Formulation, *in vitro* release and transdermal diffusion of acyclovir and ketoconazole for skin conditions in HIV/AIDS patients

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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 appendixes containing relevant literature and experimental results and discussion. 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

The aim of this *in vitro* study was to investigate the efficacy of the novel Pheroid™ technology system in a semi-solid dosage form, for the topical delivery of acyclovir (5% w/w), an anti-viral agent and ketoconazole (2% w/w) an anti-fungal agent.

The human immununodeficiency virus (HIV) had an immense impact on the spectrum of diagnosis of cutaneous diseases since its first manifestation in the late 1970's (Yen-More *et al.,* 2000:432). The skin is the most commonly affected organ in HIV infected individuals with skin manifestations present in up to 92% of HIV-positive patients. According to Ramdial (2000:113) the skin may also be the first or the only organ affected throughout the course of the HIV/AIDS disease. HIV/AIDS patients are more susceptible to infections due to their compromised immune systems (Durden & Elewski, 1997:200) and an exceptionally wide range of infectious skin manifestations presents in HIV/AIDS infected individuals, some of which are viral and fungal. Acyclovir is an anti-viral active against herpes simplex virus type 1 and type 2, varicella-zoster virus, Epstein-Barr virus and the cytomegalovirus (Hayden, 2001:1317). The anti-fungal drug, ketoconazole has activity against the majority of pathogenic fungi which include *Candida species* and *Histoplasma capsulatum* (Bennett, 2001:1301). It is appropriate to formulate a topical product containing both acyclovir and ketoconazole because viral and fungal cutaneous manifestations are regularly encountered in combination in HIV/AIDS infected individuals,. This combination topical product may be useful in the treatment of viral and fungal opportunistic skin manifestations. Curing these skin lesions may also assist to improve the state of mind and wellbeing of infected individuals.

The skin, however, acts as a barrier against diffusion of substances through the underlying tissue. The main problem in transdermal and dermal delivery of actives is to overcome the stratum corneum, the skin's natural barrier (Menon, 2002:4). The Pheroid™ delivery system can promote the absorption and increase the efficacy of a selection of active ingredients in dermatological preparations (Grobler *et al.,* 2008:284). The aim of this study was to formulate a stable semi-solid product containing Pheroid™ to determine whether Pheroid™ technology would enhance the flux and/or delivery of acyclovir and ketoconazole to the epidermal and dermal layers of the skin.

In vitro studies and tape stripping were used to determine the effect that the Pheroid™ delivery system had on skin permeation of acyclovir and ketoconazole in semi-solid formulations. The formulae containing no Pheroid™ were used as a control against which the efficacy of the formulations containing Pheroid™ was measured.

The stability of the formulated semi-solid products was examined over a period of 6 months according to the International Conference of Harmonisation (ICH) Tripartite Guidelines (2003) and the Medicines control council (MCC) of South Africa (2006). The formulated products were stored at three different temperatures. The stability tests included the assay of the actives and other attributes in the formulation, pH, viscosity, mass loss and particle size observation. These tests were conducted at 0, 1, 2, 3 and 6 months.

The results demonstrated that the transdermal flux, epidermal and dermal penetration of acyclovir was enhanced by the Pheroid™ cream formulation. Ketoconazole's transdermal flux as well as delivery to the epidermal and dermal layers of the skin was improved by the Pheroid™ emulgel formula. The topical delivery of ketoconazole and acyclovir was thus enhanced by Pheroid™ technology. The Pheroid™ formulations, however, did not meet the requirements for stability according to the ICH and MCC.

Keywords: acyclovir, ketoconazole, topical delivery, HIV/AIDS, Pheroid™ technology, formulation

UITTREKSEL

Die doel van hierdie *in vitro* studie was om die doeltreffendheid van Pheriod™ tegnologie vir die topikale aflewering van asiklovir (5% w/w), 'n antivirale geneesmiddel en ketokonasool (2% w/w), 'n antifungale geneesmiddel in 'n semi-soliede doseervorm te bestudeer.

Die menslike immuniteitgebrek virus (MIV) het 'n enorme impak op die spektrum van diagnose van vel infeksies gehad sedert sy eerste manifestasie in die laat 1970's (Yen-More *et al.,* 2000:432). Die vel is die mees geaffekteerde orgaan in MIV geinfekteerde individue. Vel manifestasies is teenwoordig in tot meer as 92% van alle MIV-positiewe pasiënte. Volgens Ramdial (2000:113) is die vel ook die eerste of enigste orgaan wat deur die MIV/VIGS siekteverloop geaffekteer word. MIV/VIGS pasiënte is meer vatbaar vir infeksies aangesien hulle immuniteit stelsels onderdruk is (Durden & Elewski, 1997:200) en 'n groot verskeidenheid infektiewe vel manifestasies kom voor in MIV/VIGS individue, dit sluit virale en fungale infeksies in. Asiklovir is 'n aniti-virale middel wat aktief is teen herpes simplex virus tipe 1 en tipe 2, varicella-zoster virus, Epstein-Barr virus en die cytomegalovirus (Hayden, 2001:1317). Die anti-fungale middel, ketokonasool, het effektiwiteit teen die grootste meerderheid patogeniese fungus infeksies wat *Candida* spesies en *Histoplasma capsulatum* insluit (Bennett, 2001:1301). Virale en fungale vel manifestasies kom algemeen in kombinasie voor in MIV/VIGS geinfekteerde individue, dus is dit toepaslik om 'n topikale produk the formuleer wat beide asiklovir en ketokonasool bevat. Hierdie gekombineerde topikale produk kan nuttig wees vir die behandeling van opportunistiese virus en fungus manifestasies op die vel. Genesing van hierdie vel letsels kan ook help om die geinfekteerde individue se gemoedstoestand en welstand te verbeter.

Die vel bied uitstekende weerstand teen die aflewering van middels deur die vel na die onderliggende weefsel. Die vernaamste probleem in die transdermale aflewering van aktiewe bestandele is om die stratum corneum, die vel se natuurlike versperring, te oorkom (Menon,2002:4). Die Pheroid™ geneesmiddel aflewerings sisteem kan die absorpsie en effektiwiteit van 'n verskeidenheid aktiewe bestanddele in dermatologiese preparate verhoog (Grobler *et al.*, 2008:284).

Die doel van die studie was om 'n stabiele semi-soliede produk te formuleer wat Pheroid™ bevat en te bepaal of die Pheroid™ tegnologie die vloed en/of die aflewering van asiklovir en ketokonasool na die epidermis en dermis lae van die vel kan verbeter.

In vitro studies en 'tape stripping' metodes was gebruik om the bepaal wat die effek van die Pheroid™ geneesimiddel aflewering sisteem was op die penetrasie van asiklovir en ketokonasool in semi-soliede formulerings. Die formulerings wat geen Pheroid™ bevat het nie,

het as kontroles gedien, waarteenoor die effektiwteit van die formulerings met die Pheroid™ gemeet was.

Die stabiliteit van die geformulgeerde semi-soliede produkte was oor 'n periode van 6 maande getoets in ooreenstemming met ICH Tripartite Guidelines (2003) en die Medisyne beheer raad van Suid-Afrika (2006). Die geformuleerde produkte was by drie temperature gestoor. Die stabiliteits toetse het die toets van die aktiewe bestandele en hulp bestandele, pH, viskositeit, massa verlies en deeltjiegrootte observasie ingesluit. Die toetse was uitgevoer oor 0, 1, 2, 3 en 6 maande.

Die resultate het getoon dat die transdermale vloed, asook die epidermis en dermis penetrasie van asiklovir verhoog was deur die Pheroid™ room formulering. Ketokonasool se transdermale vloed en die aflwering na die epidermis en dermis lae van die vel is verbeter deur die Pheroid™ emulgel formule. Die topikale aflewering van ketokonasool en asiklovir is dus verhoog deur Pheroid™ tegnologie. Die Pheroid™ formulerings het egter nie aan die vereistes vir stabiliteit van die ICH en Medisyne beheer raad voldoen nie.

Sleutelwoorde: asiklovir, ketokonasool, topikale aflewering, MIV/VIGS, Pheroid™ tegnologie, formulering

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ABBREVIATIONS

AIDS	-	Acquired immunodeficiency syndrome
BHA	-	Butylated hydroxyanisole
BHT	-	Butylated hydroxytoluene
CLSM	-	Confocal laser scanning microscope
CMV	-	Cytomegalovirus
DNA	-	Deoxyribonucleic acid
EBV	-	Ebstein-Barr virus
HHV	-	Human herpes virus
HIV	-	Human immunodeficiency virus
HPLC	-	High performance liquid chromatography
HPV	-	Human papilloma virus
HSV	-	Herpes simplex virus
ICH	-	International conference on harmonisation
MCC	-	Medicines control council
RH	-	Relative humidity
RNA	-	Ribonucleic acid
RSD	-	Relative standard deviation
SD	-	Standard deviation
STD	-	Sexually transmitted disease
VZV	-	Varicella zoster virus

CHAPTER 1

INTRODUCTION AND STATEMENT OF THE PROBLEM

The acquired immunodeficiency syndrome (AIDS) pandemic and the human immunodeficiency virus (HIV) have had a significant effect on the extent and diagnosis of cutaneous disorders. The skin is the most commonly affected organ in HIV infected individuals (Ramdial, 2000:113). There is an incredibly wide range of skin manifestations that present in HIV/AIDS infected individuals because of their compromised immune systems. Cutaneous disorders in HIV-positive individuals can also be more severe and disabling than in normal individuals (Yen-More *et al.*, 2000:429).

There are mainly three types of viral infections that occur cutaneously in HIV/AIDS patients and these viruses include the human herpes viruses, *Human papilloma* virus and *Molluscum contagiosum.* The herpes simplex virus (HSV) has always been associated with immunodepression. Many opportunistic viral infections in HIV/AIDS patients have similar manifestations as those in healthy individuals, but infections in this immunocompromised population are seen in greater prevalence and are more difficult to treat (Yen-More *et al.*, 2000:432).

HIV/AIDS has also increased the worldwide prevalence of fungal infections. Fungal infections are a major cause of morbidity and mortality in patients infected with the HIV virus. The yeast *Candida* spesies and *Cryptococcus neoformans,* the dimorphic fungi *Histoplasma capsulatum*, *Sporothrix schenckii* and dermatophyte fungi are the most common pathogenic fungi in patients infected with HIV (Durden & Elewski, 1997:200).

Acyclovir is the anti-viral drug of choice in the treatment of many types of herpes virus infections. Acyclovir, a synthetic purine nucleoside analogue, is active against HSV type 1 and type 2 and against the varicella zoster virus. Acyclovir also affects the Epstein-Barr virus and cytomegalo virus, but to a lesser extent (Hayden, 2001:1317; Sweetman, 2008).

Ketoconazole is a broad spectrum imidazole anti-fungal agent (Skiba *et al.,* 2000:2) and has an antifungal activity against fungal pathogens which include *Candida* species, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii* (Bennett, 2001:1301).

The transdermal delivery of drugs offers a number of advantages over the traditional oral dosage forms such as minimising the first-pass metabolism, avoiding the acidic environment of the stomach responsible for degrading drugs and decreasing the variability in the plasma concentration. The transdermal route also provides a more controlled, non-invasive method for

delivering drugs. Patient compliance may be improved because of the reduced frequency of administration. The trauma associated with parenteral therapy is also avoided (Roberts *et al.*, 2002:90). Considering the physicochemical properties of acyclovir, transdermal diffusion may be possible, but poor transdermal delivery can be anticipated for ketoconazole (McEvoy, 2002:11; British Pharmacopoeia, 2009).

Pheroid™ technology will be used in this study as a carrier system for acyclovir and ketoconazole to potentially improve dermal and transdermal delivery of the active ingredients. The Pheroid™ delivery system consists of a colloid system that contains stable lipid-based submicron- and micron-sized structures, called Pheroid™. These Pheroid™ are uniformly distributed in a dispersion medium. The basic Pheroid™ has a vesicular structure with sizes ranging from 200 - 440 nm (Grobler *et al.*, 2008:283). The main essential fatty acids used in Pheroid™ are linolenic acid and linoleic acid as well as oleic acid. These essential fatty acids are emulsified in water and saturated with nitrous oxide (Grobler *et al.*, 2008:305). The Pheroid™ itself also contains anti-fungal activity (Grobler, 2004:12), hence a further reason to formulate ketoconazole within Pheroid™.

