., 2008) and their formulation in a watery and oily environment, respectively, as hydrophilic compounds is more stable in hydrophilic environments and lipophylic compounds in lipophylic environments.

The pH of all four formulations stayed between 5 and 6 for the duration of stability testing, ideally in the same range as the physiological pH of skin (Elsner, 2006).

The maximum weight loss was 1.4 % for the emulgel formulation after 24 weeks, which is insignificant. At 40°C/75 % RH weight loss was less, possibly because the container might not be suitable for use at such a high temperature and water might have been absorbed from the environment.

Viscosity of all four formulations decreased significantly due to the high temperatures, the most for the lotion – from 30 cP to just 12 cP after 24 weeks. The emulgel formulation had the least decrease in viscosity – from 210 cP to 180 cP – possibly due to the less oily base. Viscosity changes in stability tests are acceptable if the uniformity of the formulation remain unchanged (Vaughan, 1997). The oil droplets of the lotion at 25°C did not change during the 24 week period. The uniformity of the lotion at both 25 and 30°C only changed after 24 weeks. After 8 weeks at 30°C the oil droplets of the lotion aggregated to larger than 10 µm and according to Barry (2002) particles of this size cannot permeate skin. After 4 weeks at 40°C the lotion was no longer uniform and the oil droplets aggregated to larger than 10 µm. The uniformity of the Pheroid™ lotion remained unchanged at all temperatures during the 24 weeks of testing. After 24 weeks at 25°C and after 12 weeks at both 30 and 40°C the oil droplets of the Pheroid™ lotion aggregated to larger than 10 µm. After 24 weeks at 25 and 30°C the uniformity of the emulgel remained unchanged and no significant aggregation of the oil droplets could be noticed. After 24 weeks at 40°C the oil droplets of the emulgel aggregated to larger than 10 µm, the emulgel was no longer uniform and the phases separated. The Pheroid™ emulgel was the most stable formulation.

There was no aggregation and the formulation remained uniform at all temperatures for the full 24 weeks of stability testing. All four formulations had a bright orange colour due to rifampicin. Colour changes might be an indication of the breakdown of rifampicin, as was seen in the HPLC analysis. It might also indicate possible incompatibilities between ingredients (Vaughan, 1997). After 24 weeks at 25 and 30°C there was only a slight colour change in the lotion. At 40°C the lotion started darkening after 4 weeks and after 24 weeks precipitation of rifampicin and phase separation of the lotion was found. After 24 weeks at 25°C the Pheroid™ lotion had a slight colour change. At 30°C significant colour changes in the Pheroid™ lotion was visible after 8 weeks and at 40°C after only 4 weeks. Slight colour changes were visible in the emulgel after 24 weeks at 25°C, significant changes after 24 weeks at 30°C and after 4 weeks at 40°C. After 24 weeks at 40°C slight phase separation of the emulgel was found. Slight colour changes

were visible in the Pheroid™ emulgel after 24 weeks at 25°C, significant changes after 24 weeks at 30°C and after 8 weeks at 40°C. After 24 weeks at 40°C significant phase separation of the Pheroid™ emulgel was found.

3.2 Franz cell diffusion

Both isoniazide and rifampicin permeated through the membrane. The average cumulative concentration (μg/cm^2) of isoniazide after 12 h (Figure 1) was the highest for the Pheroid™ emulgel (average 2.249 % diffused) and this is possibly due to the hydrophilic nature of isoniazide and the oily base of the formulation; allowing isoniazide to diffuse freely into the watery solution.. The lowest permeation of isoniazide was from the lotion (average 0.593 % diffused), 3.79 times less than the Pheroid™ emulgel. The Pheroid™ emulgel had the largest variance for isoniazide and the lotion had the least variance as depicted in the box-plots (Figure 3). The median of the cumulative concentration of isoniazide as shown in Figure 3 (lotion 57.35 μg/cm^2, emulgel 146.25 μg/cm^2, Pheroid™ lotion 148.36 μg/cm^2 and Pheroid™ emulgel 207.83 μg/cm^2) did not differ much from the average as shown in Figure 1 (lotion 55.17 μg/cm^2, Pheroid™ lotion 146.53 μg/cm^2, emulgel 148.04 μg/cm^2 and Pheroid™ emulgel 209.09 μg/cm^2) and therefore both median and average can be used to determine the cumulative concentration in this case.

The cumulative concentration of rifampicin after 12 h (Figure 2) was the highest for the Pheroid™ lotion (average 5.049 % diffused), possibly due to the lipophilic nature of rifampicin and the watery base of the formulation, allowing rifampicin to diffuse freely into the organic solution. The lowest permeation of rifampicin was from the lotion (average 2.991 % diffused), 1.852 times less than the Pheroid™ lotion. The Pheroid™ lotion had the largest variance for rifampicin and the lotion had the least variance as depicted by the box-plots (Figure 4). The median of the cumulative concentration of rifampicin as shown in Figure 4 (lotion 549.46 μg/cm^2, Pheroid™ lotion 909.22 μg/cm^2, emulgel 489.40 μg/cm^2 and Pheroid™ emulgel 610.14 μg/cm^2) was different from the average as shown in Figure 2 (lotion 560.65 μg/cm^2, Pheroid™ lotion 938.64 μg/cm^2, emulgel 506.94 μg/cm^2 and Pheroid™ emulgel 609.88 μg/cm^2). The

median and average cumulative concentration values, when compared to those of isoniazide, differ more. The aforementioned is probably due to greater variation in the data and for that reason the median is a more reliable and accurate method (Gerber et al., 2008).

There was no diffusion (through the skin) or penetration (into the skin) of the actives from any of the formulations.

3.3 Statistical analysis

The p-values showed no difference in the diffusion of isoniazide from the emulgel and Pheroid™ lotion ($p = 1.0$) and significant differences between the other formulations ($p < 0.05$) (Lang and Secic, 1997). The p-values for rifampicin showed no difference between the emulgel and lotion ($p = 1.0$), significant differences between the emulgel and the Pheroid™ lotion, the Pheroid™ lotion and the lotion ($p < 0.05$) and small differences between the rest of the comparisons ($p > 0.05$) (Lang and Secic, 1997). It is clear that the release of isoniazide and rifampicin from the formulations and membrane permeation were improved by the Pheroid™ delivery system, but the formulations needs to be altered to increase the amount of actives released.

4 Conclusion

In this study the isoniazide and rifampicin were formulated into Pheroid™ and non-Pheroid™ formulations to be used in diffusion studies and stability testing. It can be concluded that the Pheroid™ formulations were more stable than their non-Pheroid™ equivalents as is evident in the stability results and of the four formulations the Pheroid™ emulgel is most stable. According to the concentration assay isoniazide was more stable in the non-Pheroid™ emulgel and rifampicin more stable in the Pheroid™ emulgel. Membrane diffusion studies showed that the release of isoniazide was best from the Pheroid™ emulgel and rifampicin from the Pheroid™ lotion. Thus it can be concluded that the Pheroid™ improved the release of the actives and membrane permeation. The inability of isoniazide and rifampicin to permeate skin and the minute percentages that permeated the membrane indicates poor release from the formulations and this can be improved by further alterations to the formulations.

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FIGURE LEGENDS

Figure 1: Average cumulative concentration (μg/cm^2) of isoniazide through cellulose acetate membranes after 12 h (n=10).

Figure 2: Average cumulative concentration (μg/cm^2) of rifampicin through cellulose acetate membranes after 12 h (n=10).

Figure 3: Box-plots depicting the median cumulative concentration (μg/cm^2) of isoniazide through cellulose acetate membranes after 12 h.

Figure 4: Box-plots depicting the median cumulative concentration (μg/cm^2) of rifampicin through cellulose acetate membranes after 12 h.

FIGURES

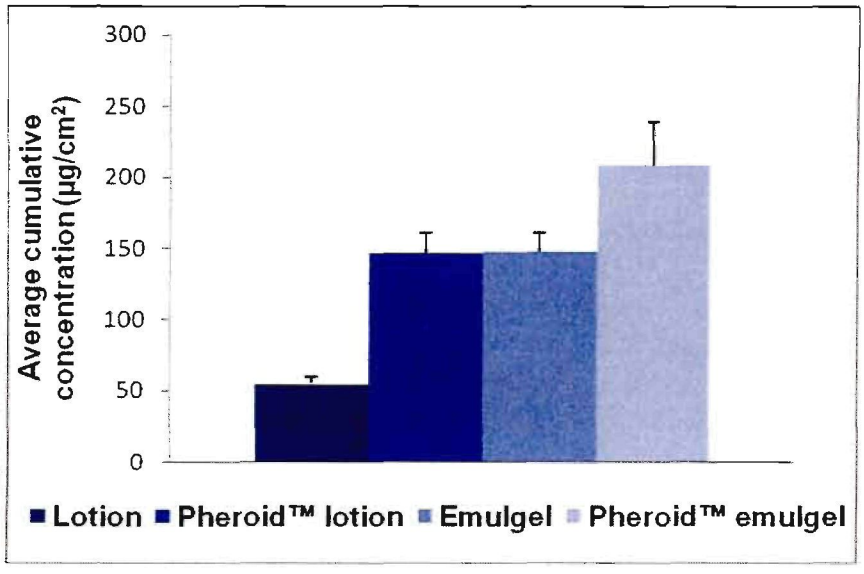

Figure 1: Average cumulative concentration (µg/cm^2) of isoniazide through cellulose acetate membranes after 12 h (n=10).

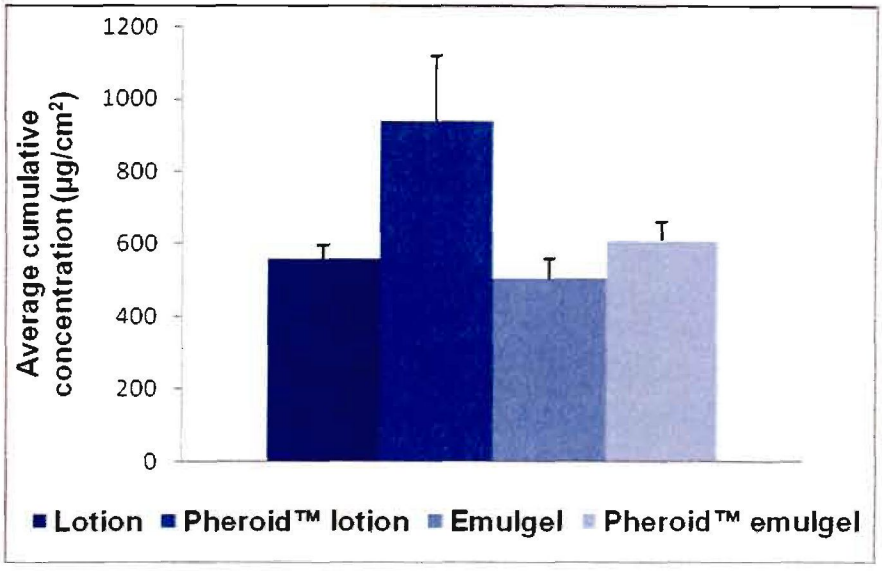

Figure 2: Average cumulative concentration (µg/cm^2) of rifampicin through cellulose acetate membranes after 12 h (n=10).

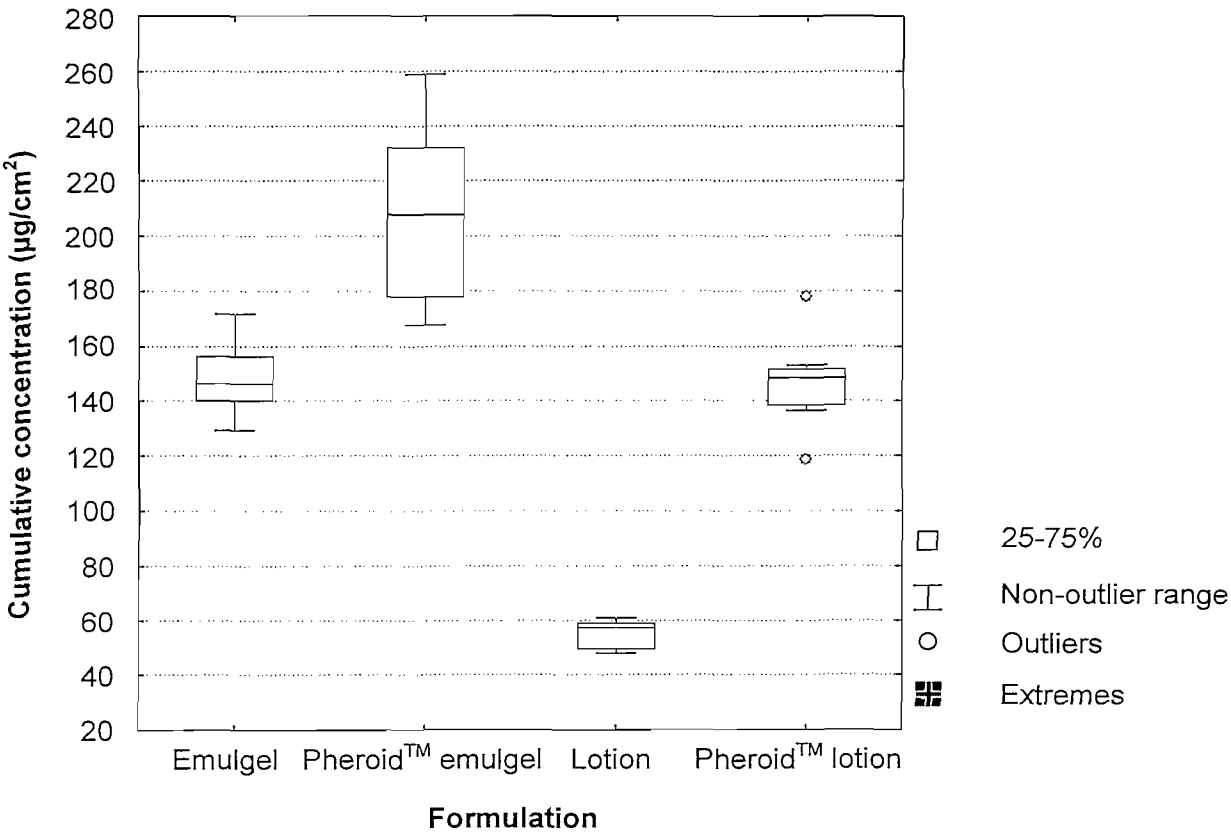

Figure 3: Box-plots depicting the median cumulative concentration (µg/cm^2) of isoniazide through cellulose acetate membranes after 12 h.