The objective of this study was to formulate a semi-solid product with the use of the Pheroid™ delivery system, containing acyclovir and ketoconazole that can be used for the treatment of viral and fungal opportunistic cutaneous infections. The aims of this study included the:

- formulation of two types of semi-solid products containing acyclovir and ketoconazole;
- formulation of the same two types of semi-solid products containing acyclovir and ketoconazole with the inclusion of Pheroid™;
- determination of the stability of the formulated products in accordance with the International conference on harmonisation (ICH) Tripartite Guidelines and the Medicines Control Council (MCC) of South Africa;
- development and validation of a high performance liquid chromatography (HPLC) method for the assay analysis of the active ingredients and the other attributes in the semi-solid products; and
- determining whether the Pheroid™ delivery system improves transdermal delivery and/or dermal delivery to the target sites of acyclovir and ketoconazole through *in vitro* and tape stripping methods.

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CHAPTER 2

PERCUTANEOUS DELIVERY OF ACTIVES FOR HIV/AIDS SKIN MANIFESTATIONS

2.1 INTRODUCTION

The skin is both a challenge and an opportunity for the delivering of drugs (Menon, 2002:4). The natural function of the skin is protection of the body against the loss of endogenous substances such as water and undesired influences from the environment caused by exogenous substances. This implies that the skin acts as a barrier against diffusion of substances through the underlying tissue. Overcoming this natural barrier is the main problem in dermal or transdermal delivery of drugs.

2.2 THE STRUCTURE AND FUNCTION OF HUMAN SKIN

The skin is the largest organ of the human body, accounting for approximately 16% of total body weight (Wickett & Visscher, 2006:99). An average square centimetre of skin contains 10 hair follicles, 15 sebaceous glands, 12 nerves, 100 sweat glands, 360 cm of nerves and 3 blood vessels (Asbill & Michniak, 2000:36). The skin forms a fascinating and unique interface between our bodies and the outside world seeing that it is the only organ in the human body that is constantly exposed to the external environment (Hadgraft, 2004:291).

The skin simply outweighs any other organ in the human body, in terms of the number of functions performed. Its role is to prevent loss of water and other components of the body to the environment and to protect the body from a variety of environmental insults (Wickett & Visscher, 2006:99). The primary function of the skin is protection of the body against physical, chemical, immunological and pathogenic factors, as well as ultraviolet radiation and free radicals. It is also a major participant in thermoregulation and pigment production. The skin functions as a sensory organ and performs endocrine functions such as Vitamin D synthesis and peripheral conversion of pro-hormones (Menon, 2002:4).

Normal skin is divided into three main structural layers, namely the stratified epithelium called the epidermis, the connective-tissue dermis and the subcutaneous layer (Hunter, 1973:340). A simplified diagram demonstrating the skin structure with its three layers is shown in Figure 2.1.

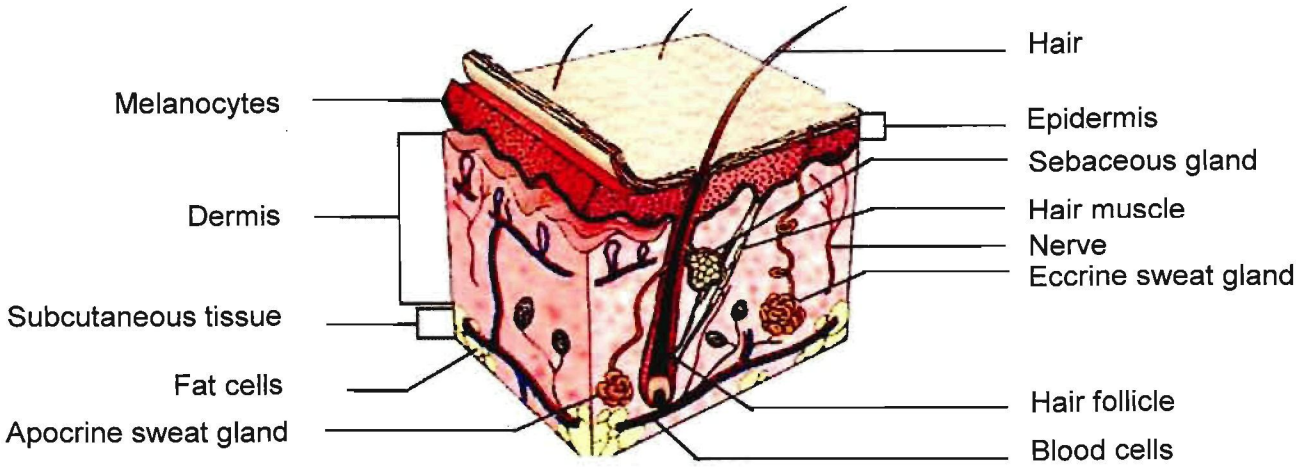


Figure 2.1: Simplified diagram of skin structure with the three main layers of the skin (Adapted from the American skin association, 2009)

2.2.1 Epidermis

The epidermis is the relatively thin outer layer of the skin (Mac Neal, 2006), serves as protection against the environment and assists in maintaining homeostasis (Wickett & Visscher, 2006:98). The epidermis also has immunologic functions and provides some protection from ultraviolet radiation through the pigment system (Wickett & Visscher, 2006:98).

The epidermis itself is generally considered to be subdivided into five separate layers, namely the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The various layers of the skin work in concert to provide strength, flexibility and perform the multiple functions of the skin (Wickett & Visscher, 2006:98).

There are four important cell types in the epidermis: keratinocytes, melanocytes, Merkel cells and Langerhans cells, with the keratinocytes as the predominant cell type (Wickett & Visscher, 2006:98). Keratinocytes originate from cells in the layer of the epidermis called the basal layer. The new cells slowly migrate up toward the surface of the epidermis at which they transform to corneocytes of the stratum corneum. Once the keratinocytes reach the skin surface, they are gradually shed and are replaced by younger cells pushed up from below. Scattered throughout the basal layer of the epidermis are melanocytes, which produce the pigment melanin, one of the contributors of skin colour. This pigment system protects the skin against ultraviolet radiation (Mac Neal, 2006). Merkel cells are involved in sensory perception and Langerhans cells are important for antigen presentation and immune response (Asbill & Michniak, 2000:36).

The dermal-epidermal junction is not a flat surface; and distinct papillae can be observed in this area using light microscopy. The surface area of the dermal-epidermal layer decreases with age. This layer facilitates the diffusion of some substances such as xenobiotics. The dermal-epidermal junction is also thought to play a role in the permeation of large molecular weight

proteins and peptides (Asbill & Michniak, 2000:36). The basement membrane zone separates the epidermis and the dermis. Underneath the epidermal layers and beneath the basement membrane zone, lies the dermis, which is the thickest layer of the skin (Convatec, 2008:1).

2.2.2 Dermis

The connective-tissue dermis supports the epidermis and separates it from the fatty layer below. The dermis is a thick layer that consists mainly of fibroblasts, endothelial cells and mast cells. In conditions involving inflammatory reactions, macrophages, lymphocytes and leucocytes may also be observed in this layer. The cells are embedded in connective tissue composed of mainly collagenous fibres; and this accounts for approximately 70% of the dry weight of the skin. These fibres form a scaffold which provides the skin's mechanical strength. The elasticity is provided by the elastic connective tissue, which is the second most significant component. The proteins associated with this layer are elastin, fibrillin, vitronectin, tissue-amyloid P, only to name a few (Asbill & Michniak, 2000:36).

The highly vascular dermi contains nerve endings, sweat glands, pilobaceous units, hair follicles and blood vessels (Menon, 2002:4). Nerve endings sense pain, touch, pressure and temperature. Sweat glands produce sweat in response to heat and stress. Sebaceous glands secrete sebum into hair follicles that helps keep skin moist and soft and act as a barrier against foreign substances. Hair follicles produce various types of hair found throughout the body (Mac Neal, 2006). Blood vessels supply oxygen and nutrients to the epidermis and dermis, nerves, sweat and sebaceous glands, hair follicles and lymph vessels, and help to regulate body temperature (Hunter, 1973:342).

2.2.3 Subcutaneous tissue

The deepest layer of the skin is the subcutaneous tissue or hypodermis. The hypodermis acts as a heat isolator, a shock absorber and an energy storage region. This layer is a network of fat cells arranged in lobules and linked to the dermis by interconnecting collagen en elastin fibres. The fat cells (possibly 50% of the body's fat) as well as the other main cells in the hypodermis are fibroblasts and macrophages. One of the major roles of the hypodermis is to hold the skin's vascular and neutral systems. It also anchors the skin to the underlying muscle. Fibroblasts and adipocytes can be stimulated by the accumulation of interstitial and lymphatic fluid within the skin and subcutaneous tissue (Szuba & Rockson, 1997:326).

2.3 DRUG TRANSPORT THROUGH THE SKIN

2.3.1 The skin as the barrier to percutaneous absorption

The vital barrier function of the skin resides primarily in the top stratum of the epidermis, the stratum corneum. The stratum corneum forms a relatively impermeable layer at the surface of human skin giving it its remarkable ability to defend the body against both chemical and microbiological attack, as well as dehydration (Wickett & Visscher, 2006:98). This barrier function is the result of the unique structure and composition of the stratum corneum (Pilgrim *et al.*, 1998:1669).

On most body sites the stratum corneum is typically 12 - 16 layers thick (Wickett & Visscher, 2006:98). Layers of the stratum corneum consists of flat, roughly hexagonally shaped, partly over lapping cells, called corneocytes (Benson, 2005:24). Individual corneocytes are 0.2 - 1.5 µm thick and 20 - 40 µm in diameter as opposed to a diameter of 6 or 8 µm for the basal cell (Menon, 2002:5).

Knowledge of the stratum corneum lipid organization is important for understanding the barrier functions of the skin (Bouwstra, 1997:403). Peter Elias championed the classic brick and mortar organization and it is still the most simplistic organizational description of the stratum corneum (Menon, 2002:8). Stratum corneum corneocytes, terminally differentiated keratinocyte, with their resistant cell envelopes and keratin micro-fibrils, are considered to be the bricks and the layers of lipids found between the cells are considered to be the mortar (Wickett & Visscher, 2006:98). Protein enriched corneocytes (bricks) impart a high degree of tortuosity to the path of water or any other molecule that traverses the stratum corneum, while the hydrophobic lipid, organised into tight lamellar structures (mortar), provides a water-tight barrier to the already tortuous route of permeation in the interfollicular domains (Menon, 2002:8). The intercellular matrix (mortar) is primarily composed of long chain ceramides, free fatty acids, triglycerides, cholesterol, cholesterol sulphate and sterol or wax esters (Benson, 2005:24).

It is however important to view this model in the context that the corneocytes are not brick shaped, but are polygonal, elongated and flat (Benson, 2005:24). Both the bricks and the mortar of the stratum corneum are produced by keratinocytes at the stratum granulosum where keratinocytes release the lipids of the mortar into the space between the cells as they are being transformed into the corneocytes bricks. The bricks are linked by desmosomes (Wickett & Visscher, 2006:98).

2.3.2 Penetration pathways across the skin

A drug molecule on the surface of the skin can penetrate to the viable tissue by means of two main routes of penetration, namely the transepidermal and transappendageal routes.

2.3.2.1 Transepidermal route

The transepidermal penetration route is once more subdivided into the transcellular route and the intercellular pathway.

2.3.2.1.1 Transcellular route

This route is regarded as a polar route through the keratinocytes of the stratum corneum. The cellular components that the solute diffuses through provide an essential aqueous environment. Hydrophilic molecules, thus, rapidly move through these keratinocytes. The keratin-filled cells do not exist in isolation and they are bound to polar lipids that connect to the intercellular multiple bilayered lipid domains (Williams, 2003:33). A molecule crossing directly through the continuous stratum corneum by means of the transcellular route faces numerous hurdles. First, there is partitioning into the keratinocyte, followed by diffusion through the hydrated keratin. In order to leave the cell, the molecule must partition into the bilayer lipids before diffusing across the lipid bilayer to the next keratinocyte (Williams, 2003:33). The pathway is directly across the stratum corneum and hence, the path length for permeation is usually related to the thickness of the stratum corneum (Williams, 2003:34).