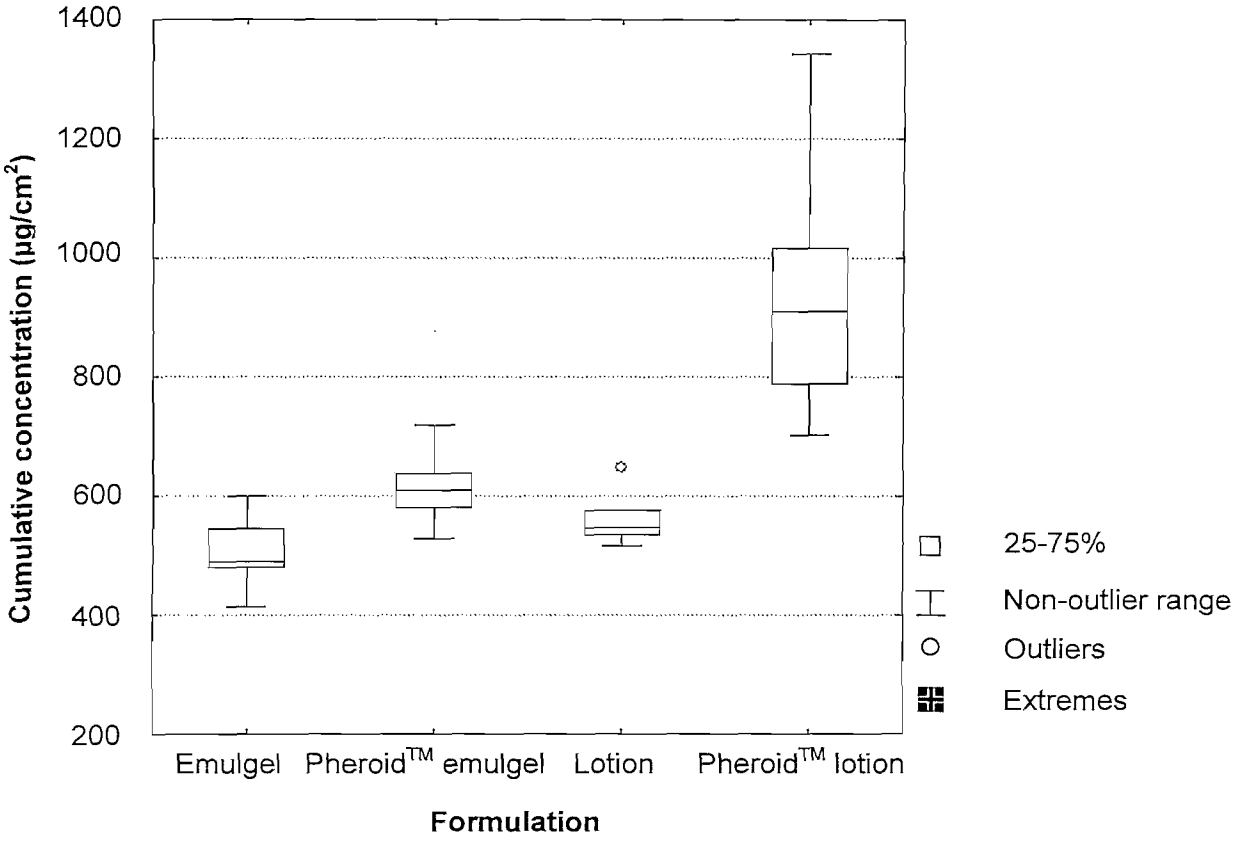

Figure 4: Box-plots depicting the median cumulative concentration (µg/cm^2) of rifampicin through cellulose acetate membranes after 12 h.

CHAPTER 4: CONCLUSION AND FUTURE PROSPECTS

The scope of this study was to investigate the transdermal delivery of isoniazide and rifampicin in four different topical formulations, to see if the Pheroid™ improved drug delivery of these preparations. It was also important to test the stability of the formulations and of the actives in them over six months.

Neither isoniazide nor rifampicin possesses the ideal physicochemical characteristics to cross the stratum corneum and therefore the Pheroid™ drug delivery system was employed to entrap both actives and carry them across the stratum corneum. The Pheroid™ drug delivery system is a stable, skin-friendly carrier, consisting of a submicron sized emulsion and it can penetrate skin, the vascular system, keratinised tissue, intestinal lining, fungi and even parasites.

Isoniazide and rifampicin were formulated into two Pheroid™ and two non-Pheroid™ spray formulations: lotion, Pheroid™ lotion, emulgel and Pheroid™ emulgel. *In vitro* permeation studies were performed with vertical Franz cells, using female abdominal skin as membrane, for a 12 hour periods at pH 7.4. The donor phase consisted of the formulation, containing 5 mg/ml of isoniazide and 10 mg/ml of rifampicin.

The formulations were placed in stability chambers at 25°C/60 % RH, 30°C/60 % RH and 40°C/75 % RH for a period of 6 months. Stability tests were performed at 4, 8, 12 and 24 weeks and consisted of concentration assays, pH, weight loss, viscosity, particle size, physical appearance and colour change tests.

The reason for the chosen drug combination and the topical formulations was to develop a product which could serve as an additional therapy to current systemic treatment regimens for CTB.

CTB is pathological lesions of the skin caused by *Mycobacterium tuberculosis, mycobacterium bovis* or the BCG vaccine. It can develop when systemic tuberculosis spreads or after direct inoculation with *Mycobacterium tuberculosis*. The diagnosis of CTB is confirmed by acid-fast bacilli (AFB) staining, culture or polymerase chain reaction (PCR) of the infected tissues. CTB lesions may include ulceration, plaque formation or necrosis and this will influence the diffusion of isoniazide and rifampicin from the topical formulation to their intended sites of action on the tubercle bacilli.

Current treatment for CTB consists of the same standard three/four drug oral regimen as for pulmonary TB. CTB does improve with this treatment, but a full recovery can take up to 6 months. The lesions of CTB can cause deformities and the implementation of a topical preparation might show quicker healing and prevent the formation of scars and disfigurement.

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

- CLSM showed that the formulations were homogenous with no crystals. Oil droplets smaller than 10 µm formed, enabling the permeation through skin. Thus it proved that isoniazide and rifampicin was successfully formulated into these topical preparations.
- *In vitro* permeation studies showed no permeation of isoniazide or rifampicin from any of the formulations through the skin and no isoniazide or rifampicin was found in the skin after 12 h.
- Membrane studies showed the release of the actives from all formulations and very small concentrations permeating the membrane, indicating poor formulation.
- Membrane studies proved that both actives were best released from the Pheroid™ emulgel formulation and that the Pheroid™ formulations showed better release and permeation than their non-Pheroid™ equivalents.
- Six month stability testing showed rifampicin to be more stable than isoniazide in these formulations. The concentration of isoniazide decreased to less than 50 % within 8 weeks for all four formulations even at 25°C and minimal concentrations were left after 24 weeks at 40°C. Rifampicin concentrations only decreased to less than 50 % after 12 weeks at 25°C and after 24 weeks at 40°C noticeable concentrations were left.
- Stability testing showed that the Pheroid™ formulations were more stable than their non-Pheroid™ equivalents and the Pheroid™ emulgel was the most stable formulation of the four.

Aspects identified which might render further investigation:

- Modify the formulations to improve skin permeation and prevent degradation of the actives, especially isoniazide.
- *In vitro* efficacy testing of the proposed Pheroid™ emulgel formulation using reference strains of *Mycobacterium tuberculosis*.
- Clinical trials in patients with CTB to determine the efficacy of the isoniazide and rifampicin Pheroid™ emulgel formulation as well as duration of treatment necessary to

achieve resolution of CTB lesions and thus total eradication of *Mycobacterium tuberculosis*.

- Development of similar topical Pheroid™ formulations with rifampicin in combination with other drugs e.g. dapsone, to be used in leprosy patients.

APPENDIX A: INTERNATIONAL JOURNAL OF PHARMACEUTICS

GUIDE FOR AUTHORS

Scope of the journal

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Crowe, J.H., Crowe, L.M., Carpenter, J.F., Aurell Wistrom, C., 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem. J., 242, 1-10.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J., Anchordoguy, T.J., 1988. Interactions of sugars with membranes. Biochim. Biophys. Acta, 947, 367-384.

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Timsina, M.P., Martin, G.P., Marriott, C., Ganderton, D., Yianneskis, M., 1994. Drug delivery to the respiratory tract using dry powder inhalers. Int. J. Pharm., 101, 1-13.

Gibaldi, M. and Perrier, D., 1982. Pharmacokinetics, 2nd Ed., Dekker, New York.

Deppeler, H.P., 1981. Hydrochlorothiazide. In: Florey, K. (Ed.), Analytical Profiles of Drug Substances, Vol. 10, Academic Press, New York, pp. 405-441.

US Pharmacopeia XXII, 1990. US Pharmacopeial Convention, Rockville, MD, pp. 1434-1435.

Mueller, L.G., 1988. Novel anti-inflammatory esters, pharmaceutical compositions and methods for reducing inflammation. UK Patent GB 2 204 869 A, 23 Nov.

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APPENDIX B: FORMULATION AND STABILITY TESTING

B.1 INTRODUCTION

Four different topical formulations containing the actives, isoniazide and rifampicin, were prepared - two with Pheroid™ and two without, namely a lotion, a Pheroid™ lotion, an emulgel and a Pheroid™ emulgel. It was decided that spray formulations would be appropriate due to the nature of the skin condition of patients with cutaneous tuberculosis as discussed in Chapter 2.

Stability testing of a new cosmetic product is performed to ensure that requirements are met regarding physical and chemical properties and that the product remains functional and aesthetically pleasing under specified conditions (COLIPA, 2008:1). In this study, four topical formulations were prepared and a six month stability testing was performed on them. The following tests were performed during the allocated time frame: concentration assays; pH; weight loss; viscosity; particle size; appearance and colour tests.

B.2 METHODS AND MATERIALS USED

Large batches (700 ml) of the four formulations were prepared and placed in 30 ml white plastic containers, a different container for each set of stability tests. Each formulation (250 ml) was placed in a glass Consol container for the purpose of viscosity measurement. The containers were then placed in Labcon stability chambers at 25°C/60 % RH (relative humidity), 30°C/60 % RH and 40°C/75 % RH. The glass containers were placed in a 25°C stability chamber. Tests were performed at 4, 8, 12 and 24 weeks.

B.2.1 Formulation

Rifampicin ({2,7-(epoxypentadeca[1,11,13]-trienimino)naphtha[2,1-b]furan-1,11](2H)-dione,5,6, 17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,{22-heptamethyl-8-[N-(4-methyl-1-piperazinyl)formidoyl]-}21-acetate), MW = 822,95 g/mol and isoniazide (pyridine-4-carbohydrazide), MW = 137.14 g/mol, were obtained from Aventis (South-Africa). Water was purified by a Milli-Q Academic purification system (Millipore, Milford, USA). Pheroid™ constituents were supplied by The Unit for Drug Research and Development, North-West

University. All other materials were obtained from Merck Chemicals (Pty.) Ltd., South-Africa. Micrographs were taken with a Nikon PCM 2000 confocal laser scanning microscope (CLSM).

B.2.1.1 Lotion method

Table B.1: Lotion spray formula.

Ingredients	% m/m	Activity
Rifampicin	1.00 %	Active
Oleic acid	14.00 %	Base, penetration
Cetylalcohol	2.50 %	Thickening agent
Span 60	1.50 %	Emulsifier
Tween 80	5.00 %	Emulsifier
Methylparaben	0.40 %	Preservative
Propylparaben	0.08 %	Preservative
Butylated hydroxytoluene (BHT)	0.20 %	Antioxidant
Isoniazide	0.50 %	Active
Distilled water	74.80 %	Base

- Heat rifampicin and oleic acid together to 70°C, stirring continuously.
- Add the cetylalcohol, emulsifiers, preservatives and antioxidant.
- Heat isoniazide and the distilled water to 70°C.
- Add the water-phase to the oil-phase and homogenise at 13500 rpm until cooled to 40°C.
- Shake until cooled to 25°C.

B.2.1.2 Pheroid™ lotion method

Table B.2: Pheroid™ lotion spray formula.

Ingredients	% m/m	Activity
Rifampicin	1.00 %	Active
Oleic acid	14.00 %	Base, penetration enhancer
Cetylalcohol	2.50 %	Thickening agent
Span 60	1.50 %	Emulsifier
Tween 80	5.00 %	Emulsifier
Methylparaben	0.40 %	Preservative
Propylparaben	0.08 %	Preservative
BHT	0.20 %	Antioxidant
Isoniazide	0.50 %	Active
Vitamin F-ethyl ester	2.80 %	Pheroid™ ingredient
Cremophor RH 40	1.00 %	Pheroid™ ingredient
dl-α-Tocopherol	0.20 %	Pheroid™ ingredient
N_2O-water	70.80 %	Pheroid™ ingredient

- Heat rifampicin and oleic acid together to 70°C, stirring continuously.
- Add the cetylalcohol, emulsifiers, preservatives, antioxidant, vitamin F-ethyl ester and cremophor RH 40.
- Cool to 55°C and add *dl*-α-tocopherol.
- Heat isoniazide and the distilled water to 70°C.
- Add the oil-phase to the water-phase while homogenising at 13500 rpm and homogenise until cooled to 40°C.
- Shake until cooled to 25°C.

B.2.1.3 Emulgel method

Table B.3: Emulgel spray formula.

Ingredients	% m/m	Activity
Rifampicin	1.00 %	Active
Oleic acid	14.00 %	Base, penetration enhancer
Metolose	0.35 %	Gelling agent
Glycerol	2.50 %	Thickening agent
Span 60	2.50 %	Emulsifier
Tween 80	6.50 %	Emulsifier
Methylparaben	0.40 %	Preservative
Propylparaben	0.08 %	Preservative
BHT	0.20 %	Antioxidant
Isoniazide	0.50 %	Active
Distilled water	71.90 %	Base

- Heat rifampicin and oleic acid together to 70°C, stirring continuously.
- Add the emulsifiers, preservatives and antioxidant and cool to 55°C.
- Mix the metolose and glycerol well and add to the oil phase.
- Heat isoniazide and the distilled water to 70°C.
- Add the oil-phase to the water-phase and homogenise at 13500 rpm until cooled to 40°C.
- Shake until cooled to 25°C.

B.2.1.4 Pheroid™ emulgel method

Table B.4: Pheroid™ emulgel spray formula.

Ingredients	%m/m	Activity
Rifampicin	1.00 %	Active
Oleic acid	14.00 %	Base, penetration enhancer
Metolose	0.35 %	Gelling agent
Glycerol	2.50 %	Thickening agent
Span 60	1.50 %	Emulsifier
Tween 80	5.00 %	Emulsifier
Methylparaben	0.40 %	Preservative
Propylparaben	0.08 %	Preservative
BHT	0.20 %	Antioxidant
Isoniazide	0.50 %	Active
Vitamin F-ethyl esther	2.80 %	Pheroid™ ingredient
Cremophor RH 40	1.00 %	Pheroid™ ingredient
dl-α-Tocopherol	0.20 %	Pheroid™ ingredient
N_2O-water	70.50 %	Pheroid™ ingredient

- Heat rifampicin and oleic acid together to 70°C, stirring continuously.
- Add the emulsifiers, preservatives, antioxidant, vitamin F-ethyl ester and cremophor RH 40.
- Cool to 55°C and add *dl*-α-tocopherol.
- Mix the metolose and glycerol well and add to the oil phase.
- Heat isoniazide and the distilled water to 70°C.
- Add the oil-phase to the water-phase and homogenise at 13500 rpm until cooled to 40°C.
- Shake until cooled to 25°C.