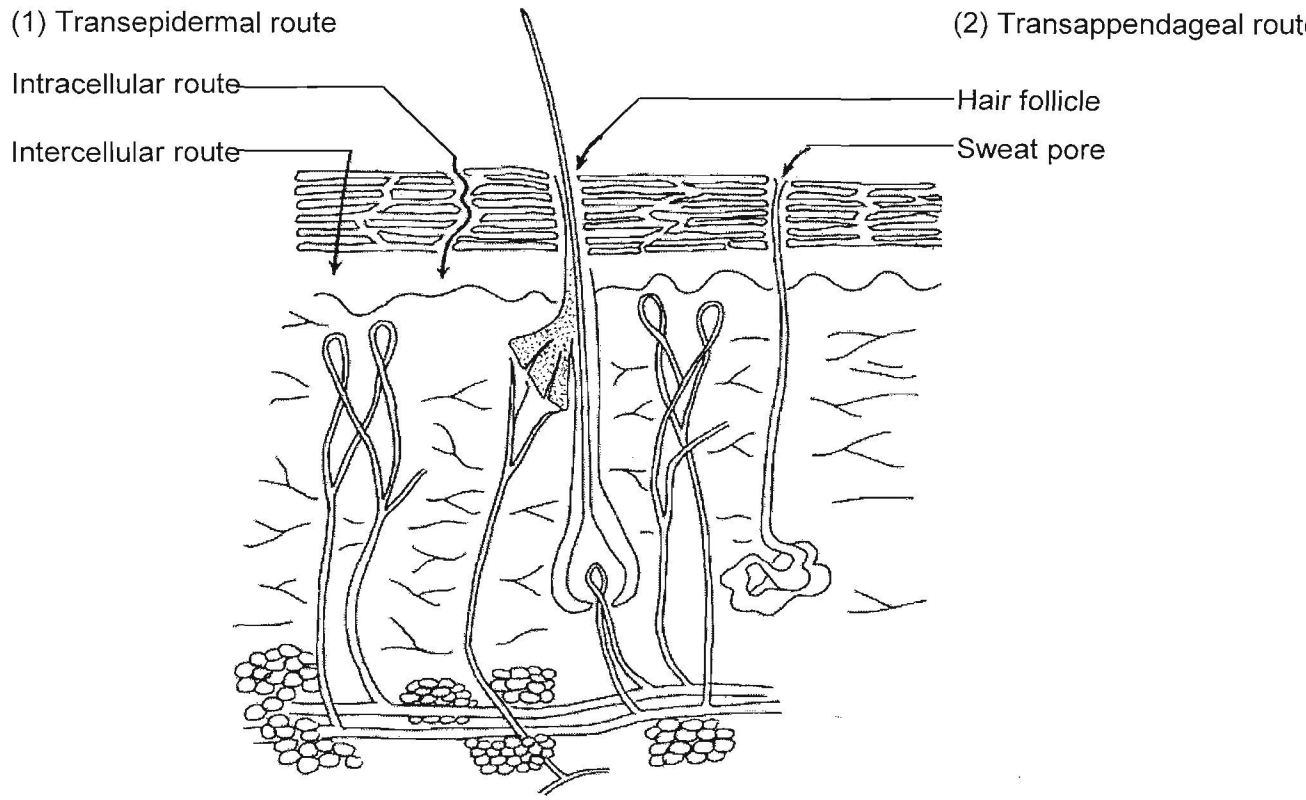
Experimental evidence suggests that, under normal circumstances, the predominant route of penetration is through the intercellular spaces. The diffusional pathway is therefore longer than the thickness of the stratum corneum (20 µm). It has been found to be approximately 500 µm long. The nature of the stratum corneum is, thus, very heterogeneous and it is remarkable that the diffusion through it can be described by Fick's law of diffusion (Hadgraft, 2004:292).

2.3.2.1.2 Intercellular pathway

It is generally accepted that the intercellular lipid pathway provides the principal route by which most small, uncharged molecules cross the stratum corneum (Roberts *et al.*, 1996:24). The molecules move within the lipid bilayer of the stratum corneum. It is the only continuous phase within the stratum corneum and it comprises 1% of the diffusional area in the membrane. The intercellular permeation distance is estimated to be 150 - 500 µm (Williams, 2003:34).

2.3.2.2 Transappendageal route

The transappendageal route consists of the sweat ducts and the hair follicles with their sebaceous glands. Appendages essentially offer pores that bypass the barrier of the stratum corneum. The appendageal area available for transport is only about 0.1% of the total skin surface. This route usually contributes negligibly to steady state flux. The pathway may be important for large polar molecules and ions that struggle to cross the intact stratum corneum (Barry, 2001:101). A diagram of the skin structure and the routes of penetration are shown in Figure 2.2.



(1) Directly across the stratum corneum: intracellular or intercellular route
(2) Transappendageal: via the sweat ducts and through the hair follicles with their sebaceous glands

Figure 2.2: Diagram of skin structure and routes of penetration (Adapted from Sinko & Martin, 2006:346)

2.4 FACTORS THAT INFLUENCE PERCUTANEOUS ABSORPTION

The rate or extent of absorption of a drug through the skin has been shown to be influenced by many physiological factors as well as the physicochemical properties associated with the drug itself. These factors will be discussed in the following section.

2.4.1 Physiological factors influencing transdermal drug delivery

Biological variability of skin complicates the prediction of the rate of percutaneous penetration of topically applied drugs (Riviere, 1993:113). The following biological factors influence drug delivery across the skin.

2.4.1.1 Skin hydration

The barrier function of the stratum corneum is decreased by hydration. When water saturates the skin, the tissue swells, softens and wrinkles and its permeability increases. It is also well known that hydration of the skin is one of the most important methods for penetration enhancement (Hunter, 1973:342).

2.4.1.2 Skin age

There are obvious structural and functional alterations that occur to the skin membrane as it ages, but it is difficult to determine if it is because of age or environmental damages. It is generally accepted that the stratum corneum remains basically unchanged during a normal lifespan (Williams, 2003:14).

Children have a greater surface area per unit body weight, thus, they are more susceptible to the toxic effects of applied drugs. Some premature infants are born without a stratum corneum. This can be very dangerous, but can also be turned to an advantage during the treatment of breathing difficulties with caffeine and pain with buprenorphin, through topical application instead of intravenous injection through the tiny veins (Barry, 2002:510).

Hydration of the skin also decreases with age and this factor can alter drug permeation, since the moisture content plays an important role in drug delivery. The amount of topically applied drug entering the systemic circulation can be affected by the blood flow in the skin. Blood flow tends to reduce with age and can thus decrease transdermal drug flux (Williams, 2003:14).

2.4.1.3 Race and gender

Studies done by Berardesca *et al.* (1991:89) depicted no differences in transepidermal water loss in the delivery of aspirin, benzoic acid or nicotine between African, Asian and European skin. Differences in drug absorption could be anticipated, because of the significant differences in the stratum corneum water content between races. There are no significant reports of a difference in penetration of drugs through the skin of different sexes, although the keratinocytes of males (34 - 44 μm) are somewhat smaller than in females (37 - 46 μm) (Williams, 2003:17).

2.4.1.4 Regional skin sites

It is obvious that the skin structure varies to some degree over the human body. The stratum corneum is typically 12 - 16 layers thick, but it can vary from as little as 9 cell layers of the forehead or eyelids to as much as 25 on the skin of the palms and up to 50 or more on the soles of the feet (Wickett & Visscher, 2006:98). The permeability of the different skin sites is not simply a function of the stratum corneum thickness as different permeants display varied rank orders through different skin sites. Numerous authors report that scrotal skin is highly permeable (Hunter, 1973:342) and that genital tissue usually provides the most permeable site for transdermal drug delivery (Williams, 2003:16).

There is, thus, a considerable variation in the permeation of a drug or permeant across a specific body site of an individual, as well as a variation between the same body site in different individuals. A 30% variation of penetration across an individual's abdominal skin can occur; with a 40% variation between the penetration across the abdominal skin of two different individuals (Williams, 2003:17). During this study the abdominal skin of two individuals was used.

2.4.1.5 Drug-skin metabolism

According to Barry (2002:510) a topically applied compound's therapeutic efficiency may be determined by the metabolism that takes place in the skin. It has been estimated that the skin can metabolise a quantity of 5% of topically applied drugs.

2.4.1.6 Temperature

A rise in the skin temperature is responsible for an increase in penetration of permeants (Hunter, 1973:342), because of structural alterations within the stratum corneum that occur with an increase in the temperature of the skin. The permeant diffusion coefficient decreases with a decrease in temperature. Thus, when temperature rises there is an increase in the passive diffusion through the stratum corneum (Williams, 2003:18).

2.4.1.7 Skin condition

The skin's main function is to protect the body from the external environment. It is difficult to penetrate the stratum corneum, but when the skin is damaged, whether it is by an alkali, acid, abrasions or cuts, penetration through the skin increases (Williams, 2003:22). Diseases commonly alter the barrier properties of the skin. Penetration will increase if the skin is inflamed, has a loss of stratum corneum or there is an altered keratinisation. Some diseases may cause thickening of skin, corns, calluses or warts in which case the drug penetration will

decrease (Barry, 2002:509). The effects of HIV/AIDS on skin condition are discussed in Section 2.7.

2.4.2 Physicochemical factors influencing transdermal drug delivery

Various physical and chemical factors such as the diffusion coefficient, partition coefficient, molecular size and shape, pH, pK_a and ionisation, drug concentration, hydrogen bonding, drug solubility and the melting point of an active, influence the transdermal delivery of drugs.

2.4.2.1 Diffusion coefficient (D)

The diffusion through the stratum corneum is expressed as the diffusion coefficient (D) which is a property of the permeant in the membrane and is a measure of how easily a drug will transverse through the tissue. It is expressed in units of area/time, usually cm^2/h or cm^2/s (Williams, 2003:27). At a constant temperature, the properties of a drug and the diffusion medium, and the interaction between these two factors, determine the diffusion coefficient of a drug present in a topical vehicle or in the skin. The value of D indicates the penetration rate of a molecule under specific conditions (Barry, 2002:512).

2.4.2.2 Partition coefficient (P)

The partition coefficient can also be described as the solvent to water quotient of drug distribution. The partition coefficient (P) is an indication of the lipid solubility of a drug, and therefore, whether that drug is likely to be transported across membranes. It is given by its ability to partition between a lipid-like solvent and water or an aqueous buffer. This is known as a drugs' partitioning coefficient and it is a measure of its lipophilicity. The value of the partition coefficient (P) is determined by measuring the drug partitioning between water and a suitable solvent at a constant temperature. Octanol, an organic solvent, is usually selected to mimic the biological membrane because of its similar properties (Aulton, 2002:243). Octanol is usually used to determine log D (octanol-buffer partition coefficient) in transdermal delivery studies as a guide to how well the drug spread between the stratum corneum lipids and water. The partition coefficient of a drug is important during the establishment of the flux of the drug through the stratum corneum (Barry, 2002:512).

For optimum penetration a balanced partition coefficient with an adequate solubility in oil and water are needed to ensure that the concentration gradient in the membrane is high. Intermediate partition coefficients (octanol-water partition coefficient (log P) between 1 and 3) give molecules sufficient lipid solubility within the domains of the stratum corneum to allow diffusion through the domain, but still have a hydrophilic nature to allow partitioning into the viable tissues of the epidermis (Benson, 2005:25). Compounds with high log P values exhibit low permeability, because they are not able to partition out of the stratum corneum (Thomas &

Finnin, 2004:699). Compounds with a low log P show low permeability, because there is little partitioning into the tissue of the epidermis (Benson, 2005:26).

Ketoconazole, for example, has a log P of 3.73 (Männistö *et al.*, 1982:730) and acyclovir has a log D of 0.018, which were calculated by using *n*-octanol and 0.2 M phosphate buffer solution (PBS) (Dollery, 1999:A39). These values do not fall in the ideal range, which means the skin penetration is likely to be poor. A penetration accelerant namely Pheorid™ vesicles will therefore be incorporated into the formulae of the cream and emulgel to promote permeation of acyclovir and ketoconazole.