B.2.2 Concentration assay

The analytical instrument used was the Agilent® series 1100 high performance liquid chromatography (HPLC) with a pump, auto sampler, UV detector and Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software and the column used was a Phenomenex®, Jupiter 5u, C18, 300A, 250 x 4.6 mm, 5 µm column. The two actives were separated by gradient elution. The mobile phase was composed of:

- A degassed mixture of octane-sulphonic acid ($C_8H_{17}NaO_3S$) in HPLC (deionised) water with the pH adjusted to 3.5 with 10 % phosphoric acid (H_3PO_4).
- Methanol (HiperSolv for HPLC).

Table B.5: Mobile phase composition - monobasic ammonium phosphate buffer (A) and methanol (B).

Mobile phase composition A:B		Duration (min)
50	50	1
0	100	8
0	100	24
50	50	25

The flow rate was 1.0 ml/min, the injection volume 10 µl and the stop time after 25 min. Both isoniazide and rifampicin was detected with UV at 254.4 nm at retention times of 3.4 and 9.8 min, respectively.

B.2.3 pH

A Mettler Toledo SevenMulti pH meter (Switzerland) was used to measure pH. The pH meter was calibrated before use with Mettler Toledo buffer solutions at pH 4.01, 7.00 and 10.01 with a slope at 25°C.

B.2.4 Weight loss

Filled containers were weighed with a Mettler Toledo AB204 balance (Switzerland) to determine product loss due to evaporation or breakdown of the formulation. The containers were first cooled to room temperature to avoid fluctuations of the readings on the balance.

B.2.5 Viscosity

Viscosity was measured with a Brookfield LVDVII+ viscometer on a helipath stand D (Brookfield engineering labs, U.S.A) with samples adjusted in a Brookfield temperature controller TC202 to 25°C. The low viscosity spindle (LV1 61) was used for the lotion and the Pheroid™ lotion and the small sample adaptor for the emulgel and Pheroid™ emulgel. The speed was set at 30 and 60 rpm respectively, taking care to keep the torque above 10 %. 32 data points were collected at intervals of 10 sec.

B.2.6 Particle size

CLSM micrographs were taken with a CLSM Nikon PCM 2000 with DMX 1200 digital camera with a He/Ne laser (543 nm) and a Argon ion laser (457 – 517 nm) and a CLSM Nikon D-eclipse C1 si with a violet diode laser (400 – 405 nm), a He/Ne laser (543 nm) and a Argon ion laser (457 – 514 nm) was also used. Microscopy immersion oil – type A (Nikon, Japan) was used for the polarized light microscopy. A tab was added to the micrographs to indicate particle size in µm. These micrographs were also used to assess the physical appearance of the emulsions, as the oil and water phases can easily be distinguished. The slides for CLSM were prepared as follows:

- Measure 50 µl of the formulation and add 1 µl of Nile red.
- Shake well and incubate in the dark for 15 min.
- Drop 20 µl of the mixture on a microscopic slide and cover with a glass cover slip.
- Add a drop of immersion oil on the reverse side of the slide and view on the CLSM.

B.2.7 Colour and appearance

Photos were taken with a Kodak EasyShare C503 camera (setting: Close-up) and the appearances were compared with the initial colour and appearance of the particular formulation. Care was taken to ensure the photos were taken in the exact same location with the same lighting every time.

B.3 RESULTS AND DISCUSSION

B.3.1 Formulation

The formulation for the lotion was attempted with a variety of bases and thickening agents, until the appropriate combination was found. The percentages of oil and thickening agents were then altered in order to achieve the optimal consistency that would result in a "light" feeling on the skin. The formulation was sprayable and non-greasy. CLSM micrographs (Figure B.1 a) showed that the formulation was uniformly dispersed and no crystals were visible.

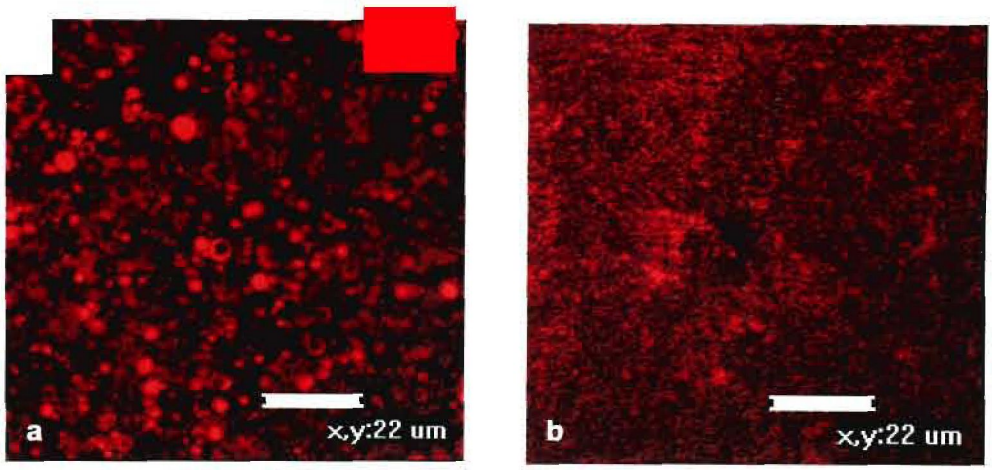


Figure B.1: CLSM micrographs after formulation of the **a)** lotion and **b)** Pheroid™ lotion.

The Pheroid™ lotion formulation was obtained from the lotion and predetermined constituents were added to ensure the formation of Pheroid™ vesicles. The formulation had the same consistency as the lotion and it was also sprayable and non-greasy. CLSM micrographs (Figure B.1 b) showed that the formulation was uniformly dispersed and no crystals were visible.

The formulation for the emulgel spray was attempted with different bases, gelling agents and thickening agents, until the appropriate combination was found. Natural clays, such as veegum and trachacant were decided against as it might cause a blockage in the pipe of the spray bottle. The percentage of metolose was then altered in order to achieve the wanted consistency and "light" feeling on the skin. The formulation was sprayable and non-greasy. CLSM micrographs (Figure B.2 a) showed that the formulation was uniformly dispersed and no crystals were visible.

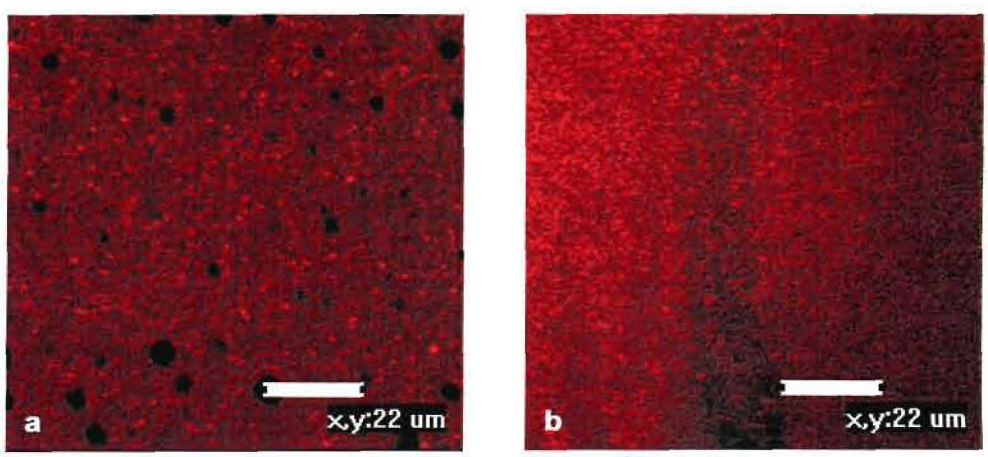


Figure B.2: CLSM micrographs after formulation of the **a)** emulgel and **b)** Pheroid™ emulgel.

The Pheroid™ emulgel formulation was derived from the emulgel formula and ingredients were added to ensure the formation of Pheroid™ vesicles. The formulation had the same consistency as the emulgel and it was also sprayable and non-greasy. CLSM micrographs (Figure B.2 b) showed that the formulation was uniform and no crystals were visible.

All four formulations were homogenous and no crystals were visible, implying that the actives were fully dissolved and methods were successful. According to Barry (2002:31) particles smaller than 3 µm can cross the stratum corneum and particles between 3 and 10 µm can penetrate skin via the transfollicular route. The size of the oil droplets was smaller than 3 µm, except for the lotion where it was still not larger than 10 µm. Thus transdermal penetration can be expected for all four formulations and for the lotion by means of the transfollicular route only.

B.3.2 Concentration assay

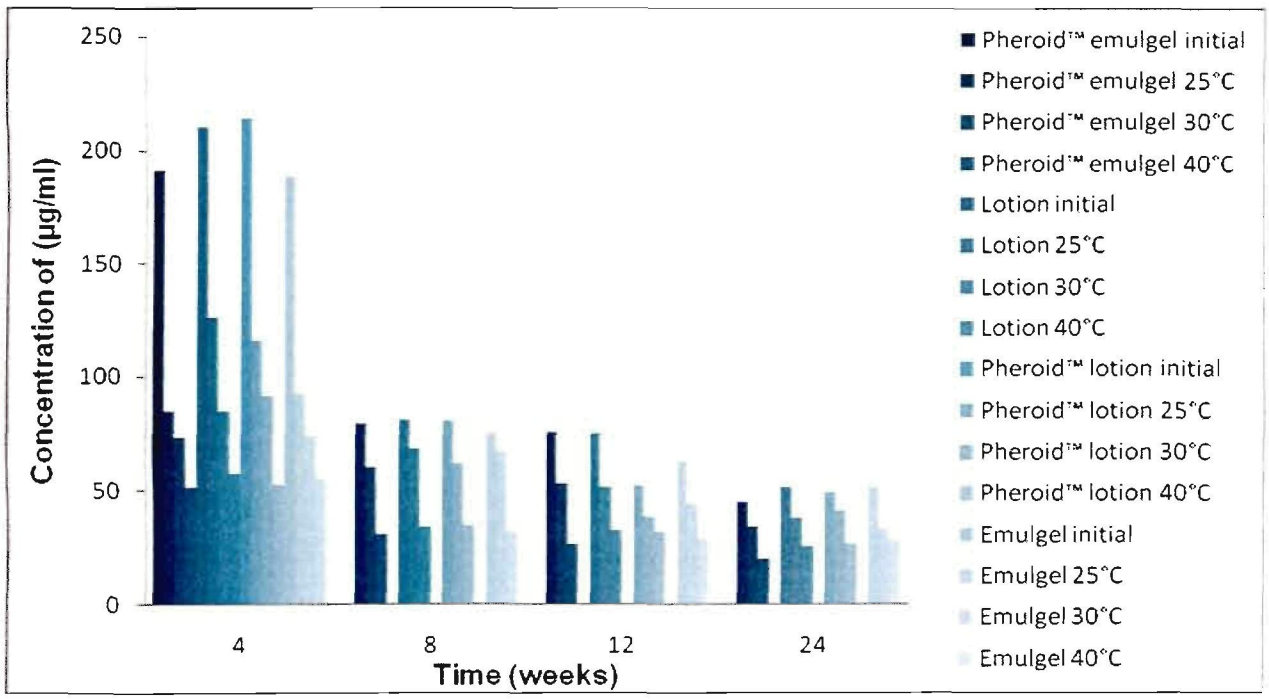


Figure B.3: Concentration (µg/ml) of isoniazide in the four formulations over 24 weeks.

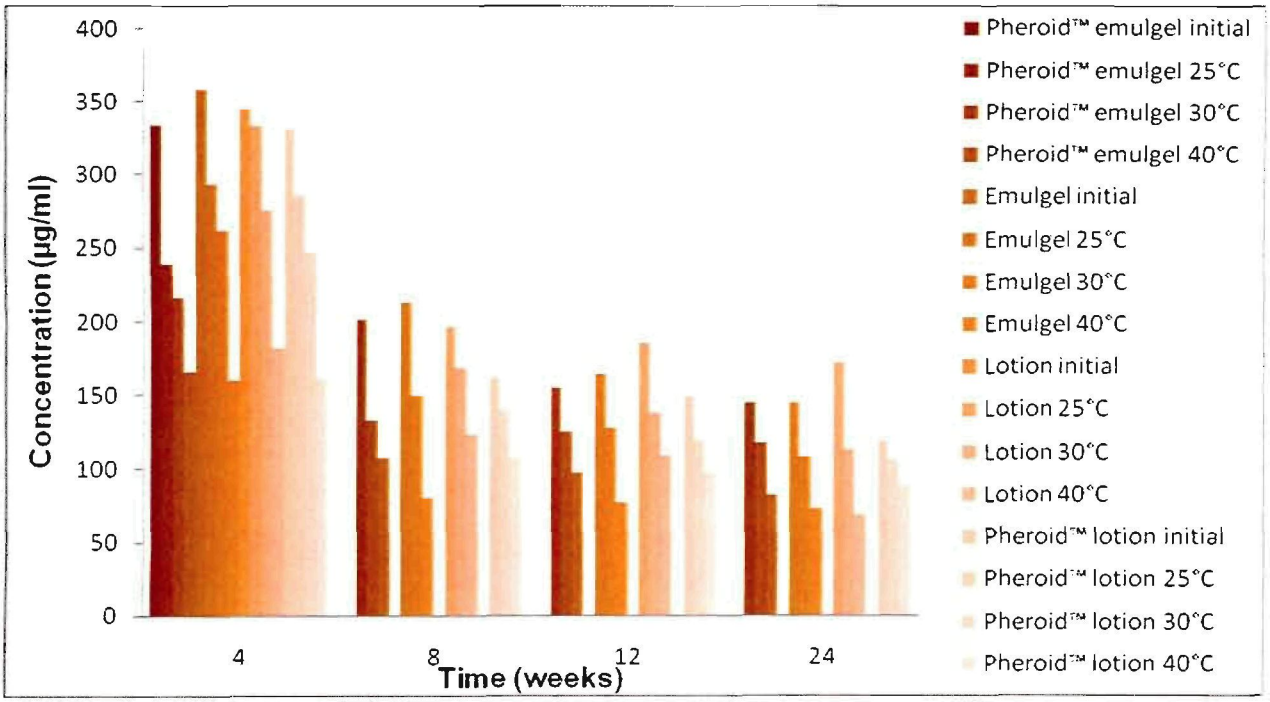


Figure B.4: Concentration (µg/ml) of rifampicin in the four formulations lotion over 24 weeks.

The ICH (2003:13) stipulates that a 5 % difference in assay concentration can be considered to be a "significant" change". At 4 weeks the breakdown of isoniazide was already significant and for the Pheroid™ formulations it was well below 50 % of the initial concentration. Isoniazide was more stable in the non-Pheroid™ formulations, perhaps due to the less oily base and the hydrophilic character of isoniazide (Stephan *et al*, 2004:4167). At 25°C the concentration of rifampicin only dropped below 50 % after 12 weeks. The Pheroid™ has an oily base and rifampicin is lipophylic (Sousa, 2008:873). This might be the reason rifampicin was more stable in the Pheroid™ formulations and did not break down as much as isoniazide in any of the formulations.

B.3.3 pH

Over the 24 week period the pH never dropped below 5 and never rose above 6 in all four formulations. This makes it acceptable as it is in the same range as the pH of skin that physiologically is between 5 and 6 (Elsner, 2006:36).

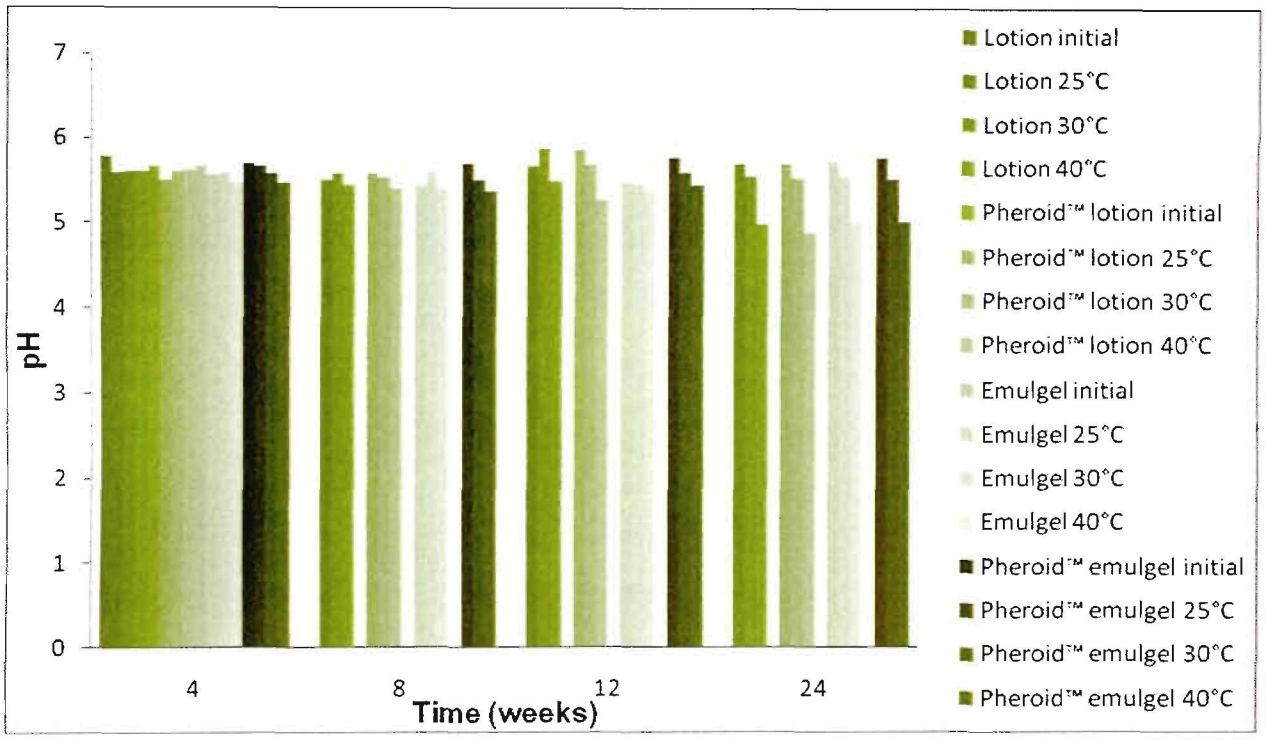


Figure B.5: pH stability results over 24 weeks.

B.3.4 Weight loss

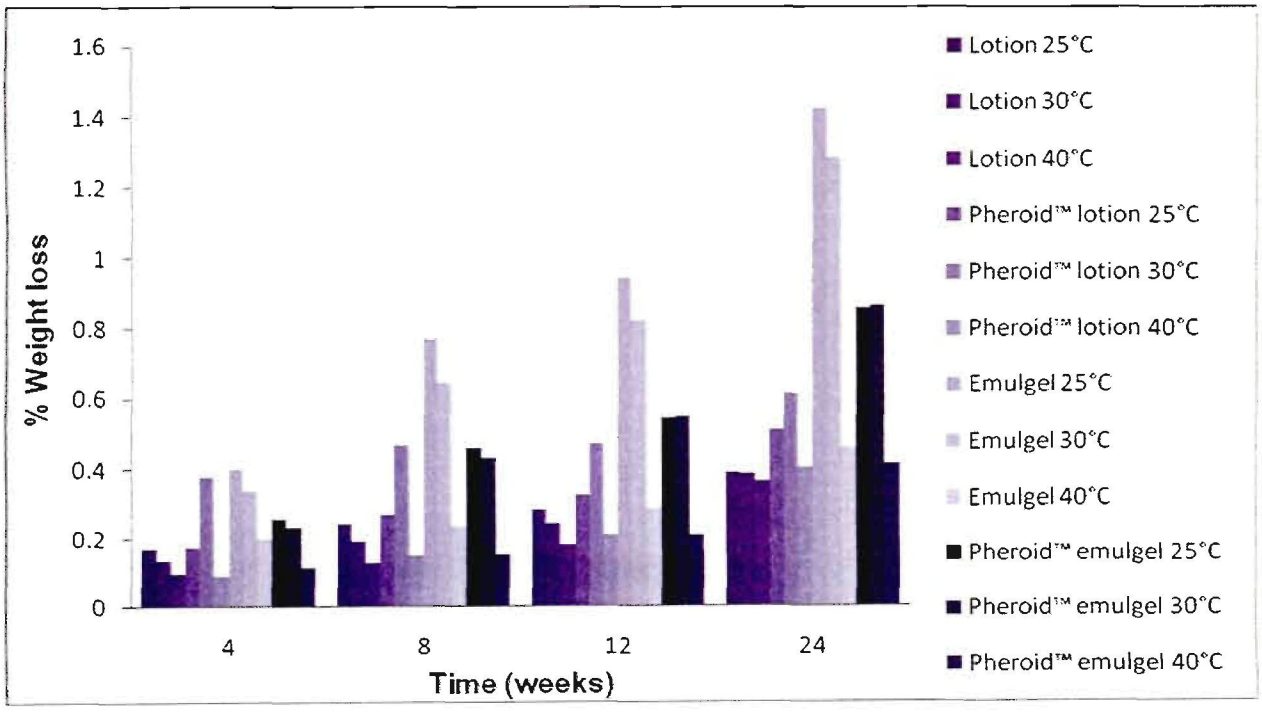


Figure B.6: Weight loss stability results over 24 weeks.

Weight loss over the 24 week period was not significant in any of the four formulations as the maximum was 1.4 % for the emulgel after 24 weeks. Weight loss at 40°C/75 % RH was less than at the lower temperatures and this could be due to the high humidity and possible porosity of the containers, letting water or steam through.

B.3.5 Viscosity

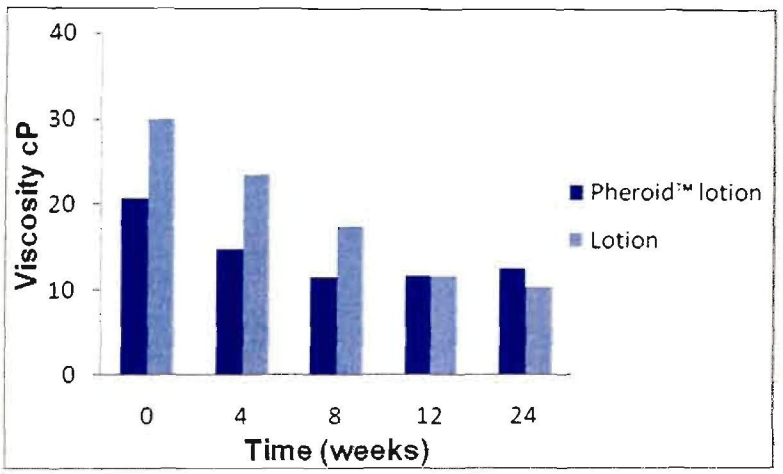


Figure B.7: Viscosity stability results for lotion and Pheroid™ lotion over 24 weeks.

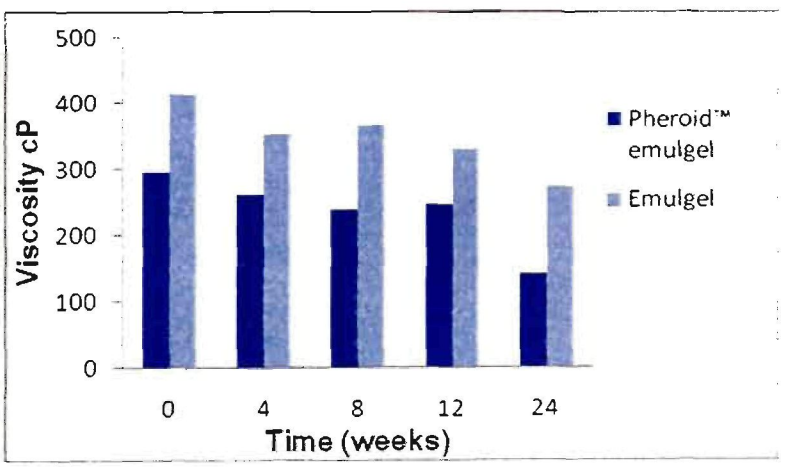


Figure B.8: Viscosity stability results for emulgel and Pheroid™ emulgel over 24 weeks.

According to Vaughan (1997:201), if a formulation remains uniform during stability testing, viscosity changes are acceptable. The viscosity of all four formulations dropped significantly over the 24 week period, mainly due to the elevated temperatures. Because there were minimal changes in the uniformity of all the formulations as was noted in the CLSM microscopy, it can be accepted that the formulations were stable. The lower viscosities can be ascribed to changes in the formulation, such as the breakdown of the actives, as found in the assay, and

possibly incompatibilities between constituents or breakdown of the Pheroid™. The viscosity of the emulgel seemed to be least affected by temperature, perhaps due to a higher initial viscosity and a less oily base.

B.3.6 Particle size

Particles smaller than 3 µm can cross the stratum corneum, paticles between 3 and 10 µm can enter follicles and particles larger than 10µm stay on the skin surface (Barry, 2002:31). All four formulations initially had uniformly distributed particles with no visible crystals and the particle sizes of the oil droplets were smaller than 10 µm, indicating that they would be able to cross the skin barrier. The droplet size of the lotion was somewhat larger than for the other formulations and thus they would only be able to cross the skin by the transfollicular route. For the lotion at 25°C the particle size showed no change in the 24 week period and the distribution was only altered after 24 weeks. The oil droplets of the lotion at 30°C started to increase after 8 weeks to larger than 10 µm, meaning they would stay on the skin surface and the distribution was only altered after 24 weeks. At 40°C the droplets of the lotion started to increase to over 10 µm and the distribution was altered after only 4 weeks

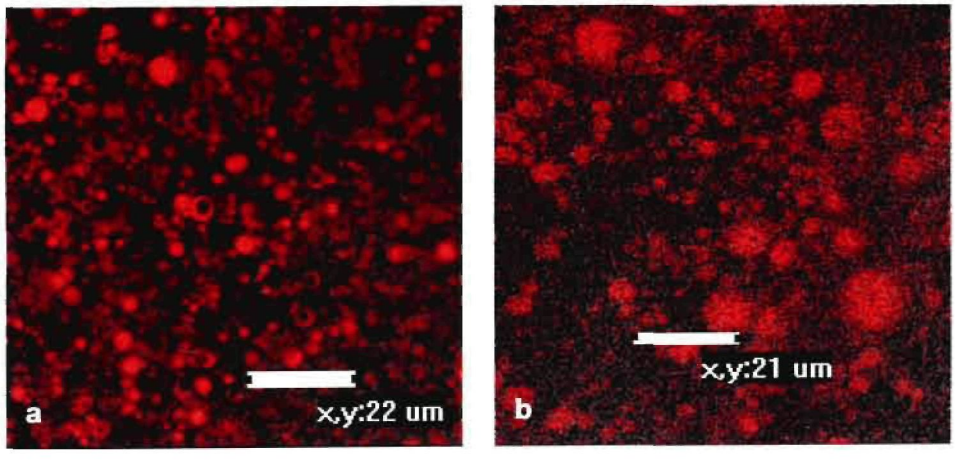


Figure B.9: CLSM micrographs of lotion **a)** initially and **b)** after 24 weeks at 40°C.

For the Pheroid™ lotion at 25°C the distribution showed no change during the 24 week period and the oil droplets only started to increase in size after 24 weeks to larger than 10 µm. The oil droplets of the Pheroid™ lotion at 30°C started to enlarge after 12 weeks to bigger than 10 µm and the distribution was not altered in the 24 week period. At 40°C the droplets of the Pheroid™ lotion started enlarging to over 10 µm after 12 weeks and the distribution was not altered after 24 weeks.

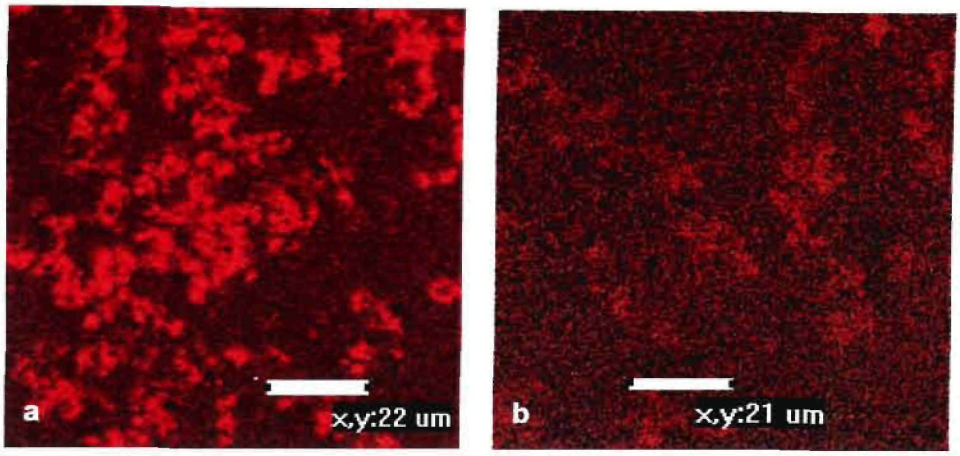


Figure B.10: CLSM micrographs of Pheroid™ lotion **a)** initially and **b)** after 24 weeks at 40°C.

For the emulgel the particle distribution showed no change during the 24 week period and the oil droplets did not enlarge after 24 weeks to bigger than 10 µm at 25°C or 30°C. At 40°C the droplets of the emulgel started enlarging to over 10 µm after 24 weeks and the emulsion seemed to separate.