2.4.2.3 Molecular size and shape

Theoretically, absorption of a drug is inversely related to its molecular weight. For transcellular passive diffusion a molecular weight of less than 500 Da is preferable. Drugs with molecular weights above this may be absorbed less efficiently. For intercellular absorption the molecular weight should ideally be less than 200 Da. There is, however, samples where larger molecules with molecular weights up to 400 Da have been absorbed via this route (Aulton, 2002:243).

The effect of the size of a molecule on penetration can only be determined if the effect of size could be isolated from the drug's solubility characteristics. This is difficult to do without changing the partitioning coefficient, which plays a dominant role during percutaneous absorption (Barry, 2002:513).

It is even more difficult to determine the effect of molecular shape of a drug on penetration separate from the partition coefficient domination. Thus, nothing is known about this factor in skin permeation (Barry, 2002: 513).

2.4.2.4 pH, pK_a and ionisation

The stratum corneum is extremely resistant to change in pH, tolerating a pH range of 3 - 9. According to the pH-partition hypothesis, only unionised molecules pass readily across lipid membranes. Weak acids and bases dissociate to different degrees, depending on the pH and their pK_a or pK_b values. The proportion of unionised drug in the applied phase, mainly determines the effective membrane gradient, and this fraction depends on the pH (Barry, 2002:511).

However, ionised molecules penetrate the stratum corneum to a limited extent, because they usually have a much greater aqueous solubility than the neutral species and in saturated or near saturated solutions, they may make a significant contribution to the total flux. Although the partition coefficient (K) may be small for ionised species, the constant concentration of the drug may be very high (Barry, 2002:512).

2.4.2.5 Drug concentration

The flux of a solute is proportional to the concentration gradient across the entire barrier phase. The concentration differential is considered to be the driving force for diffusion. One requirement for maximal flux in a thermodynamically stable situation is that the donor solution should be saturated (Barry, 2002:512).

2.4.2.6 Hydrogen bonding

The number of hydrogen bonds is important in the permeability of a permeant. Too many hydrogen bonds within a molecule are unfavourable to its permeation. In general, no more than 5 hydrogen bond donors and no more than 10 hydrogen bond acceptors should be present if the molecule is to be well absorbed (Aulton, 2002:243).

2.4.2.7 Drug solubility and solubility parameter

An adequate solubility in oil and water is needed in order for the concentration gradient of the membrane to be as high as possible for optimum penetration (Barry, 2002:513). For highly hydrophilic molecules the transcellular route is the predominant route for the molecule or permeant to follow (Williams, 2003:33).

2.4.2.8 Melting point

For optimum penetration a low melting point is needed which correlates with ideal solubility (Barry, 2002:513).

2.5 MATHEMATICAL MODEL OF SKIN PERMEATION

An understanding of the basic principles of permeation through membranes is valuable in all areas of biopharmaceutics. Fundamental mathematics is also important in dosage form design (Barry, 2002:505). By nature of the stratum corneum is very heterogeneous and it is perhaps surprising that the diffusion through it can be described by the simple solutions of Fick's laws of diffusion (Hadgraft, 2004:292).

2.5.1 Fick's first law of diffusion

The amount of material (M) passing through a unit area (S) per unit time (t) is termed the flux (J) (Equation 2.1).

$$J = \frac{dM}{S.dt} \qquad \textbf{(Equation 2.1)}$$

Drug permeation across the stratum corneum obeys Fick's first law (Benson, 2005:24). Fick's first law of diffusion states that the rate of transfer of diffusing substance through a unit area of a section is proportional to the concentration gradient measured normal to the section (Williams, 2003:41) (Equation 2.2).

$$J = -D\frac{\partial C}{\partial x} \qquad \textbf{(Equation 2.2)}$$

Where J is the flux of the permeant, C is the concentration (g/cm^3) of the diffusing substance, x is the space coordinate (cm) measured normal to the section and D is the diffusion coefficient (Williams, 2003:41). The negative sign indicates that the flux is in the direction of decreasing concentration, thus down the concentration gradient (Barry, 2002:506).

2.5.2 Fick's second law of diffusion

Fick's second law of diffusion can be derived from equation 2.2. When a topical permeant is applied on to the skin, it is assumed that the diffusion is unidirectional, which means the concentration gradient is from the surface into the tissue. This reduces the number of variables by one. Fick's second law expresses unidirectional diffusion in an isotropic medium mathematically as:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad \textbf{(Equation 2.3)}$$

where C is the concentration, t is the time, D is the diffusion coefficient in the stratum corneum and x is the distance from the surface of the skin (Williams & Barry, 2004:42).

2.6 PENETRATION ENHANCERS

There is considerable interest in the skin as a site of drug application, both for local and systemic effect. However, the skin possesses a formidable barrier to drug penetration thereby limiting topical and transdermal bioavailability. In this section the skin penetration enhancement techniques that have been developed to improve bioavailability and increase the variety of drugs for which topical and transdermal delivery is a viable option, are described (Benson, 2005:23). According to Barry (2002:522) an ideal penetration enhancer should have all of the following characteristics:

- tasteless, odourless, colourless and inexpensive;
- the material should be pharmacologically inert;
- the material should be chemically and physically stable;

- it should be non-allergenic, non-irritant and non-toxic;
- its action should be specific, immediate and the effect should be suitable and predictable;
- upon removal of the material, the skin should immediately and fully recover its normal barrier properties;
- the enhancer should not cause loss of body fluids, electrolytes or other endogenous materials;
- it should be compatible with all drugs and excipients;
- the substance should be a good solvent for drugs;
- the material should be cosmetically acceptable; and
- the chemical should be able to be formulated into all the variety of preparations used topically.

No single material possesses all of these attributes; however, there are many substances that have several of these properties. A brief overview of the best-known enhancement techniques will be discussed in this section, which includes chemical enhancers, physical enhancers and Pheroid™ technology.

2.6.1 Chemical enhancers

Accelerants or sorption promoters can enhance drug flux, because these substances temporarily diminish or disturb the barrier of the skin. The mechanism of action of chemical penetration enhancers falls into two major categories, namely, those who impact diffusion across the stratum corneum, by disrupting the lipid organization of the stratum corneum, rendering it more permeability; and those that alter partitioning into the stratum corneum. The latter increases the solubility of the drug within the stratum corneum by affecting the solubility properties of the skin (Thomas & Finnin, 2004:700).

A summary of chemical penetration enhancers include: water, hydrocarbons, sulphoxides and its analogues, pyrrolidones, fatty acids and alcohol, azone and its derivates, alcohols and glycols, essential oils, terpenes and its derivates, as well as synergistic mixtures (Barry 2002:523). Water, fatty acids and Pheroid™ technology were the chemical enhancers used during this study.

2.6.1.1 Water

For safety and effectiveness, the best penetration enhancer is water. Most substances penetrate better through hydrated stratum corneum than through dry tissue, because water opens up the compact layer of the horny layer (Barry 2001:106).

During this study oil-in-water emulsions were used. According to Barry (2001:105) this may donate water to the skin with a slight hydration increase, which may have a small enhancement on the skin's permeability.

2.6.1.2 Fatty acids

Fatty acids, as chemical enhancers of transdermal diffusion, disrupt the ordered nature of the skin's lipids, increasing the fluidity and hence, assisting in permeation of the drug applied (Thomas & Finnin, 2004:700). Fatty acids have been used to improve the transdermal delivery of acyclovir, estradiol, progesterone, salicylic acid and 5-flurorouracil, indicating that these enhancers promote the delivery of both lipophilic and hydrophilic permeants (Williams & Barry, 2004:610).

2.6.1.3 Pheroid™

Pheroid™ technology was used during this study as the main penetration enhancer and will be discussed in detail in Section 2.6.3.

2.6.2 Physical enhancers

The most noticeable advancements in physical enhancement methods are approaches such as iontophoresis and electroporation. These methods are typically used for the delivery of large molecular weight or highly potent compounds. Enhancing drug delivery by low-frequency sonophoresis using ultrasound, has also shown to be valuable. This technique, which was originally used in physiotherapy, uses ultrasonic energy that disturbs the lipid packing in the stratum corneum (Barry, 2001:108; Thomas & Finnin, 2004:700).

Another alternative approach involves the use of fabricated micro-needles. A device of 400 micro-needles was designed to penetrate only the outer layer of the skin when it is inserted onto the skin, producing a channel for drug transport across the stratum corneum. This method has proved to be painless, because the nerves are located much deeper within the skin (Barry, 2001:108; Thomas & Finnin, 2004:700). More techniques, which are still experimental, include photomechanical waves and magnetophoresis (Barry, 2001:109). During this study no physical enhancement techniques were used.

2.6.3 Pheroid™ technology

Pheroid™ technology, formerly known as Emzaloid™ technology, is able to enhance the absorption and/or efficacy of various substances, while reducing unwanted side effects. The North-West University of South Africa purchased the Pheroid™ technology in December of 2003 from MeyerZall (Pty) Ltd, who initially developed this technology. Registered patents of Pheroid™ technology describe the use of Pheroid™ as a delivery system to promote the absorption and increase the efficacy of dermatological, biological and oral medicines in various pharmacological groups (Grobler *et al.*, 2008:284). Pheroid™ are mainly composed of modified plants and essential fatty acids (Grobler, 2004:4).

2.6.3.1 Structural characteristics of Pheroid™

The Pheroid™ delivery system consists of a colloid system that contains stable lipid-based submicron- and micron-sized structures, called Pheroid™ (Grobler *et al.*, 2008:284). These Pheroid™ are uniformly distributed in a dispersion medium. The dispersed phase can be manipulated in terms of morphology, structure, size and function (Grobler *et al.*, 2008:285). The basic Pheroid™ has a vesicular structure with sizes ranging from 200 - 440 nm (Grobler *et al.*, 2008:283).

Various types of Pheroid™ structures can be manufactured (Grobler *et al.*, 2008:285). When deciding on the type and diameter of the Pheroid™ to use, the following parameters are considered: (1) the amount and size of the active compound to be entrapped; (2) the rate of delivery and (3) the route of administration (Grobler *et al.*, 2008:285). Some of these types include bilayer membrane vesicles, highly elastic or fluid bilayered vesicles, pro-Pheroid™ reservoirs, micro-sponges and depots (Grobler, 2004:5).

Pheroid™ consist largely of ethylated and pegylated polyunsaturated fatty acids, which include omega-3 and omega-6 fatty acids, but excludes arachidonic acid (Grobler *et al.*, 2008:285). The fatty acids are compatible with the orientation of fatty acids found in humans, because they are in the *cis*-formation (Grobler *et al.*, 2008:285).

Essential fatty acids are used mainly in the Pheroid™ in an effort to make a skin friendly barrier. The primary essential fatty acids used in every formulation are linolenic acid and linoleic acid (Grobler *et al.*, 2008:305). All topical Pheroid™ formulations currently contain vitamin E or vitamin E derivates, also known as tocopherol. These molecules are used as anti-oxidants and as emulsion stabilisers (Grobler *et al.*, 2008:293).

Nitrous oxide (N_2O) is a unique component of Pheroid™. It is distributed throughout the continuous phase, adding another dimension to the basic Pheroid™. N_2O within the dispersed

phase contributes to the miscibility of the fatty acids, the self-assembly process of the Pheroid™ and to the stability of the formed Pheroid™.

N_2O is a volatile compound that is both water and fat soluble, which enables free movement of the gas through the epidermal and dermal layers of the skin (Grobler *et al.*, 2008:289). The N_2O essential fatty acid (NOEFA) matrix provides a functional model for the transport of hydrophobic and hydrophilic drugs (Grobler *et al.*, 2008:290).

In the design of the Pheroid™ one or more characteristics of colloidal dosage forms, commonly used, have been included (Grobler *et al.*, 2008:287). Pheroid™ generally contain a lipid bilayer as is the case with liposomes, but it contains no phospholipids or cholesterol (Grobler, 2004:7; Grobler *et al.*, 2008:288). The Pheroid™ are formed by a self-assembly process similar to that of low-energy emulsions and micro-emulsions, but no lyophilisation or hydration of the lipid components is necessary. Pheroid™ are dispersed within a dispersion medium as in emulsions, but in addition it contains a dispersed gas phase associated with the fatty acid dispersed phase.

The design of the Pheroid™ allows for the manipulation of structural and functional characteristics. These characteristics can be manipulated by:

- changing the composition or concentration of the fatty acids and the hydration medium (ionic strength, pH);
- changing the method of preparation;
- changing the character and the concentration of the active compound;
- addition of sunscreen formulations;
- addition of charge-inducing agents;
- addition of cryo-protectants; and
- addition of non-fatty acids or phospholipids i.e., cholesterol (Grobler *et al.*, 2008:292).

2.6.3.2 Functional characteristics of Pheroid™

The components of Pheroid™ are manipulated in a very specific manner to ensure its high entrapment capabilities, fast rate of transport, delivery and stability. The absorption capabilities and drug release characteristics of the Pheroid™ can thus be controlled (Grobler, 2004:4).

2.6.3.2.1 Pliability and versatility

Versatility is one of the requirements for an acceptable carrier due to the different target cells in the human body (Grobler *et al.*, 2008:293). The functional versatility of the Pheroid™ is shown

in the different routes of administration that have been investigated, the enhanced efficacy of a variety of compounds and the variety of dosage forms that have been used. Pheroid™ are extremely pliable and elastic due to the use of gas, as well as the pliable pegylated tails added to the fatty acids (Grobler *et al.*, 2008:294).

2.6.3.2.2 Entrapment efficiency

An entrapment of 90% is the aim for all Pheroid™ products. Due to the pliable design of the Pheroid™ the number of colloidal particles per volume can be increased or decreased to suite the required concentration of active compound. Size, charge and solubility of the active compound determine the number of molecules trapped within one Pheroid™ (Grobler *et al.*, 2008:294). Entrapment of actives within the Pheroid™ generally creates a safer, more effective formulation as well as the maintenance of the membrane integrity of cells of the epidermis (Saunders *et al.,* 1999:99; Grobler, 2004:4).

2.6.3.2.3 Penetration efficiency

According to Grobler (2004:4), studies show that Pheroid™ technology increases the percentage active compound delivered to the skin. A comparative investigation, where an existing commercial product is compared to the enhancement caused by the carrier, is an easy way to determine the penetration efficiency (Grobler *et al.*, 2008:296).

2.6.3.2.4 Uptake of Pheroid™ and compounds by cells

The stratum corneum offers a challenge for the efficient dermal and transdermal delivery of active compounds. The Pheroid™ are stearically stabilised by electro-chemical interaction, which makes the Pheroid™ an extremely elastic vesicular structure. Fluidity of the Pheroid™ membrane contributes to the efficient delivery of topical drugs. The mechanism of uptake by the cells as well as the formulation of the Pheroid™ may influence the uptake of the Pheroid™. Factors that influence the permeation of Pheroid™ formulation include:

- size of the Pheroid™;
- morphology of the Pheroid™;
- molecular geometry;
- concentration and ration of the various fatty acids;
- hydration medium;
- pH of the preparation;
- character and concentration of the active compound;

- state of the Pheroid™; and the
- presence of charge-changing molecules or molecules that influence the electrostatic milieu (Grobler *et al.*, 2008:299).

2.6.3.2.5 Metabolism, targeting and distribution

Pheroid™ are released after metabolism in either the mitochondria or the peroxisomes of the cell, depending on the composition of the Pheroid™ (Grobler *et al.*, 2008:300). Pheroid™ are also known to protect drugs from metabolism (Grobler, 2004:7). Cellular uptake of the Pheroid™ is based on various interactions between the fatty acids and cells (Grobler *et al.*, 2008:300). There exists an affinity between the Pheroid™ and the cell membrane due to the fact that Pheroid™ consist of fatty acids, which results in enhanced penetration and delivery (Grobler, 2004:6). Distribution of Pheroid™ is influenced by the type and extent of the fatty acid modifications (Grobler *et al.*, 2008:300).

2.6.3.3 Therapeutic enhancement of anti-viral and anti-fungal compounds with Pheroid™ technology

The performance of a delivery system can be indicated by the efficiency of entrapment and the rate of transport and release of active compounds into, and across the skin. However, the effect of a carrier should ideally be measured by its contribution to therapeutic efficacy (Grobler *et al.*, 2008:300). According to Grobler (2004:9), both *in vitro* and *in vivo* studies showed that the percentage active compound delivered, was enhanced by the entrapment in Pheroid™. Membrane diffusion studies have also shown an increase in the percentage release of acyclovir (anti-viral) and miconazole nitrate (anti-fungal) to human skin, compared to commercial available products (Grobler, 2004:9). The Pheroid™ itself also contains anti-fungal activity (Grobler, 2004:12), thus the more reason to formulate ketoconazole within Pheroid™.

2.7 SKIN DISORDERS ASSOCIATED WITH HIV/AIDS

2.7.1 HIV/AIDS

2.7.1.1 Pathogenesis

HIV infection is an infection by one of two viruses, HIV-1 and HIV-2. The HIV viruses progressively destroy some types of white blood cells called lymphocytes. Lymphocytes are an important part of the body's immune defences. When lymphocytes are destroyed, the body becomes susceptible to attack by many other infectious organisms. Many of the complications of HIV infection, including death, are usually the result of these other infections and not of the HIV infection itself. AIDS is the most severe form of HIV infection. A person with HIV infection is considered to have AIDS when at least one complicating illness develops or his ability to

defend against infection, significantly declines as measured by a low CD4+ lymphocyte count (Mac Neal, 2006).

The HIV virus surrounded by a lipid envelope, is more than 100 nm in diameter and has a dense cylindrical nucleoid containing core proteins, genomic ribonucleic acid (RNA) and the reverse transcriptase, which classifies HIV as a retrovirus. HIV, however, is unique among retroviruses, because it has at least five genes in addition to the standard genes, which encode for core proteins, envelope protein and reverse transcriptase (Penneys, 1989:1).

The virus invades active monocytes, with particular affinity for T-helper cells. Patients develop both a decreased number of T-lymphocytes and decreased function of CD4+ T-cells. Response to T-cell mitogens such as pokeweed mitogen and Concanavalin A is poor. As might be expected, patients also manifest with inversion of the T4/T8 ratio and show either abnormal or no delayed type hypersensitivity to common agents. Defects of natural killer (NK) cells and monocytes are also present. Consequently, the patient with HIV disease is rendered susceptible to organisms, which usually cause little or no morbidity or mortality in the immunocompetent host (Durden & Elewski, 1997:200).

2.7.1.2 Geographical distribution

AIDS was first reported in 1981, when it was observed that homosexual individuals appeared to be at risk for diseases associated with impaired cell-mediated immunity. Subsequently, other high risk groups were identified, and a retrovirus known as HIV-1 was found to be the causative agent (Durden & Elewski, 1997:200).

Clinical manifestations of HIV were first observed in the late 1970's and HIV was first isolated in the 1980's. After many years of studying this deadly virus, researchers and clinicians agreed that early treatment is the key to retarding disease progression. Highly active antiretroviral therapy (HAART) has been effective in prolonging survival, especially when used early in the disease process (Yen-More *et al.*, 2000:423).

Infections with HIV-1 and HIV-2 are serious, and tend to occur in different regions. HIV-1 is most common in the Western Hemisphere; Europe; Asia; and Central, South and East Africa. HIV-2 is common in West Africa, although many people there are infected with HIV-1 (Mac Neal, 2006).

2.7.1.3 Modes of transmission

The transmission of HIV requires contact with a body fluid that contains the virus or infected cells. HIV can appear in nearly any body fluid, but transmission mainly comes from blood, semen, vaginal secretions, and breast milk. Although low concentrations of HIV are also

present in tears, urine and saliva; transmission from these fluids is extremely rare. HIV is commonly transmitted in the following ways:

- Sexual contact with an infected person, during which the mucous membrane lining the mouth, vagina, penis or rectum is exposed to contaminated body fluids (unprotected sex).
- The injection or infusion of contaminated blood, as occurs with blood transfusion.
- Sharing of needles or an accidental prick from an HIV-contaminated needle.
- Transfer of the virus from an infected mother to child before birth, during birth or after birth through the mother's milk (Mac Neal, 2006).

Susceptibility to HIV infection increases when the skin or mucous membrane is torn or damaged, even minimally, as can happen during vigorous vaginal or anal intercourse. Sexual transmission of HIV is more likely if either partner has herpes, syphilis or another sexually transmitted disease (STD) that produces breaks in the skin or inflammation of the genitals. However, HIV can be transmitted even if neither of the partners have other STD's or obvious breaks in the skin (Mac Neal, 2006).

2.7.2 Early cutaneous signs and symptoms of HIV

Patients may remain asymptomatic for a prolonged period of time with little evidence of immunosuppression. However, 50% of patients with early infection have had skin eruptions described as macular, maculopapular, red and predominantly located at the trunk (Penneys, 1989:7).

HIV and the AIDS epidemic have had a profound impact on the spectrum of diagnosis of cutaneous disease. Skin disorders may be present in up to 92% of HIV-positive patients, and may be the first or only organ affected throughout the course of the disease (Ramdial, 2000:112).

2.7.3 Opportunistic infections in HIV/AIDS

According to Ramdial (2000:113), the skin is the most commonly affected organ in HIV infected individuals, thus, the reason why skin manifestations of HIV infection have been the subject of intense scrutiny. Infectious and non-infectious HIV-induced skin diseases may not only serve as a marker of HIV infection, but also as a marker of the stage of the HIV disease (Ramdial, 2000:113). There is an incredibly wide range of skin manifestations that presents in HIV/AIDS infected individuals. These skin manifestations are summarised in Figure 2.3.

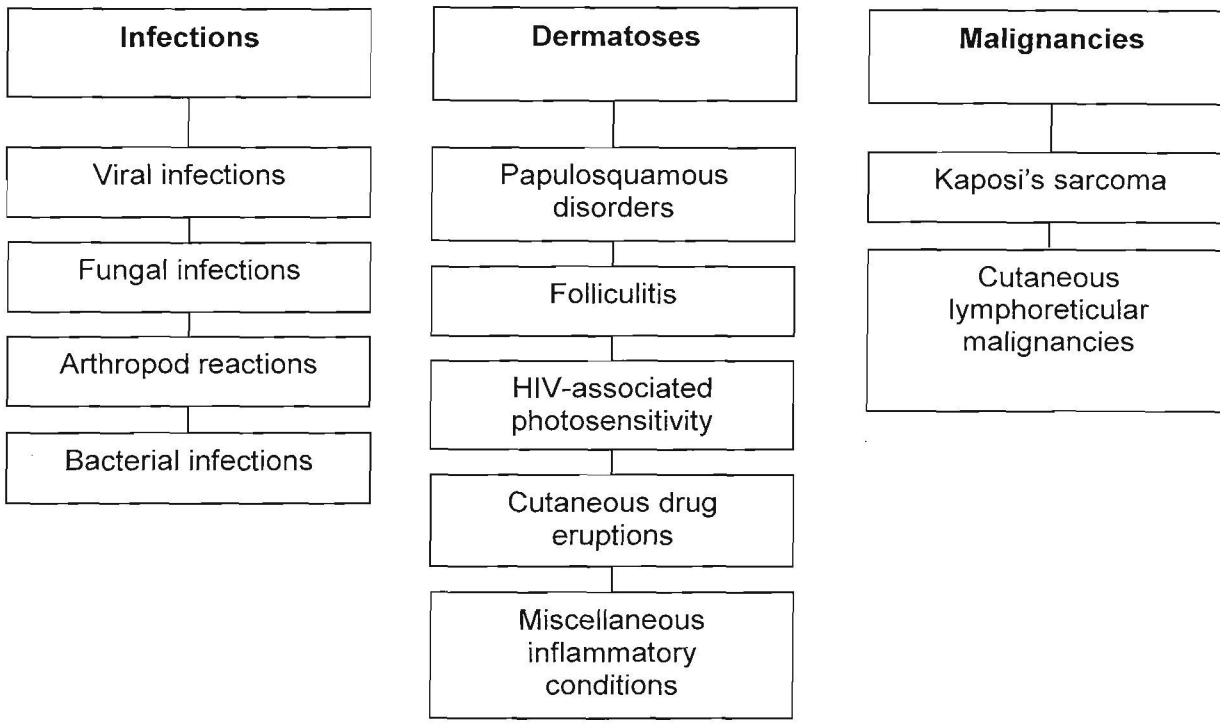


Figure 2.3: Manifestations present in HIV/AIDS infected individuals

This study will focus on viral and fungal infections. The opportunistic infections that occur in HIV/AIDS patients are summarised in Figure 2.4.