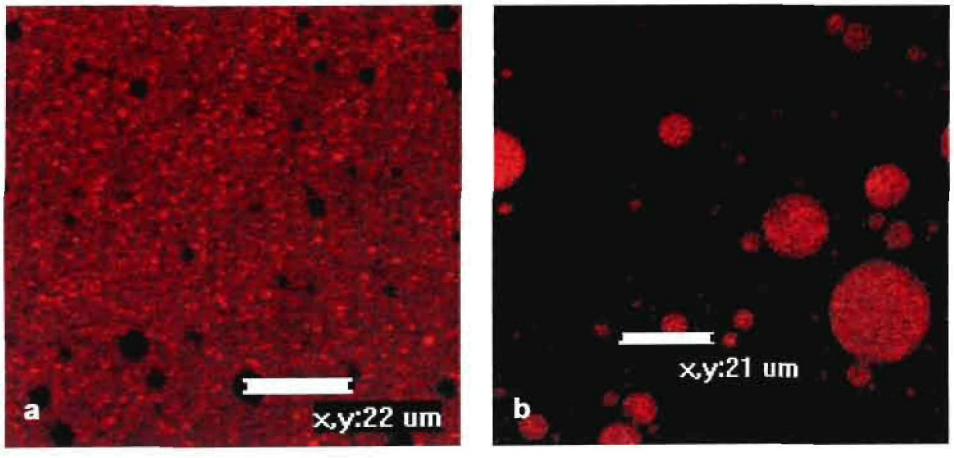


Figure B.11: *CLSM micrographs of emulgel* **a)** *initially and* **b)** *after 24 weeks at 40°C.*

For the Pheroid™ emulgel the particle distribution showed no change during the 24 week period and the oil droplets did not enlarge after 24 weeks to bigger than 10 µm at 25, 30 or 40°C.

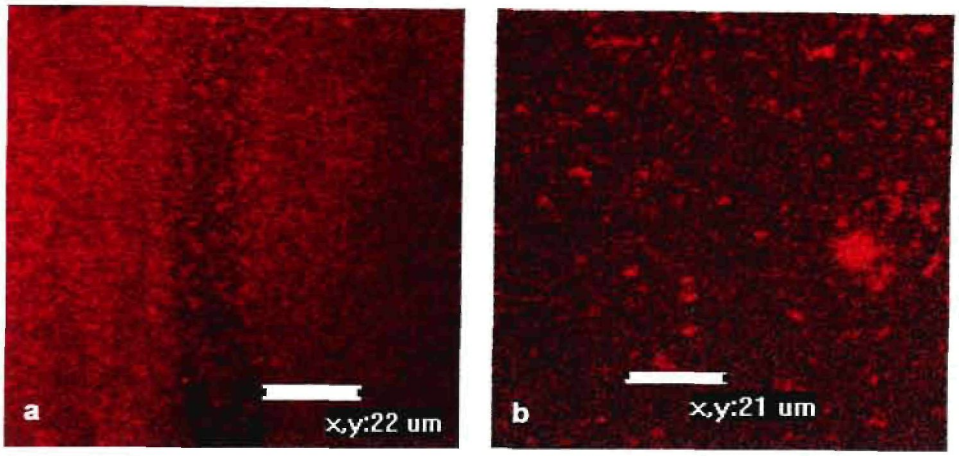


Figure B.12: CLSM micrographs of Pheroid™ emulgel **a)** initially and **b)** after 24 weeks at 40°C.

The size of the oil droplets of all four formulations as well as the droplet appearance and distribution was stable at 25°C. It was clear that the Pheroid™ improved the homogeneity of the formulations and prevented the aggregation of oil droplets. The Pheroid™ emulgel proved to be the more stable formulation, even at 40°C.

B.3.7 Colour changes

The colour of the lotion started to change after 24 weeks at 25°C, slightly more after 24 weeks at 30°C and significantly changed after only 4 weeks at 40°C. At 24 weeks at 40°C rifampicin precipitated as dark reddish-brown particles on top of the lotion. No phase separation was found, except at 40°C after 24 weeks.

Table B.6: Colour changes of lotion over 24 weeks.

	Initial	4 weeks	8 weeks	24 weeks
25°C				
30°C				
40°C				

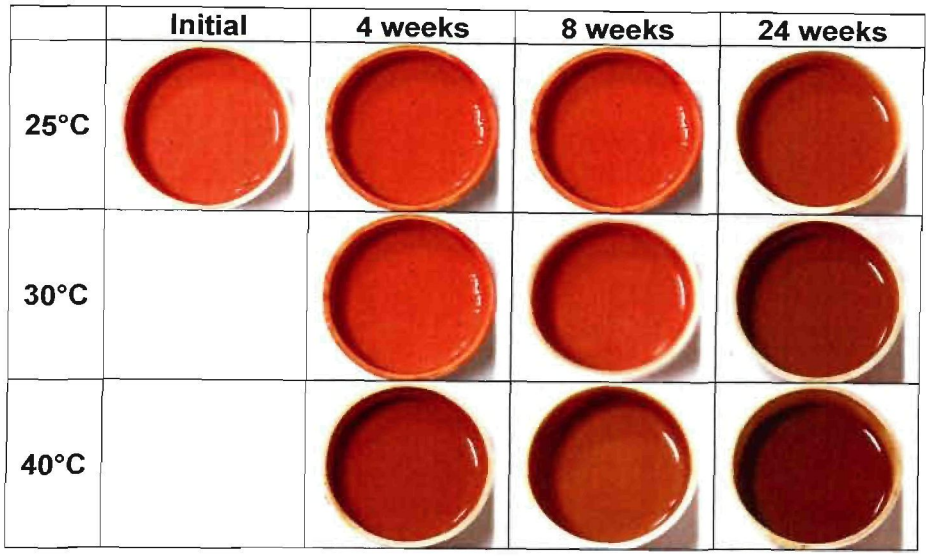
The colour of the Pheroid™ lotion changed after 24 weeks at 25°C, slightly more after 8 weeks at 30°C and started changing significantly after only 4 weeks at 40°C. No precipitation or phase separation was observed.

Table B.7: Colour changes of Pheroid™ lotion over 24 weeks.

	Initial	4 weeks	8 weeks	24 weeks
25°C				
30°C				
40°C				

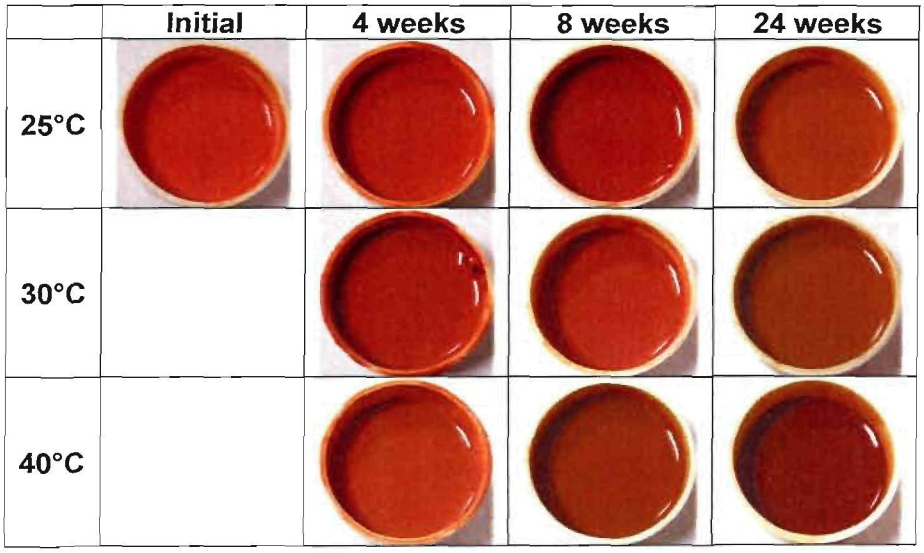
The colour of the emulgel changed after 24 weeks at 25°C, slightly more after 24 weeks at 30°C and started changing significantly after only 4 weeks at 40°C. No precipitation was found and slight phase separation after 24 weeks at 40°C could be seen.

Table B.8: Colour changes of emulgel over 24 weeks.



The colour of the Pheroid™ emulgel changed after 24 weeks at 25°C, slightly more after 24 weeks at 30°C and started changing significantly after 8 weeks at 40°C. No precipitation was found and definite phase separation after 24 weeks at 40°C could be seen.

Table B.9: Colour changes of Pheroid™ emulgel over 24 weeks.



The strong orange colour of the formulations is attributed to the inclusion of rifampicin, thus the changes in colour may indicate the breakdown of the compound, as is evident in the HPLC analyses, or it might possible be an indication of incompatibilities between constituents (Vaughan, 1997:202). Phase separation due to the aggregation of oil droplets was only visible

after 24 weeks and at 40°C, making all four formulations relatively stable. Again the Pheroid™ emulgel seemed to be the more stable formulation.

B.4 CONCLUSION

A combination of isoniazide and rifampicin was formulated into Pheroid™ and non-Pheroid™ lotions and emulgels and these formulations were used in the diffusion studies and stability testing. Stability testing proved the Pheroid™ formulations to be more stable than their non-Pheroid™ equivalents and the Pheroid™ emulgel holds some promise if altered to prevent the breakdown of the actives, especially isoniazide.

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APPENDIX C: VALIDATION OF THE HPLC EXPERIMENTAL METHOD FOR ISONIAZIDE AND RIFAMPICIN IN PHEROID™ AND NON-PHEROID™ FORMULATIONS

C.1 INTRODUCTION

The purpose of this validation is to demonstrate that the HPLC method implemented is reliable and sensitive enough to determine the amounts of isoniazide and rifampicin in the four different topical formulations during the six months of stability testing.

C.2 SUMMARY

Table C.1: Summary of validation of isoniazide and rifampicin.

Test	Isoniazide	Rifampicin
Specificity	Complies	Complies
Range	140-200 µg/ml	280-400 µg/ml
Linearity	$r^2 = 0.998$	$r^2 = 0.996$
Accuracy	100.58 %	96.61 %
Precision	RSD 0.35 %	RSD 0.74 %

C.3 CHROMATOGRAPHIC CONDITIONS

Analytical instrument

Agilent series 1100 HPLC with a pump, auto sampler, UV detector and Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software.

Column

Phenomenex®, Jupiter 5u, C18, 300A, 250 x 4.6 mm, 5 µm column.

Mobile phase

Isoniazide and rifampicin were separated by means of gradient elution. The two solvents of the mobile phase were:

- A degassed mixture of octane-sulphonic acid ($C_8H_{17}NaO_3S$) in HPLC (deionised) water. The pH was adjusted to 3.5 with 10 % phosphoric acid (H_3PO_4)

- Methanol (HiperSolv for HPLC)

The composition of the mobile phase is listed in table C.2.

Table C.2: Mobile phase with monobasic ammonium phosphate buffer (A) and methanol (B).

Mobile phase composition A:B		Duration (min)
50	50	1
0	100	8
0	100	24
50	50	25

Flow rate: 1.0 ml/min

Injection volume: 10 µl

Detection: UV at 254.4 nm

Retention time: Isoniazide – 3.4 min

Rifampicin – 9.8 min

Stop time: 25 min

Solvent: Mobile phase

C.4 SAMPLE PREPARATION

The formulation (2 g) was accurately weighed into a 50 ml volumetric flask and filled to volume with methanol. A quantity was then transferred to amber HPLC vial and analysed.

C.5 STANDARD PREPARATION

The quantities in Table C.3 were accurately weighed in a 100 ml volumetric flask and filled to volume with methanol. A quantity was then transferred to amber HPLC vial and analysed.

Table C.3: Quantities for standard preparation.

Ingredient	Quantity (mg)
Rifampicin	40.0
Isoniazide	20.0
Methylparaben	16.0
Propylparaben	3.2
BHT	8.0
dl-α-tocopherol	8.0

C.6 VALIDATION TEST PROCEDURE AND ACCEPTANCE CRITERIA

C.6.1 Specificity

- Prepare a placebo as described in Section C.6.3.1. Prepare a sample from the placebo as described in the method under sample preparation (Section C.4).
- Inject in duplicate.
- Prepare a sample from the topical preparation as described in the method under sample preparation (Section C.4).
- Dilute the sample solution 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide.
- Store these solutions overnight in closed test tubes at 40°C to degrade.
- Inject the samples into the HPLC with a run time of 30 min.
- Examine the chromatograms to determine whether any additional peaks were formed.

ACCEPTANCE CRITERIA:

The sample should not contain any peaks that will interfere with the determination of isoniazide or rifampicin.

C.6.2 Linearity

- Prepare a standard as described in the method under standard preparation (Section C.5).
- Inject 7, 8, 9, 10, 11, 12 and 13 µl of this standard solution in duplicate into the HPLC.

ACCEPTANCE CRITERIA:

Linear regression analysis should yield a regression coefficient (r^2) of ≥ 0.99.

C.6.3 Accuracy

C.6.3.1 Placebo of formulations

- Accurately weigh 3 x 1.6 g, 3 x 2 g and 3 x 2 4 g of placebo into 100 ml volumetric flasks.
- Add 8 ml, 10 ml and 12 ml of the 500 % standard solution respectively to each flask. Fill to volume with methanol. This is done in order to obtain 9 spiked placebo samples with known amounts of active at concentrations of approximately 80 %, 100 % and 120 % of the expected sample concentration.
- Inject the samples into the HPLC in duplicate.

Table C.4: Quantities for placebo preparation.

Ingredient	Quantity (g)
Oleic acid	14.0
Cetyl alcohol	2.0
Tween 80	5.0
Span 60	1.5
Vitamin F ethyl ester	2.8
Cremophor RH 40	1.0
Distilled water	71.9

ACCEPTANCE CRITERIA:

Recovery must be between 98-102 %.

C.6.4 Precision

C.6.4.1 Intra-day precision (repeatability)

- Weigh 3 x 1.6 g, 3 x 2 g and 3 x 2.4 g of the same batch of topical formulation into 50 ml volumetric flasks (9 flasks).
- Prepare a single standard at 100 % of the expected sample concentration as described in the method.

- Inject into the HPLC in duplicate.

ACCEPTANCE CRITERIA:

Repeatability must be better than 2 % (n = 9).

C.6.4.2 Inter-day precision

Analyse the same batch of topical formulation again in triplicate as described above for intra-day precision (at 100 % of the sample concentration) on two more days to determine the between-day variability of the method. If possible, a different analyst should perform the analysis, preferably using different equipment.

ACCEPTANCE CRITERIA:

Inter- day precision must be better than 5 % (n = 9).

C.6.5 Ruggedness

C.6.5.1. Stability of sample solutions

- Prepare a sample as described under sample preparation (Section C.4).
- Inject the sample into the HPLC.
- Leave the sample in the auto sampler tray and reanalyse at hourly intervals up to 12 h to determine the stability of the sample.
- Program the pump to reduce the flow rate from 1.0 to 0.1 ml/min after elution of the peak, and reset the flow rate to 1 ml/min 5 min before injecting the next sample.

ACCEPTANCE CRITERIA:

Sample solutions should not be used for a period longer than it takes to degrade by 2 %, and in this case special precautions should be followed to compensate for the degradation.

C.6.5.2 System repeatability

Inject a sample or standard six times consecutively in order to test the repeatability of the peak area as well as the retention time.