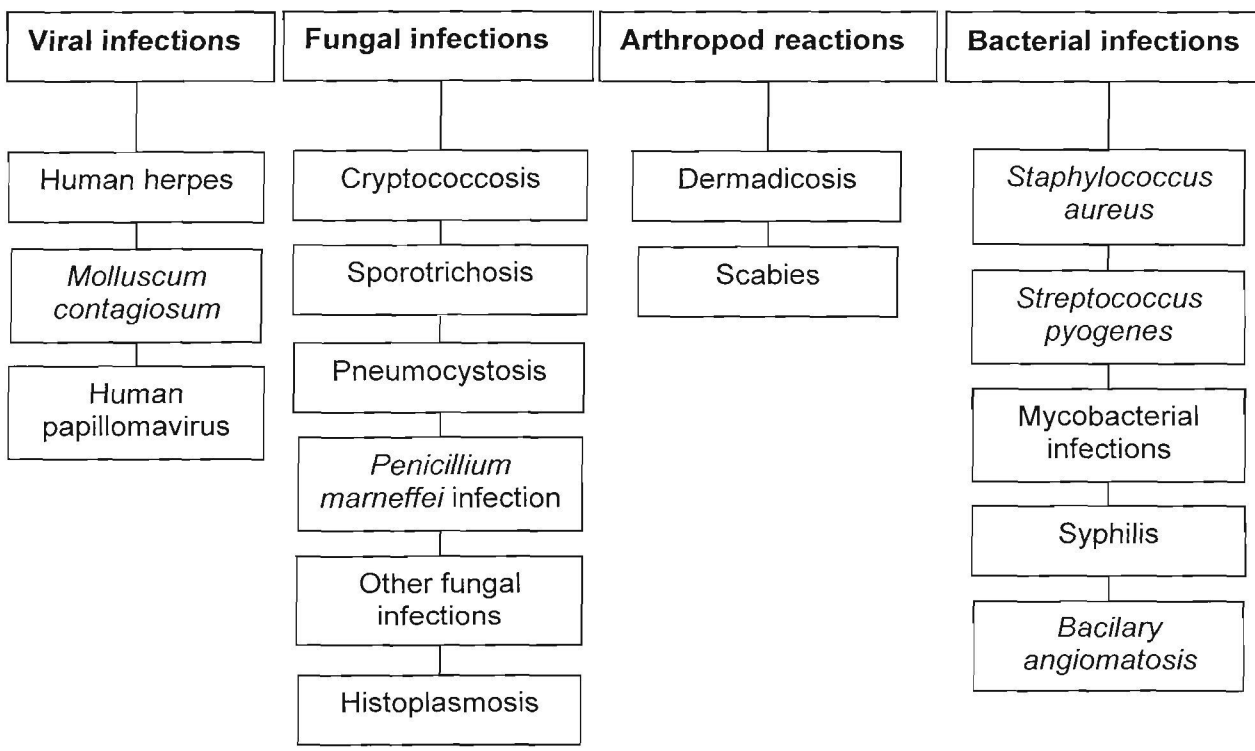


Figure 2.4: Opportunistic infections in HIV/AIDS patients

Cutaneous disorders in HIV-positive individuals can be more severe and disabling than in normal host individuals. The cutaneous manifestation itself can in some situations lead to the diagnosis of HIV or AIDS (Yen-More *et al.*, 2000:429). The severe reduction of T-helper lymphocytes in the progressive stages of HIV infection paves the way for opportunistic infections (Ramdial, 2000:113).

2.7.3.1 Viral infections

Although many opportunistic viral infections in HIV-positive persons have similar manifestations as those in otherwise healthy individuals, infections in this immunocompromised population are seen in greater prevalence, cause unique and more severe mucocutaneous lesions, are more difficult to treat, and may lead to higher rates of neoplasia (Yen-More *et al.*, 2000:429).

Opportunistic viral infections in HIV-positive patients can create both physical and psychological mortality and morbidity. Skin manifestations of these viral diseases can often lead to a diagnosis of either HIV or the underlying disease (Yen-More *et al.*, 2000:423). There are three main types of viral infections that occur cutaneously in HIV/AIDS patients. These viruses are discussed below and include: human herpes viruses, human papilloma virus and *Molluscum contagiosum*.

2.7.3.1.1 Human herpes viruses

The human herpes virus family consists of eight viruses: HSV types 1 and 2, varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human herpes viruses (HHV) 6, 7 and 8. All herpes viruses are enclosed double stranded deoxyribonucleic acid (DNA) viruses that cause either active or dormant infection continuing all the way through the lifetime of the host (Yen-More *et al.*, 2000:423).

- Herpes simplex viruses (HSV)

HSV is transmitted by direct contact with active lesions on the skin, mucus membranes, saliva, semen and cervical secretions. Primary HSV infections, in normal hosts, are usually more relentless and harsh than recurrences. Cutaneous HSV infections occur in the region of 20 - 50% of patients with HIV/AIDS. Though, up to 95% of HIV-seropositive patients have antibodies against HSV-1, HSV-2 or both. Primary infection in HIV/AIDS patients with HSV can be critical (Yen-More *et al.*, 2000:423). Reactivation of HSV occurs frequently in patients with AIDS at all levels of immunocompetency, resulting in chronic mucocutaneous disease with severe and extensive skin ulcers. Recurring HSV infection becomes persistent and more progressive with advancement in HIV disease. Ulcers usually present in the perianal, genital and lip areas (Ramdial, 2000:113). It is hypothesised that HVS can affect other areas in

HIV/AIDS patients such as the cornea, oesophagus, lungs, trachea, bronchial tree, pericardium, liver and the brain (Yen-More *et al.,* 2000:423). The differential diagnosis of HSV in HIV/AIDS patients is far reaching. Ulcerated and verrucous lesions can imitate such entities as aphthous ulcers, opportunistic atypical mycobacterial and deep fungal infections, traumatic ulcers and epithelial neoplasms (Yen-Moore *et al.*, 2000:424).

- Varicella zoster virus (VZV)

Coalescence of individual lesions, haemorrhagic bullae, extensive ulceration with epidermal necrosis and black eschars may be seen (Ramdial, 2000:114). Ramdial (2000:114) stated that the occurrence of *herpes zoster* in younger patients; recurrent attacks that are more severe, more frequent and of longer duration; as well as the resistance to acyclovir, shows to the co-existence of HIV and VZV.

- Cytomegalovirus (CMV)

Primary skin involvement by CMV is rare, although the CMV is the most commonly isolated virus in AIDS patients. Up to 90% of AIDS patients develop acute active CMV infection at some stage of their illness. CMV clinically presents as maculopapular eruptions, ulcers, nodules, vesicles, generalised morbilliform eruptions as well as verrucous and hyper pigmented indurated plaques (Ramdial, 2000:114).

2.7.3.1.2 Human papillomavirus (HPV)

The most widespread group of viral infections in the skin, is infection with HPV. It has been speculated that global NK cell deficiency and T lymphocyte defects are factors predisposing HIV infected persons to HPV infection. HPV has a wide range, from common and genital warts, to epithelial and invasive squamous carcinoma of the cervix. Common warts occur more frequently in HIV positive patients than in non-infected individuals. Different HPV types are associated with different clinical lesions. Condylomata acuminate manifestations, reported in 20% of homosexual men, range from soft lesions with smooth or rough surfaces to cauliflower like plaques. Excision of these lesions in AIDS patients are advised, because of the risk of dysplasia in perianal condyloma (Ramdial, 2000:115).

2.7.3.1.3 *Molluscum contagiosum* (MC)

MC in immunocompetent hosts occurs in the anogenital area and truck, and undergoes spontaneous resolution within a year of occurrence. In HIV-positive patients, MC presents as persistent papules over the face, neck, eyelid, intertrigininous area, axillae, perianal area and buttocks (Ramdial, 2000:114).

MC is associated with significant immunosuppression. Between 10 - 20% of HIV/AIDS patients have MC. Individual lesions may be large and solitary, measuring up to 2 cm in diameter. In HIV/AIDS infected individuals warty verrucous papules may be seen in addition to the typical umbilicated papules (Ramdial, 2000:114). Verrucous and giant MC occur in the late stage of the disease with peripheral CD4 T-lymphocyte counts of < 50 cells/mm^3 (Ramdial, 2000:115).

2.7.3.2 Fungal infections

Superficial fungal infections are among the most common cutaneous problems in patients with HIV infection (Penneys, 1989:8). Organisms formerly considered to be non-pathogenic are emerging as virulent pathogens among HIV-infected persons. Fungal infections in the patient infected with HIV are a major cause of morbidity and mortality. The yeast *Candida* and *Cryptococcus neoformans,* the dimorphic fungi *Histoplasma capsulatum* and *Sporothrix schenckii*, as well as dermatopyte fungi are the most common pathogenic fungi in patients infected with HIV (Durden & Elewski, 1997:200). Recognition of these fungal infections leads rapidly to the correct diagnosis. The presence of these unusual patterns can suggest clinical diagnosis of HIV infection in patients who are otherwise asymptomatic (Penneys, 1989:8). The AIDS epidemic has increased the worldwide prevalence of fungal infections, because the defective immune system resulting from HIV infection, provides an ideal environment for mycotic pathogens (Durden & Elewski, 1997:200).

2.7.3.2.1 *Histoplasma capsulatum*

Approximately 17% of reported HIV-infected patients with disseminated histoplasmosis have histological and/or culture-proven mucocutaneous lesions of histoplasmosis. Histoplasmosis is transmitted by inhalation; and skin involvement is usually secondary to lung involvement, but primary skin infections may occur in a solitary or disseminated form. The clinical lesions associated with histoplasmosis can be nodular, papular, macular, fistulous, pustular, herpes-like or plaque-like (Ramdial, 2000:115).

2.7.3.2.2 *Cryptococcus neoformans*

Cryptococcus is the second most common fungal infection in HIV-positive patients. Skin involvement occurs in 6 - 7% of patients with *Cryptococcus*. The mucocutaneous lesions in *Cryptococcus* are polymorphous (Ramdial, 2000:115).

2.7.3.2.3 *Sporothrix schenckii*

Sportotrichosis in HIV infected individuals occurs as a result of haematogenous dissemination of the fungus rather than from local cutaneous inoculation. Skin lesions range from pustules, ulcers, papules and erythematic nodules, to plaques (Ramdial, 2000:116).

2.7.3.2.4 *Pneumocistis carinii*

Cutaneous pneumocystosis is unusual and occurs as a consequence of the use of Pentamidine sprays in patients with prior *pneumocistis* pneumonia, or in patients receiving prophylaxis, because of CD4+ lymphocyte counts less than 200 cells/mm^3. Although these sprays sterilise the respiratory tract, they do not offer systemic protection against *Pneumocystis carinii.* Cutaneous legions manifest as molluscum-like plaques, bluish cellulitic plaques and deep-seated abscesses (Ramdial, 2000:116).

2.7.3.2.5 *Penicillium marneffei* infection

Cutaneous manifestations in *Penicillium marneffei* include generalised, crusted or necrotic papules, ulcers, molluscum-like lesions, abscesses and subcutaneous nodules (Ramdial, 2000:116).

2.7.3.2.6 *Candida*

Candidiasis is common in AIDS patients. Chronic mucocutaneous and disseminated cutaneous candidiasis occurs. The histopathological features of disseminated cutaneous candidiasis include dermal micro-abscesses and associated leucocytoclastic vasculitis. Aggregates of candidal hyphae and spores are found within micro-abscesses or in areas of vascular damage (Ramdial, 2000:116).

2.7.3.2.7 *Blastomycis dermatitidis*

Blastomycosis is an uncommon fungal infection in AIDS patients, occurring in the advanced stage of the disease (Ramdial, 2000:116).

2.8 TREATMENT OF HIV/AIDS SKIN MANIFESTATIONS WITH COSMECEUTICAL ACTIVES

The short term control of opportunistic infections requires a high index of suspicion, biopsies, and cultures as indicated and appropriate medical and/or surgical interventions. Longer term control of these infections is being facilitated by the initiation of HAART therapy (Yen-More *et al.*, 2000:429). There is a changing trend in HIV-induced cutaneous manifestations, because of the use of HAART therapy in some countries, but in developing countries where HAART is not readily available, the skin continues to be destroyed by HIV infection (Ramdial, 2000:113). The ultimate key to the management of HIV and these opportunistic infections will be by reducing transmission through better education (Yen-More *et al.*, 2000:429).

This study focuses on treating both viral and fungal infections, with a cream and emulgel, which contains a combination of an anti-viral agent, acyclovir, and an anti-fungal agent, ketoconazole. The mechanisms of action of these actives are discussed in this section.

2.8.1 Acyclovir

Acyclovir is an antiviral drug of choice in the treatment of many types of herpes virus infections, including genital HSV infections, herpetic conjunctivitis and herpes simplex encephalitis.

2.8.1.1 Chemistry

Acyclovir (9-[(2-hydroxyethoxy)methyl]-9H-guanine) is an acyclic guanine nucleoside analogue that lacks a 3'-hydroxyl on the side chain (Hayden, 2001:1317). The chemical structure of acyclovir is shown in Figure 2.5.

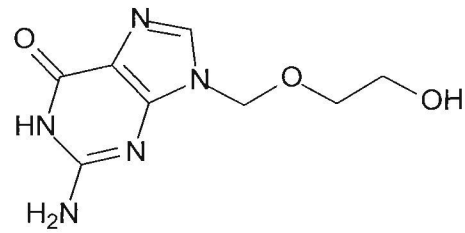


Figure 2.5: Chemical structure of acyclovir (Sweetman, 2008)

2.8.1.2 Mechanism of action

Acyclovir is active against HSV-1 and HSV-2, as well as VZV (Hayden, 2001:1317). HSV-1 (0.02 - 0.09 µg/ml) seems to be the most susceptible to acyclovir, then HSV-2 (0.03 - 2.2 µg/ml), followed by VZV (0.8 - 4.0 µg/ml). Acyclovir also effects EBV (0.8 - 4.0 µg/ml) and CMV (> 20 µg/ml), but to a lesser extent (Hayden, 2001:1317; Sweetman, 2008).

Acyclovir inhibits viral DNA synthesis. It must be phosphorylated intracellular to be active. Acyclovir is converted to the monophosphate by the viral thymidine kinase, then to diphosphate by cellular guanylate kinase, and finally to the active triphosphate by various cellular enzymes (Hayden, 2001:1317). This active form inhibits viral DNA synthesis and replication by inhibiting the herpes virus DNA polymerase enzyme, as well as being incorporated into viral DNA. This process is highly selective for infected cells (Sweetman, 2008).

There is some evidence that show that acyclovir has an effect on latent HSV at an early stage of reactivation, but it is generally considered to have no activity against latent viruses (Sweetman, 2008).

2.8.1.3 Adverse reactions

Acyclovir can be administered orally, intravenously or topically (Jiang *et al.*, 1998:103). It is generally well tolerated. Adverse effects after systemic administration include renal impairment, increased serum bilirubin and liver enzymes, haematological changes, skin rashes, fever, headache, dizziness, and gastrointestinal effects. Anaphylaxis has been reported. Neurological effects include confusion, hallucinations, tremors, agitation, psychosis, somnolence, convulsions, and coma have been reported in a small number of patients (Sweetman, 2008).

Topical application of acyclovir, especially to genital lesions, may sometimes produce stinging, itching, burning or erythema (Sweetman, 2008). The main reason for topical administration of acyclovir is because of the large number of adverse effects with oral and parenteral administration. However, topical application of acyclovir has shown to be less effective compared with oral or intravenous routes. Lack of effectiveness of topically applied acyclovir has been attributed to an inadequate delivery of the drug to the skin cells (Jiang *et al.*, 1998:103).

2.8.1.4 Physicochemical properties

Physicochemical properties an active drug can either be a preventive factor, or it can be helpful to transdermal delivery of the drug. The physicochemical properties of acyclovir are shown in Table 2.1.

Table 2.1: Physicochemical properties of acyclovir and ketoconazole

Physicochemical property	Acyclovir	Ketoconazole	References
Aqueous solubility	2.5 mg/ml in water at 25°C.	40 µg/ml in water at 25°C.	McEvoy., 2002:11.
Molecular weight	225.2 g/mol	531.43 g/mol	British Pharmacopoeia, 2009
Melting point	256.5-257°C [1]	146°C [2]	1. McEvoy., 2002:11. 2. British Pharmacopoeia, 2009
Partition coefficient (Log P)	0.018[a1]	4.31[b2]	1. Dollery, 1999: A39. 2. McEvoy., 2002:11.
pKa	2.27, 9.25	2.94, 6.51	Skiba *et al.*, 2000:2.

[a] Log P between *n*-octanol and 0.2 M PBS

[b] Log P between *n*-octanol and water

2.8.2 Ketoconazole

Ketoconazole is a broad spectrum imidazole anti-fungal agent formulated into a number of dosage forms through various routes of administration, such as oral and topical (Skiba *et al.*, 2000:2). Ketoconazole has an antifungal activity against fungal pathogens including: *Candida* species, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii* (Bennett, 2001:1301).

2.8.2.1 Chemistry

Ketoconazole (cis-1-acety1-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)1,3dioxolan-4-yl] methoxyl]phenyl]piperazine) is an imidazole derivate that is structurally related to miconazole (Männisotö *et al.*, 1982:730; Mingawa *et al.*, 1983:105). The chemical structure of ketoconazole is shown in Figure 2.6.

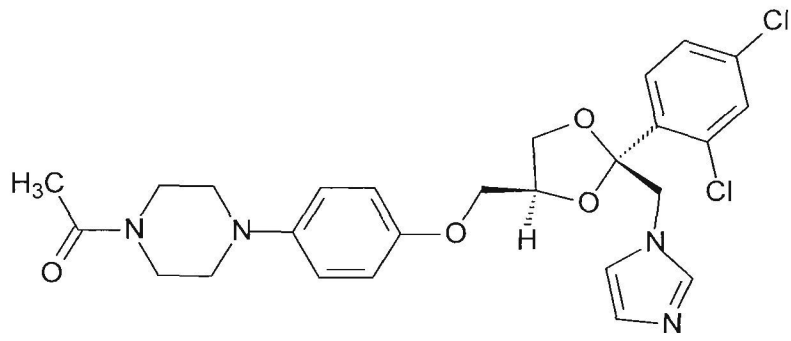


Figure 2.6: Chemical structure of ketoconazole (Bennett, 2001:1301)

2.8.2.2 Mechanism of action

Ketoconazole works by altering the fungal cell membrane. It inhibits ergosterol synthesis by interaction with 14-alpha demethylase. Lanosterol is converted by 14-alpha demethylase, a cytochrome P-450 enzyme, to ergosterol, which is an essential component of the membrane. Inhibition of ergosterol synthesis results in increased cellular permeability, which causes leakage of the cellular contents of the fungi (Bennett, 2001:1301). At higher concentrations, ketoconazole may have a direct physicochemical effect on the fungal cell membrane, which leads to a fungicidal action (Bennett, 2001:1301).

Ketoconazole possesses actions that may make it useful in conditions other than fungal infections. It can inhibit sterol synthesis in humans, including the synthesis of aldosterone, cortisol and testosterone (Bennett, 2001:1301).

2.8.2.3 Physicochemical properties

The physicochemical properties of an active drug can either be a preventive factor or it can be helpful to transdermal delivery of the drug. The physicochemical properties of ketoconazole are shown in Table 2.1.

2.8.2.4 Adverse reactions

Gastrointestinal disturbances, such as nausea and vomiting, are the most frequent adverse effects after oral use of ketoconazole. Hepatitis has been reported in some cases where the treatment with ketoconazole was continued for longer than 2 weeks. Ketoconazole interferes with steroid biosynthesis. Endocrine effects of ketoconazole include menstrual irregularities, oligospermia and gynaecomastia. Other adverse effects that have been reported include headache, dizziness, impotence and somnolence (Bennett, 2001:1302).

Topical adverse reactions include: pruritus, irritation, photosensitivity, dermatitis, a burning sensation and allergic skin reactions (Sweetman, 2008).

2.8.2.5 Drug interactions

Ketoconazole either increases or decreases the concentration of a number of drugs taken in combination. This is due to ketoconazole's effect on the enzyme cytochrome P-450. According to Bennett (2001:1303), topically applied ketoconazole does not have any significant systemic absorption, which minimises adverse effects and drug interactions. The drugs affected by ketoconazole are listed in Table 2.2.

Table 2.2: Interactions of ketoconazole with other drugs (Sweetman, 2008)

Ketoconazole concentration decreased	
Drugs that decrease gastric acidity:	• H2 receptor blockers • Proton pump blockers • Simultaneous antacids • Simultaneous didanosine (buffer)
Enzyme inducing drugs:	• Rifampicin • Efavirenz • Carbamazepine • Izoniazid • Nevirapine • Phenytoin
Other drug concentration increased	
Drugs metabolised by the liver: (Ketoconazole inhibits hepatic oxidase enzymes)	• Alfentanil • Antihistamines: loratadine, terfenadine • Benzodiazepines: alprazolam, midazolam, triazolam • Cisapride • Dihydropyridine • Calcium channel blockers • Domperidone • HIV-protease inhibitors: ritonavir, indinavir • Immunosuppressants • Methylprednisolone • Nevirapine • Quinidine • Sildenafil • Statins: atorvasatin, lovstatin, simvastatin • Verapamil
Other drugs:	• Buspirone • Busulfan • Digoxin • Reboxetine

2.9 CONCLUSION

Immunocompromised patients with HIV/AIDS are more susceptible to infections. Infectious skin manifestations are, thus, common in these patients. There is no cure for this illness, but treatment of the symptoms is possible. This study focuses on treating the fungal and viral skin manifestations seen in these patients. Curing the skin lesions will improve patient's physical wellbeing and possibly contribute to a better state of mind.

Seeing that most HIV/AIDS patients already drink a lot of medication, topical administration of anti-viral and anti-fungal drugs have the advantage of a reduced amount of drug interactions and adverse effects.

The skin is a formidable barrier to drug penetration. The main focus of a formulator is to find a way to improve drug delivery through the skin, without damaging the skin. In this study Pheroid™ technology was used as penetration enhancer to improve the topical delivery of acyclovir and ketoconazole to the skin. An increase in delivery of these actives to the skin can help to treat opportunistic infections in HIV/AIDS patients.

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CHAPTER 3

ARTICLE FOR PUBLISHING IN THE

INTERNATIONAL JOURNAL OF PHARMACEUTICS

Formulation and transdermal delivery of acyclovir and ketoconazole

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Abstract

The scope of this *in vitro* study was to investigate the efficacy of the novel Pheroid™ technology system in a semi-solid dosage form for the topical delivery of the anti-viral, acyclovir (5% w/w) and the anti-fungal, ketoconazole (2% w/w). The reason for the formulation of a product containing a combination of an anti-viral and an anti-fungal drug is that viral and fungal cutaneous manifestations are regularly encountered in immunocompromised HIV/AIDS individuals. This formulation may be useful to treat these opportunistic skin manifestations. The stability of the formulated semi-solid products was examined over a period of six months according to the International Conference of Harmonisation (ICH) Tripartite Guideline. The stability tests conducted included the assay of the actives, pH, viscosity, mass loss and particle size observation. Vertical Franz cell diffusion studies and tape stripping methods were used to determine the *in vitro*, epidermal and dermal delivery of these formulated semi-solid products. Results showed that the Pheroid™ formulae increased transdermal penetration as well as delivery to the dermal and epidermal skin layers. The Pheroid™ emulgel and the Pheroid™ cream increased the topical delivery of ketoconazole and acyclovir, respectively. Stability tests, however, showed that the Pheroid™ formulations were not stable.