ACCEPTANCE CRITERIA:

The peak area and retention times should have an RSD of 2 % or less.

C.7 VALIDATION RESULTS

C.7.1 Specificity

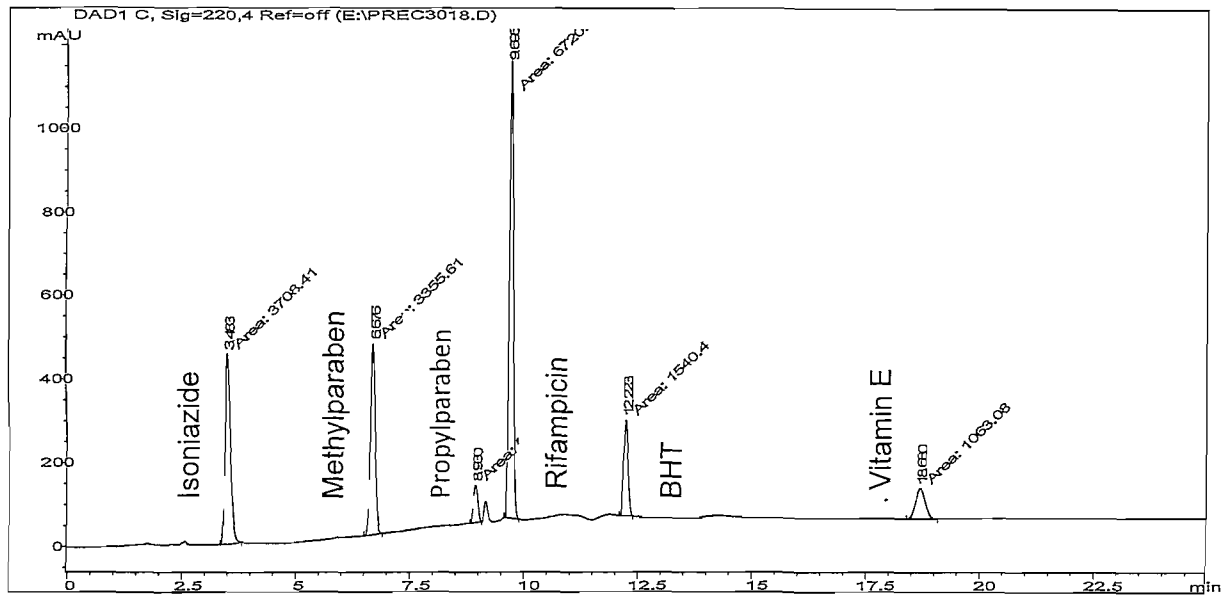

Figure C.1: Chromatogram of a standard solution.

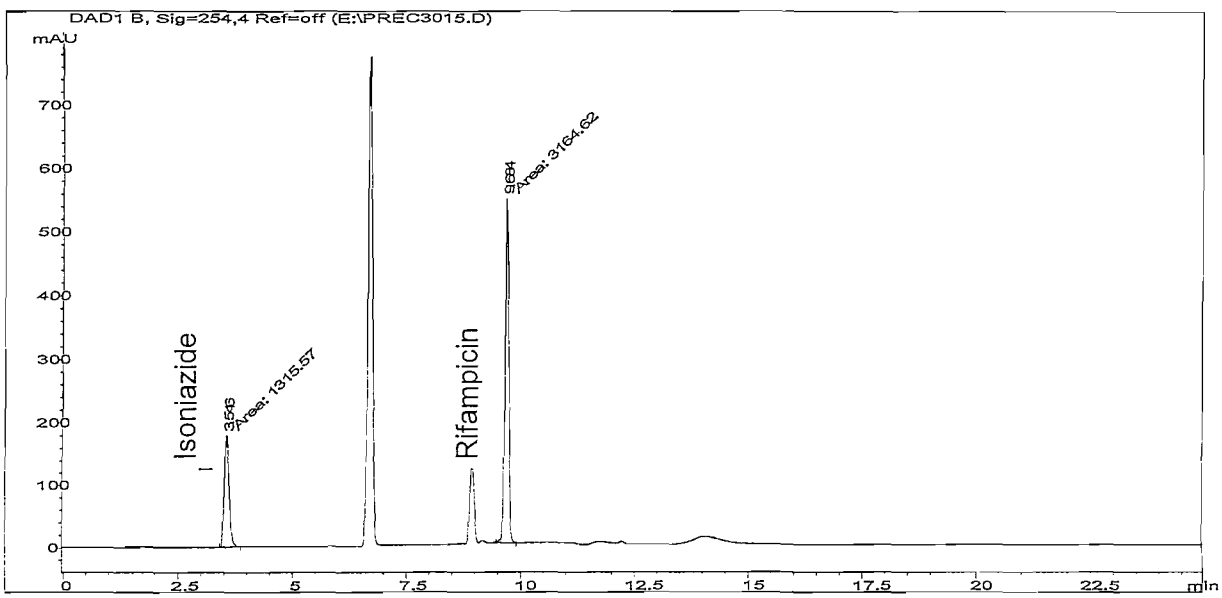

Figure C.2: Chromatogram of a sample solution stressed in water at 40°C for 24 h.

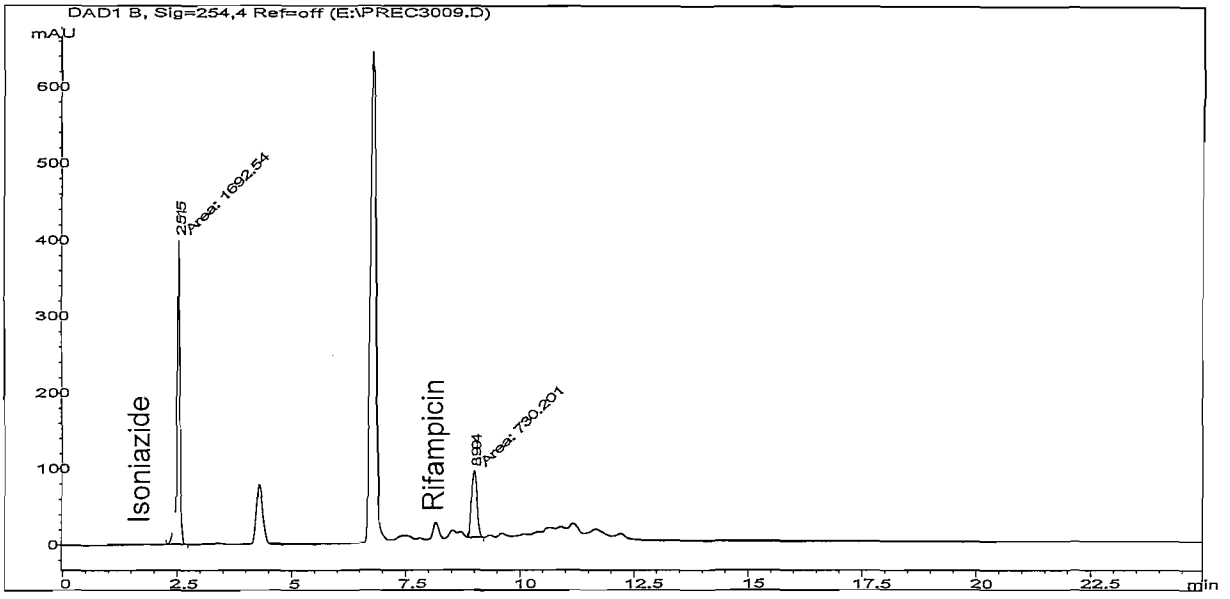

Figure C.3: Chromatogram of a sample solution stressed in 0.1 M hydrochloric acid at 40°C for 24 h.

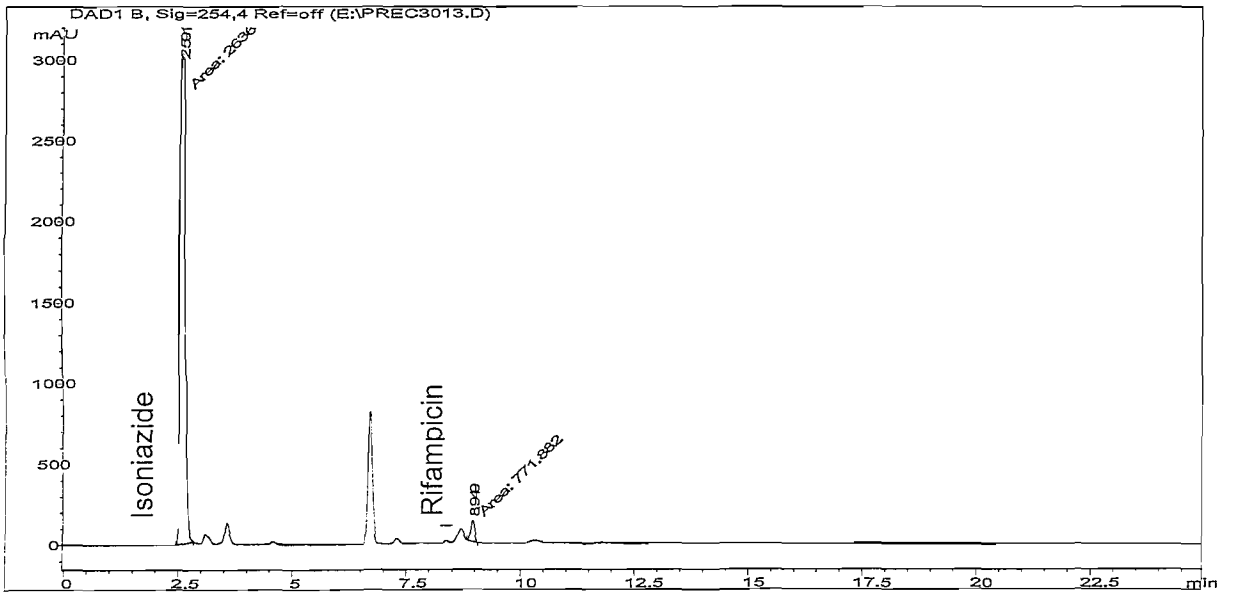

Figure C.4: Chromatogram of a sample solution stressed in 10 % hydrogen peroxide at 40°C for 24 h.

None of the ingredients in the placebo interfered with the analyte peaks. Extra peaks formed during forced degradation but it did not interfere with the remainder of the analyte peaks. Peak purity testing of the remaining peaks after forced degradation in water showed that the peaks were still pure, thus proving that the method is stability-indicating.

C.7.2 Linearity and range

C.7.2.1 Results

Table C.5: *Linearity results for isoniazide.*

Isoniazide:	Concentration (µg/ml)	Area		Mean
70 %	140	4002.0	3988.3	3995.15
80 %	160	4599.3	4607.5	4603.40
90 %	180	5197.0	5165.2	5181.10
100 %	200	5843.2	5740.1	5791.65
110 %	220	6354.5	6311.9	6333.20
120 %	240	6903.4	6753.8	6828.60

Table C.6: Linearity results for rifampicin.

Rifampicin:	Concentration (µg/ml)	Area		Mean
70 %	280	4128.4	4116.5	4122.45
80 %	320	4719.3	4651.0	4685.15
90 %	360	5354.7	5320.9	5337.80
100 %	400	5787.5	5799.7	5793.60
110 %	440	6545.9	6554.9	6550.40
120 %	480	7215.1	7180.4	7197.75

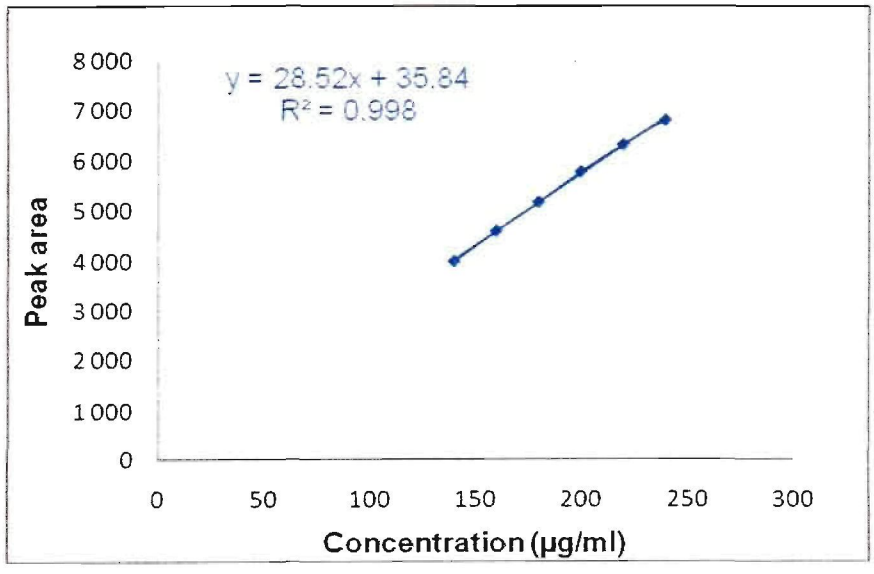

Figure C.5: Linear regression graph for isoniazide.

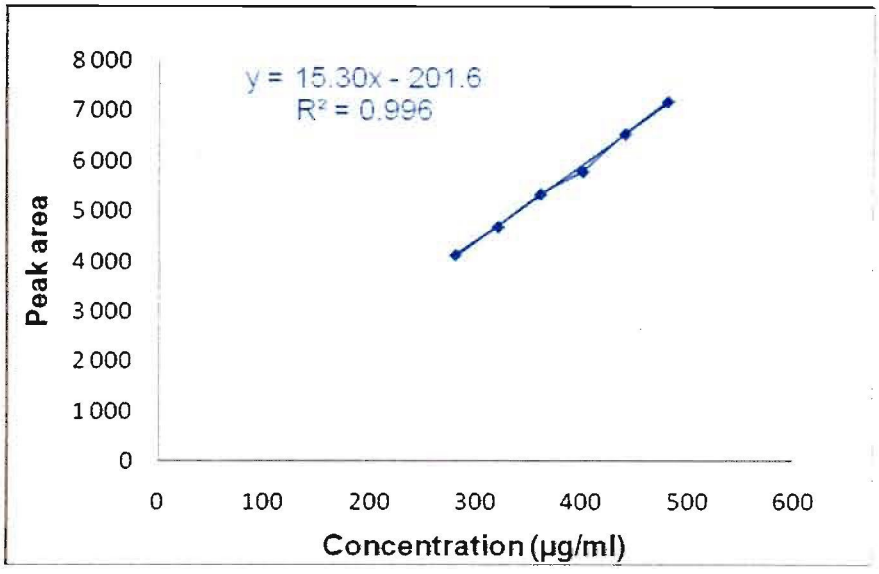

Figure C.6: Linear regression graph for rifampicin.

The method is linear for isoniazide over the concentration range 140-240 µg/ml and for rifampicin over the concentration range 240-480 µg/ml. The method is suitable for single point calibration.

C.7.3 Accuracy

Table C.7: Recovery for isoniazide.

Concentration spiked (µg/ml)	Area		Mean	Recovery (µg/ml)	%
160	2259.3	2259.2	2259.25	164.166	102.604
160	2286.8	2201.9	2244.35	163.083	101.927
160	2230.7	2227.9	2229.30	161.990	101.243
200	2806.1	2791.8	2798.95	203.383	101.691
200	2792.8	2756.8	2774.80	201.628	100.814
200	2793.1	2778.3	2785.70	202.420	101.210
240	3293.0	3239.0	3266.00	237.320	98.883
240	3213.1	3223.3	3218.20	233.847	97.436
240	3286.6	3282.7	3284.65	238.675	99.448

Statistical analysis	
Mean	100.58
SD	1.566
% RSD	1.557

Table C.8: Recovery for rifampicin.

Concentration spiked (µg/ml)	Area		Mean	Recovery (µg/ml)	%
320	5997.4	5993.0	5995.20	326.454	102.0168
320	5716.4	5708.3	5712.35	311.052	97.204
320	5852.7	5809.3	5831.00	317.513	99.223
400	7304.6	7277.3	7290.95	397.011	99.253
400	7240.2	7129.9	7185.05	391.244	97.811
400	7186.2	7156.6	7171.40	390.501	97.625
480	8208.7	8071.9	8140.30	443.260	92.346
480	7992.0	8020.1	8006.05	435.950	90.823
480	8230.9	8192.7	8211.80	447.153	93.157

Statistical analysis	
Mean	96.61
SD	3.488
% RSD	3.610

Over the range of 80-120 % of the sample concentration, the method yielded a mean recovery of 100.6 % for isoniazide and 96.6 % for rifampicin.

C.7.4 Precision

C.7.4.1 Intermediate (intra-day) precision

Table C.9: Intra-day precision for isoniazide.

Concentration spiked (µg/ml)	Area		Mean	Recovery (µg/ml)	%
200	2806.1	2791.8	2798.95	196.984	98.492
200	2792.8	2756.8	2774.80	195.285	97.642
200	2793.1	2778.3	2785.70	196.052	98.026

Statistical analysis	
Mean	98.05
SD	0.347
% RSD	0.354

Table C.10: Intra-day precision for rifampicin.

Concentration spiked (µg/ml)	Area		Mean	Recovery (µg/ml)	%
32	7304.6	7277.3	7290.95	31.761	99.253
32	7240.2	7129.9	7185.05	31.300	97.811
32	7186.2	7156.6	7171.40	31.240	97.625

Statistical analysis	
Mean	98.23
SD	0.727
% RSD	0.740

Precision was satisfactory with a RSD of 0.35 % for isoniazide and 0.74 % for rifampicin.

C.7.4.2 Inter-day precision

Table C.11: Inter-day precision for isoniazide.

	Day 1	Day 2	Day 3	Between days
	91.62	90.63	90.13	
	85.71	86.02	85.32	
	90.64	92.08	89.82	
Mean	89.32	89.58	88.42	89.11
SD	2.59	2.58	2.20	0.49
% RSD	2.90	2.88	2.49	0.56

Table C.12: Inter-day precision for rifampicin.

	Day 1	Day 2	Day 3	Between days
	94.38	92.84	92.32	
	85.78	85.77	84.88	
	94.76	95.54	94.5	
Mean	91.64	91.38	90.57	91.20
SD	4.15	4.12	4.12	0.46
% RSD	4.52	4.51	4.55	0.50

The inter-day and the intra-day variance were not significantly different. The repeatability is within acceptable limits, and the assay should perform well, even when executed by other personnel in a different laboratory.

C.7.5 Ruggedness

C.7.5.1 Stability of sample solutions

A sample was left on the auto sampler tray and re-analyzed over several time intervals to determine the sample stability.

C.7.5.1.1 Results

Table C.13: 12 h Stability of isoniazide.

Time(h)	Peak Area	%
0	4845.6	100
1	4876.7	100.642
2	4858.3	100.262
3	4900.3	101.129
4	4875.2	100.611
5	4891.6	100.949
6	4875.8	100.623
7	4832.3	99.726
8	4835.3	99.787
9	4853.0	100.153
10	4875.6	100.619
11	4832.1	99.721
12	4861.4	100.326
Mean	4862.60	100.350
SD	21.41	0.442
RSD %	0.44	0.440

Table C.14: 12 h Stability of rifampicin.

Time(h)	Peak Area	%
0	4155.4	100
1	4112.2	98.960
2	4206.3	101.225
3	4256.7	102.438
4	4275.6	102.893
5	4330.4	104.211
6	4348.9	104.657
7	4285.3	103.126
8	4386.9	105.571
9	4469.1	107.549
10	4476.6	107.730
11	4512.8	108.601
12	4560.0	109.737
Mean	4336.63	104.361
SD	134.09	3.227
RSD %	3.09	3.092

Both isoniazide and rifampicin are stable over a period of 12 h.

C.7.5.2 System repeatability

A sample was injected six times in order to test the repeatability of the peak area as well as the retention time.

C.7.5.2.1 Results

Table C.15: Repeatability for isoniazide.

	Peak area	Retention times (min)
	1170.6	2.72
	1165.5	2.72
	1158.5	2.72
	1166.6	2.72
	1176.3	2.72
	1166.2	2.72
Mean	1167.30	2.72
SD	5.39	0.00
RSD %	0.46	0.00

Table C.16: Repeatability for rifampicin.

	Peak area	Retention times (min)
	2429.4	9.73
	2392.4	9.72
	2405.8	9.73
	2438.7	9.71
	2438.8	9.70
	2388.7	9.71
Mean	2415.63	9.72
SD	20.90	0.01
RSD %	0.87	0.11

System performance proved well with RSD values of 0.46 % for peak area and 0.00 % for retention time for isoniazide and RSD values of 0.87 % for peak area and 0.11 % for retention time for rifampicin.

C.8 CONCLUSION

The method performed well and should be suitable to analyse isoniazide and rifampicin in the formulations for stability testing, quality control and batch release purposes. No interference were encountered from stressed samples or known related substances, thus the method can be regarded as being stability-indicating.

APPENDIX D: FRANZ CELL DIFFUSION STUDIES

D.1 INTRODUCTION

A membrane diffusion study was conducted to determine whether the actives were released from the formulation. Vertical Franz cell diffusion studies were implemented to determine whether the actives, isoniazide and rifampicin, could be successfully transported across the skin when formulated into topical preparations and whether the incorporation of the Pheroid™ drug delivery system would improve this transportation. Membrane studies were performed, implementing a cellulose acetate membrane, to determine drug release from the formulations, after which diffusion studies were performed with human skin to determine transdermal diffusion of the actives.

D.2 METHODS AND MATERIALS

D.2.1 Donor and receptor phase preparation

For both the skin and membrane diffusion studies the donor and receptor phases were the same. The donor phase was the four formulations: lotion, Pheroid™ lotion, emulgel and Pheroid™ emulgel. The receptor phase was a mixture of 20 % ethanol and 80 % phosphate buffer solution (PBS). The organic solvent, ethanol, was added to ensure that the lipophilic Pheroid™ and rifampicin can be fully dissolved. PBS was prepared as follows:

- Weigh 3.174 g of sodium hydroxide and 13.620 g of potassium dihydrogen orthophosphate.
- Dissolve in water and dilute to 1000 ml.
- Adjust the pH to 7.4 with 10 % phosphoric acid (British Pharmacopoeia, 2009).

D.2.2 Standard preparation

Rifampicin (20 mg) and isoniazide (10 mg) were dissolved in a volumetric flask and made up to 50 ml with 20 % ethanol-PBS.

D.2.3 Skin preparation

The excised abdominal skin of Caucasian female patients who underwent abdominoplastic surgery was used. Ethical approval for the procurement and use of the skin was provided by

the Research Ethics Committee of the North-West University under reference number 04D08. Patients gave informed consent and their identities were kept anonymous. For this study full thickness skin was used, containing all the layers – stratum corneum, epidermis and dermis. Skin obtained after surgery was frozen within 24 h and the frozen subcutaneous fatty layer then removed with a scalpel, taking care not to damage the skin. The skin was then punched into discs of approximately 15 mm in diameter, all remaining blood wiped away and frozen again. Prior to the study the discs were thawed at room temperature and mounted onto the Franz diffusion cell.

D.2.4 Franz cell diffusion method

Ten amber Franz diffusion cells were used because of the photosensitivity of both isoniazide and rifampicin (WHO, 2007:226). These cells consist of a donor compartment with a diffusion area of 1.075 cm^2 and a receptor compartment with a capacity of approximately 2 ml. The membranes were placed on the lower half of the amber vertical Franz diffusion cells, the donor compartments were placed on top, sealed with Dow-corning® vacuum grease and clamped with metal horseshoe clamps. A small magnetic stirrer bar was placed in each receptor compartment and it was filled with a solution of 20 % ethanol and 80 % PBS (pH 7.4), previously heated to 37°C. Care was taken to avoid the entrapment of air bubbles under the surface. The diffusion cells were placed in a tray on a Variomag® stirrer plate in order to continuously stir the receptor phase inside a Grant® water bath at 32°C, simulating the temperature of human skin (Azarmi *et al.*, 2007:17). The donor compartments were filled with 1 ml of the different formulations and covered with Parafilm® to prevent evaporation. The entire volume of the receptor compartment was withdrawn at 20 and 40 min, as well as at 1, 2, 4, 6, 8, 10 and 12 h and directly assayed by HPLC to determine the concentration of actives that permeated the skin.

This method was also used for skin diffusion studies, substituting the membranes with the thawed skin discs with the stratum corneum facing upwards.

D.2.5 Tape-stripping method

After the 12 h skin diffusion study, tape stripping was done to remove the stratum corneum and epidermis to determine the amounts of drug in it and also the amounts that permeated into the dermis. The Franz cells were dismounted and the donor compartments carefully removed. The skin discs were placed on Parafilm® and stapled onto a solid surface. The excess formulation was dabbed off with tissue paper and the diffusion area (approximately 11.70 mm in diameter) was still visibly indented. Pieces of tape (3M Scotch Magic® tape) were used to strip away the

epidermis, taking care to strip only the diffusion area. The first tape was discarded as it can be seen as part of the process of removing excess formulation. The following fifteen tapes were placed in 5 ml of 20 % ethanol-PBS solution, kept overnight at 4°C and a sample was then taken and analysed by HPLC. The diffusion area of the remaining skin was cut out into small pieces, placed in 2 ml of 20 % ethanol-PBS solution and centrifuged with an Eppendorf centrifuge 5804 R at 10°C and 14000 rpm for 10 min. A sample was then taken and analysed by HPLC.

D.2.6 HPLC analysis

Permeation samples were analysed using an Agilent® series 1100 HPLC with a pump, auto sampler, UV detector and Chemstation for LC 3D Systems Rev. A.08.03 [847] data acquisition and analysis software and a Phenomenex®, Jupiter 5u, C18, 300A, 250 x 4.6 mm, 5 µm column. Isoniazide and rifampicin were separated by gradient elution. The mobile phase was prepared as follows:

A: A degassed mixture of 0.01 M monobasic ammonium phosphate and 0.005 M 1-heptanesulphonic acid sodium salt in HPLC (deionised) water (pH 3.5).

B: Methanol

Table D.1: Mobile phase with monobasic ammonium phosphate buffer (A) and methanol (B).

Mobile phase composition A:B		Duration (min)
80	20	3.5
20	80	4.5
20	80	9.0
80	20	9.2

The injection volume was 20 µl, the flow rate was 1 ml/min, the total runtime was 13 min and the temperature was maintained at 25°C. Isoniazide and rifampicin were both detected at 263.4 nm with retention times were noted to be at 4.0 and 8.5 min, respectively.

D.2.7 Diffusion and statistical analysis

The average cumulative concentration for isoniazide and rifampicin for each formulation was determined by calculating the cumulative amount per area (µg/cm^2). For statistical analysis of the membrane diffusion study the median (centre of data) cumulative concentration was determined. The median is a more accurate method if there is a big variation in data (Gerber *et al.,* 2008). Statistical analysis of the data was performed by means of the Kruskal-Wallis test of

comparison. This is a nonparametric test for 3 or more groups of ordinal or continuous data and was used to calculate p-values, which measures the correctness of the statistical data. P-values are compared to the level of significance (5 % or 0.05) (Lang & Secic, 1997:71). A p-value less than the level of significance is statistically significant and implies a noticeable difference between the groups of data. A p-value of 1 would be equal to the level of significance and would imply that there is no difference between the groups. A p-value greater than the level of significance is not statistically significant, implying no noticeable difference between the groups (Lang & Secic, 1997:76). This test was two-tailed, which considers both possible endpoints in the data (Lang & Secic, 1997:72).

D.3 RESULTS AND DISCUSSION

D.3.1 Franz cell diffusion studies using cellulose acetate membranes to determine release of the drugs from the different formulations

In all four cellulose acetate membrane studies both isoniazide and rifampicin crossed the membrane, indicating drug release from all formulations. Statistical analyses were performed using the Kruskal-Wallis test for comparison and the median cumulative concentration of each formulation after 12 h was illustrated with box-plots.

D.3.1.1 Release of isoniazide from the four formulations

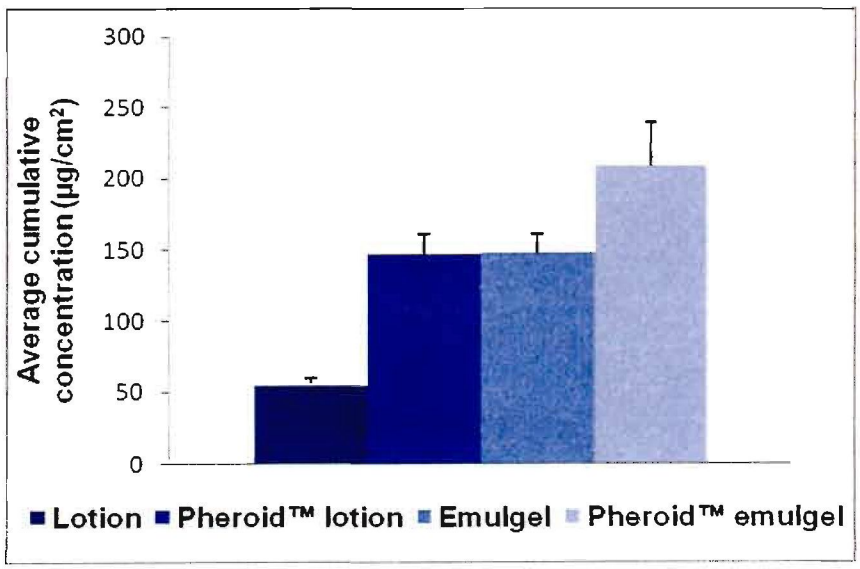

Figure D.1: Average cumulative concentration (µg/cm^2) of isoniazide diffused through cellulose acetate membranes after 12 h (n=10).

Table D.2: Kruskal-Wallis test - multiple comparisons and p-values for isoniazide.

	Emulgel	Pheroid™ emulgel	Lotion	Pheroid™ lotion
Emulgel		0.034763	0.042410	1.000000
Pheroid™ emulgel	0.034763		0.000000	0.029089
Lotion	0.042410	0.000000		0.032516
Pheroid™ lotion	1.000000	0.029089	0.032516	

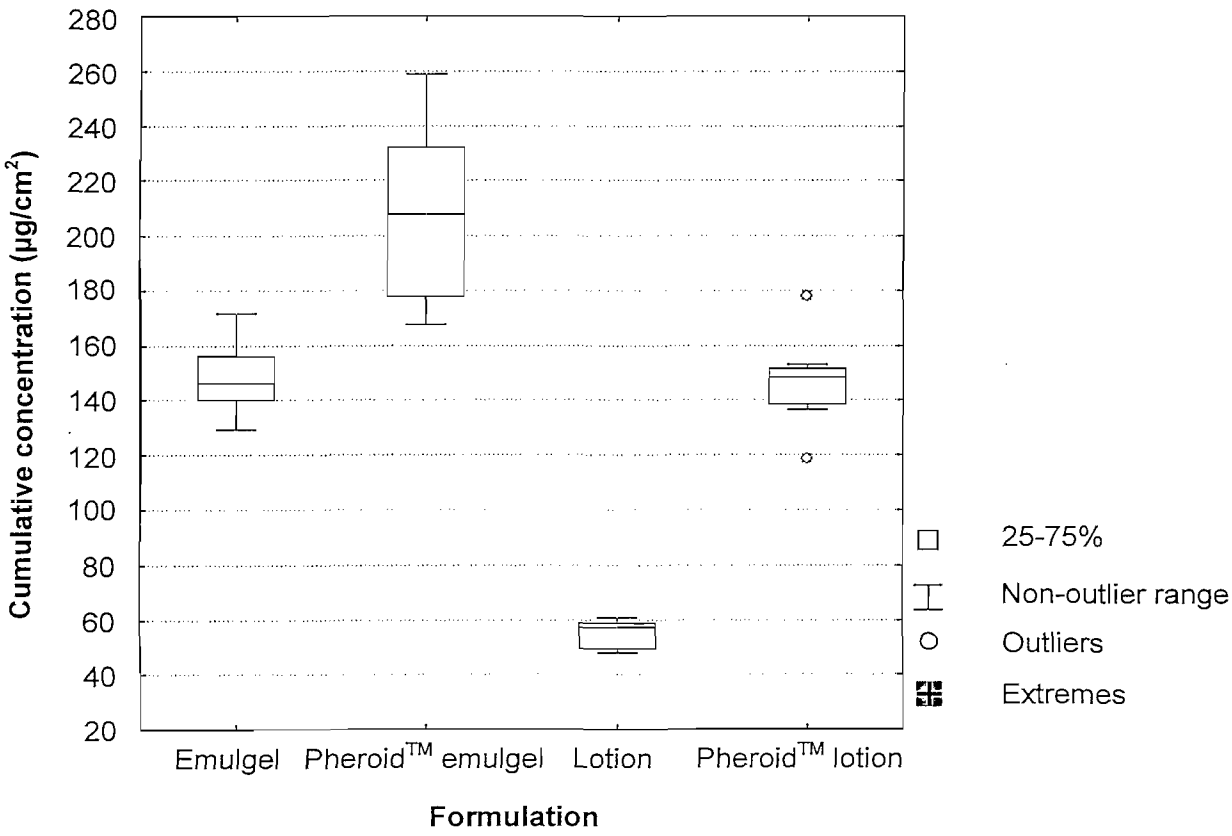

Figure D.2: Box-plots depicting the median cumulative concentration (µg/cm^2) of isoniazide diffused through cellulose acetate membranes after 12 h.

The average cumulative concentration of isoniazide in the receptor compartment after the membrane diffusion studies showed that it permeated through the membrane and thus released a percentage of the active from the formulations. The average percentage of isoniazide diffused was 0.593 % (lotion), 1.576 % (Pheroid™ lotion), 1.592 % (emulgel) and 2.249 % (Pheroid™ emulgel). Isoniazide was released 3.79 times more active from the Pheroid™ emulgel than from the lotion. The Pheroid™ emulgel clearly improved diffusion of isoniazide through the membrane. The release of isoniazide was best from the Pheroid™ emulgel and this

could be due to the oily base of the formulation and the hydrophilic nature of isoniazide, allowing isoniazide to diffuse freely into the watery solution. The box-plots clearly show the large variance in the Pheroid™ emulgel, small variance in the lotion, as well as in the Pheroid™ lotion and emulgel. The median cumulative concentration of isoniazide as shown in Figure D.2 was 57.35 µg/cm^2 (lotion), 146.25 µg/cm^2 (emulgel), 148.36 µg/cm^2 (Pheroid™ lotion) and 207.83 µg/cm^2 (Pheroid™ emulgel). The average of the cumulative concentration of isoniazide as shown in Figure D.1 was 55.17 µg/cm^2 (lotion), 146.53 µg/cm^2 (Pheroid™ lotion), 148.04 µg/cm^2 (emulgel) and 209.09 µg/cm^2 (Pheroid™ emulgel). The median and average cumulative concentration values did not differ much and therefore both median and average flux can be used to determine the cumulative concentration. The p-values confirm no differences between the emulgel and Pheroid™ lotion ($p = 1.0$) but statistically significant differences between all other formulations exist ($p < 0.05$) (Lang & Secic, 1997:76).

D.1.1.2 Release of rifampicin from the four formulations

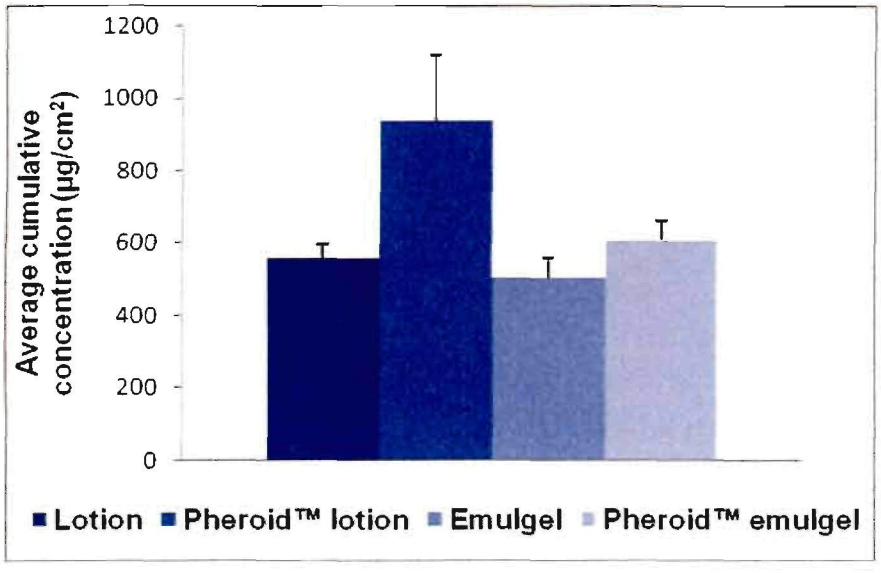

Figure D.3 Average cumulative concentration (µg/cm^2) of rifampicin diffused through cellulose acetate membranes after 12 h (n=10).

Table D.3: Kruskal-Wallis test - multiple comparisons and p values of rifampicin.

	Emulgel	Pheroid™ emulgel	Lotion	Pheroid™ lotion
Emulgel		0.076647	1.000000	0.000005
Pheroid™ emulgel	0.076647		0.913468	0.072380
Lotion	1.000000	0.913468		0.000485
Pheroid™ lotion	0.000005	0.072380	0.000485	

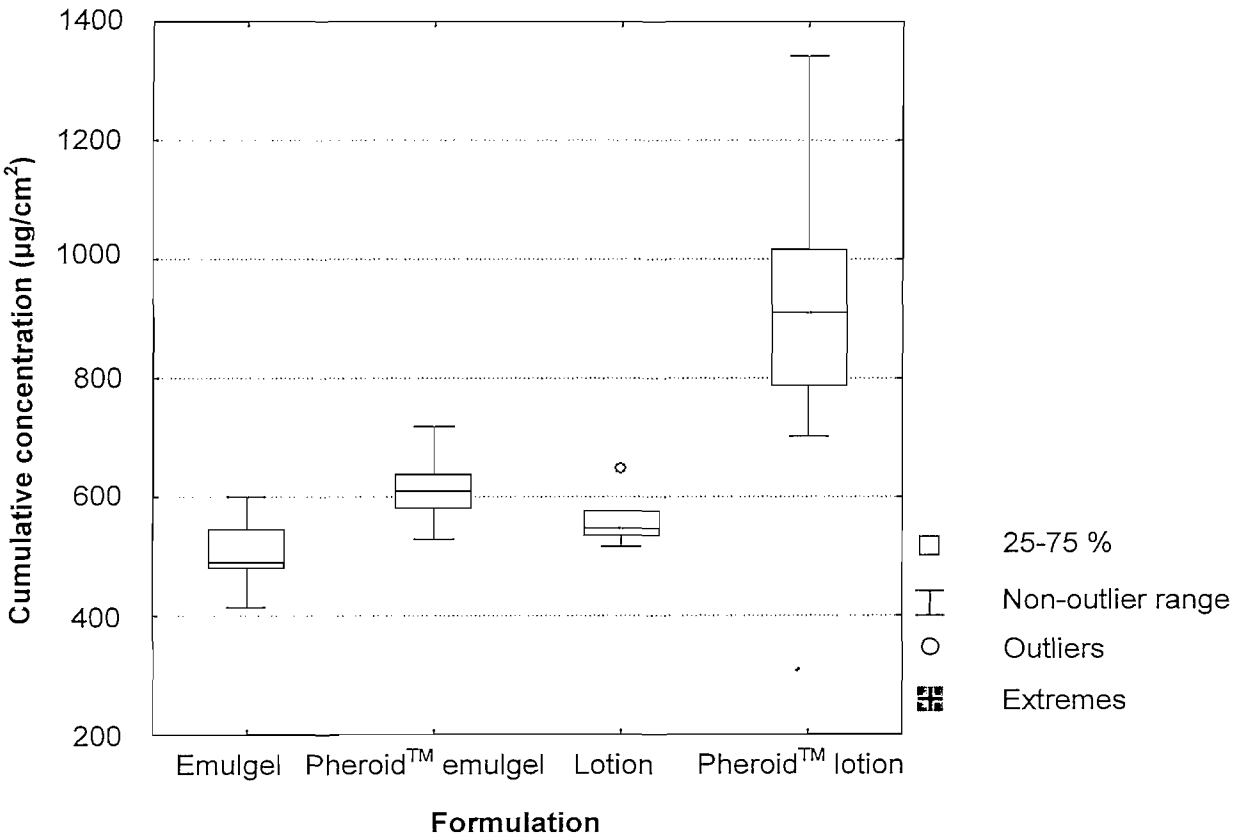

Figure D.4: Box-plots depicting the median cumulative concentration (µg/cm^2) of rifampicin diffused through cellulose acetate membranes after 12 h.

The average cumulative concentration of rifampicin in the receptor compartment after the membrane diffusion studies showed that it permeated through the membrane and that is was thus released from the formulations. The average percentage diffused of emulgel (2.726 %) was the lowest, followed by the lotion (2.991 %), the Pheroid™ emulgel (3.280 %) and lastly, by the Pheroid™ lotion (5.049 %). Rifampicin was released 1.852 times more active from the Pheroid™ lotion than from the emulgel. The Pheroid™ technology improved diffusion of rifampicin through the membrane. Release was best from the Pheroid™ lotion and this could be

due to the watery base of the formulation and the lipophilic nature of rifampicin, allowing rifampicin to diffuse freely into the organic solution. In the box-plots it can be seen that the variance is greatest for the Pheroid™ lotion, but small for the lotion, emulgel and Pheroid™ emulgel. The median of the cumulative concentration of rifampicin as shown in Figure D.4 was 489.40 µg/cm^2 (emulgel), 549.46 µg/cm^2 (lotion), 610.14 µg/cm^2 (Pheroid™ emulgel) and 909.22 µg/cm^2 (Pheroid™ lotion). The average cumulative concentration of rifampicin as shown in Figure D.3 was 506.94 µg/cm^2 (emulgel), 560.65 µg/cm^2 (lotion), 609.88 µg/cm^2 (Pheroid™ emulgel) and 938.64 µg/cm^2 (Pheroid™ lotion). The median and average cumulative concentration values, when compared to those of isoniazide, differ more. The aforementioned is probably due to greater variation in the data and it is accepted that the median is a more reliable and accurate method (Gerber *et al.,* 2008:189). The p-values of the Kruskal-Wallis test proved no difference between the emulgel and lotion ($p = 1.0$), significant differences between the Pheroid™ lotion and the emulgel, the Pheroid™ lotion and the lotion ($p < 0.05$) and small differences between the rest of the comparisons ($p > 0.05$) (Lang & Secic, 1997:76).

D.3.2 Franz cell diffusion studies through human skin

No detectable concentration of isoniazide or rifampicin was able to cross the skin after application of all of the four formulations. There was also no isoniazide or rifampicin found in the dermis, epidermis or stratum corneum, indicating no permeation of the actives into the skin.

D.4 CONCLUSION

Isoniazide was best released from the Pheroid™ emulgel and rifampicin from the Pheroid™ lotion, indicating that the Pheroid™ did improve drug release and permeation through the membrane. It can be concluded that not enough of the actives were released from the formulations. This is evident from the small percentage of isoniazide and rifampicin that diffused through the membrane and the fact that no detectable concentration of either of the actives were able to diffuse through or into the skin. Thus further attention needs to be given to the formulation of isoniazide and rifampicin into topical preparations in order to improve drug release and skin permeability.

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APPENDIX E: PHOTOS OF INSTRUMENTATION USED DURING STABILITY TESTING AND DIFFUSION STUDIES

Photo E.1: Milli-Q water purifying system

Photo E.2: Mettler Toledo balance

Photo E.3: Brookfield viscometer

Photo E.4: Agilent 1100 HPLC

Photo E.5: Labcon stability chambers

Photo E.6: Amber Franz cell receptor and donor compartment

Photo E.7: Metal horseshoe clamps

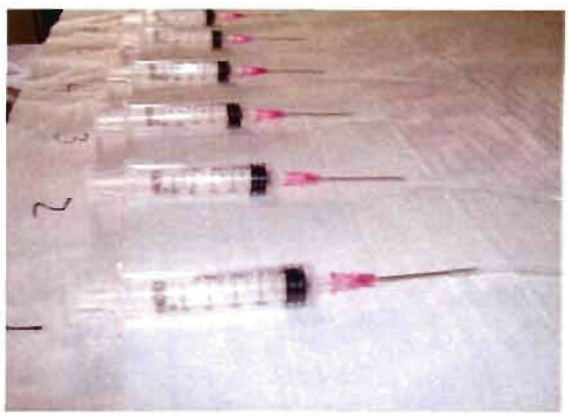

Photo E.8: Syringes for withdrawal from receptor compatments

Photo E.9: Grant water bath

Photo E.10: Assembled Franz cells