Keywords: Acyclovir, Ketoconazole, Topical delivery, HIV/AIDS, Pheroid™ technology, Formulation

1 Introduction

The acquired immunodeficiency syndrome (AIDS) and the human immunodeficiency virus (HIV) pandemic have had a profound effect on the extent and diagnosis of cutaneous disorders. According to Ramdial (2000) the skin is the most commonly affected organ in HIV infected individuals. There is an incredibly wide range of skin manifestations that presents in HIV/AIDS infected individuals. Cutaneous disorders in HIV-positive individuals can be more severe and disabling than in normal host individuals (Yen-More et al., 2000). The cutaneous manifestation itself can in some situations lead to the diagnosis of HIV or AIDS, and it can also serve as a marker of the stage of the HIV disease (Ramdial, 2000; Yen-More et al., 2000).

There are three main types of viral infections that occur cutaneously in HIV/AIDS patients, these viruses include *Human herpes viruses*, *Human papilloma virus* and *Molluscum contagiosum*. Many opportunistic viral infections in HIV/AIDS patients have similar manifestations as those in healthy individuals, but infections in this immunocompromised population are seen in greater prevalence. These infections can cause unique and more severe mucocutaneous lesions and are more difficult to treat. Viral infections may also lead to higher rates of neoplasia (Yen-More et al., 2000). The AIDS epidemic has increased the worldwide prevalence of fungal infections. Fungal infections are a major cause of morbidity and mortality in patients infected with the HIV virus. The yeast *Candida species* and *Cryptococcus neoformans,* the dimorphic fungi *Histoplasma capsulatum* and *Sporothrix schenckii*, and dermatopyte fungi are the most common pathogenic fungi in patients infected with HIV (Durden and Elewski, 1997).

Acyclovir is the anti-viral drug of choice in the treatment of many types of herpes virus infections, including genital herpes simplex infections, herpetic conjunctivitis and herpes simplex encephalitis. Acyclovir is active against herpes simplex virus (HSV) type 1 and type 2 and against the varicella zoster virus (Hayden, 2001). HSV-1 (0.02 - 0.09 µg/ml) seems to be the most susceptible to acyclovir, then HSV-2 (0.03 - 2.2 µg/ml), followed by the varicella zoster virus (0.8 - 4.0 µg/ml). Acyclovir also effects the Epstein-Barr virus (0.8 - 4.0 µg/ml) and cytomegalovirus (>20 µg/ml), but to a lesser extent (Hayden, 2001; Sweetman, 2008). Acyclovir inhibits viral deoxyribonucleic acid (DNA) synthesis. It must be phosphorylated intracellularly to be active. Acyclovir is converted to the monophosphate by the viral thymidine kinase, then to

diphosphate by cellular guanylate kinase, and finally, to the active triphosphate by various cellular enzymes (Hayden, 2001). This active form inhibits viral DNA synthesis and replication by inhibiting the herpes virus DNA polymerase enzyme as well as being incorporated into viral DNA. This process is highly selective for infected cells (Sweetman, 2008).

Ketoconazole is a broad spectrum imidazole anti-fungal agent (Skiba et al., 2000). It has an antifungal activity against fungal pathogens including: *Candida species*, *Blastomyces dermatitidis*, *Cryptocpccus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii* (Bennett., 2001). Ketoconazole alters the fungal cell membrane and inhibits ergosterol synthesis by interaction with 14-alpha demethylase. Lanosterol is converted by 14-alpha demethylase (cytochrome P-450 enzyme) to egosterol, which is an essential component of the membrane. Inhibition of ergosterol synthesis results in increased cellular permeability, which causes leakage of the cellular contents of the fungi (Bennett, 2001).

The target site for acyclovir and ketoconazole is the basal epidermis and the stratum corneum, respectively. Adequate concentrations of each drug should be delivered to the target site of the skin to inhibit the growth of viral and fungal pathogens (Pershing et al., 1994; Jiang et al., 1998). The transdermal diffusion of acyclovir may be possible when considering the aqueous solubility (2.50 mg/ml in water at 25 °C) and molecular weight (225.20 g/mol) of acyclovir, but the high melting point (256.5 - 257 °C) and low octanol-buffer partition coefficient (log D; 0.018) could be a preventive factor (Dollery, 1999; McEvoy, 2002; British Pharmacopoeia, 2009). When considering the melting point (146 °C) of ketoconazole the penetration of the drug may be possible, but it has a very low aqueous solubility (40 µg/ml in water at 25 °C), high molecular weight (531.43 g/mol) and high octanol-water partition coefficient (log P; 4.31). This could have a negative impact on the transdermal penetration of the ketoconazole and poor transdermal delivery can be anticipated (McEvoy, 2002; British Pharmacopoeia, 2009). According to Thomas and Finnin (2004), compounds with high log P values exhibit low permeability, because they are not able to partition out of the stratum corneum. This can be a positive factor for the delivery of ketoconazole to the stratum corneum.

The patented Pheroid™ delivery system, formerly known as Emzaloid™, was employed in the formulation of the semi-solids as a carrier system for acyclovir and ketoconazole. This was done in order to establish whether the Pheroid™ will enhance penetration to the target sites. The Pheroid™ delivery system consists of a colloid system that contains stable lipid-based submicron- and micron-sized structures, called Pheroid™. These Pheroid™ are uniformly distributed in a dispersion medium. The basic Pheroid™ has a vesicular structure with sizes ranging from 200 - 440 nm (Grobler et al., 2008). The main essential fatty acids used in the Pheroid™ are linolenic acid and linoleic acid, as well as oleic acid. These essential fatty acids are emulsified in water and saturated with nitrous oxide (Saunders et al., 1999) in an effort to make a skin friendly barrier. All registered topical Pheroid™ formulations currently contains vitamin E or vitamin E derivates, also known as tocopherol. These molecules are used as anti-oxidants and as emulsion stabilisers (Grobler et al., 2008). Previously conducted membrane diffusion studies have shown an increase in the percentage release of acyclovir and miconazole nitrate (anti-fungal) to human skin, in comparison to commercially available products (Grobler, 2004). The Pheroid™ itself also contains anti-fungal activity (Grobler, 2004), thus the more reason to formulate ketoconazole within Pheroid™.

Skin penetration studies play an essential role in the optimisation of formulation design in dermal and transdermal delivery. *In vitro* permeation studies and tape stripping techniques are therefore highly important (Leveque, 2004). The vertical Franz type diffusion cell is widely used, because of its reproducibility. This method has also been used to assess drug release from semi-solid dosage forms (Chattaraj, 1995). The use of *in vitro* methods to determine if the active ingredient released from a semi-solid is increasingly gaining attention from the pharmaceutical industry and regulatory authorities. No compendium method exists for determining the *in vitro* release of a drug from topical products such as creams, gels and ointments (Chattaraj, 1995).

Formulated semi-solid products were tested for stability. The purpose of stability testing was to provide evidence on how the quality of a drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. Stability testing also facilitates to establish a shelf-life for the drug product and recommended storage conditions.

Stability tests should be done on all attributes that can influence the quality, safety and efficacy of the product, which are likely to change during storage (ICH Tripartite Guidelines, 2003).

2 Materials and methods

2.1 Materials

Acyclovir 9-[(2-hydroxyethoxy)methyl]-9H-guanine (MW = 225.2) and ketoconazole (cis-1-acetyl-4-[4-[2-(2,4-dichlorophenyl-2-(1H-imidazol-1ylmethyl)-1,3-dioxalon-4-yl]methoxy]phenyl]piperazine) (MW = 531.4), were obtained by DB Fine Chemicals (Pty) Limited (Rivonia, Johannesburg, South Africa). Potassium dihydrogen orthophosphate (Merck, Wadeville, South Africa), and sodium hydroxide (Merck, Wadeville, South Africa) were used during the preparation of PBS which served in conjunction with methanol AR (Merck , Darmstad, Germany) as components of the receptor phase during the *in vitro* permeation studies. Ammonium phosphate monobasic (Sigma, St Louis, USA) and acetonitrile AR (Merck, Darmstadt, Germany) were used in the mobile phase of the transdermal high performance liquid chromatography (HPLC) analysis. The mobile phase and samples were degassed on an Elma® Transsonic TS 540 ultrasonic bath (Germany). An Agela Venusil XBP C-18 (4.6 x 150 mm, 5 µm) HPLC column was used. Vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) and a type JB5 Grant Water bath (Grant instruments, Cambridge, England) were used during the permeation studies. Tape stripping dermis samples were centrifuged with an Eppendorf centrifuge 5804R (Merck, Hamburg, Germany).

The liquid paraffin™, PEG 400, tween 80, cetyl alcohol, methylparaben and butylated hydroxyanisole (BHA) were obtained from Merck chemicals (Wadeville, South Africa). Sigma-Aldrich, St. Louis, USA supplied the butylated hydroxytoluene (BHT) and the glycerine. The span 60, xanthan gum and tocopherol were obtained for Fluka Analytical, Warren Chem Specialities and Chempure, respectively. Water used during all preparations and formulations was purified by a Milli-Q Academic purification system (Millipore, Milford, USA). The formulated products were stored in Labcon® humidity chambers (Johannesburg, South Africa). Assay determination of the formulated products was done with an Agilent 1100 Series HPLC system. In the HPLC analysis of the attributes in the formulated products, methanol AR (Merck, Darmstadt, Germany) and octane-1-sulfonic acid sodium salt (Merck, Darmstadt, Germany) were used as the mobile phase. A Luna C-18 (250 x 4.60 mm, 5 µm) column was used. The samples of the semi-solid formulated products where diluted with methanol supplied by Merck

(Wadeville, South Africa). The pH of the formulated products was determined by a Mettler Toledo Seven Multi pH meter (China), with an In Lab 410 NTC electrode 9823 (Switzerland). Viscosity was measured with a Brookfield Model DV – II+ viscometer (Massachusetts, USA), Brookfield circulating water bath with temperature controller (Massachusetts, USA), and a Helipath D20733 (Massachusetts, USA) with Helipath spindle set (Massachusetts, USA). The particle size was observed with a confocal laser scanning microscope (CLSM) Nikon PCM2000 with a digital camera DMX1200; with a He/Ne laser-543 nm and a Argon ion laser (457 - 517 nm) as well as an 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 with 457 - 514 nm. Immersion oil for microscopy, type A (Nikon, Japan) was also utilised in conjunction with polarised light microscopy procedures. The mass loss was determined on a Shimadzu AUW 120 D scale (Japan).

2.2 Methods

2.2.1 Formulation of the cream and emulgel

Acyclovir and ketoconazole were incorporated into existing basic formulae obtained in the literature. These formulae were then altered as necessary. An assay method was developed to analyse the main ingredients of each formulation. After the final formulae were perfected, the products were prepared in bulk for storing and testing purposes. A stability program was followed according to the ICH Tripartite Guideline (2003) at three storage temperatures for 6 months. The final formulations were made in sufficient quantity for storage at different temperatures during stability testing.

2.2.2 Stability program

The formulated products were subjected to long-term, intermediate and accelerated stability testing. Tests were performed at time intervals of 0, 1, 2, 3 and 6 months. The formulated products were stored in different humidity chambers at 25 °C/60% RH (relative humidity); 30 °C/60% RH and 40 °C/75% RH.

2.2.3 Assay with HPLC

HPLC analysis was done to determine the concentrations of acyclovir, ketoconazole, methylparaben, propylparaben, BHA, BHT and tocopherol in all the formulated products. HPLC

samples were prepared by weighing 1 g of each formulation into a 100 ml volumetric flask by using a syringe with a tube attached to the tip. Methanol was then added as solvent for the semi-solids. The samples were ultrasonificated for approximately 20 min or until completely dispersed. The samples were filled to volume in the volumetric flask after it cooled to room temperature. All samples were filtered with a 0.45 µm syringe filter and transferred into HPLC vials.

2.2.4 pH measurement

The pH meter was calibrated with pH buffer solutions of pH 4.01, 7.00 and 10.10. Calibration was done each day, directly prior to the pH measurements of batches. To ensure accuracy three measurements were taken on each batch at different temperatures and time intervals.

2.2.5 Viscosity

The viscosity of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel was determined at 0, 1, 2, 3 and 6 months. Measurements were done in glass containers, containing 100 ml of the product. The viscosity was only determined on the batches stored at a stability temperature of 25 °C/60% RH. Viscosity was measured for 5 min, where a total of 32 viscosity readings were taken every 10 s for each formulation. The viscosity measurements at 0, 1, 2, and 3 months were done on the same formulated product in the same container, but with the viscosity measurement done at 6 months; the viscosity of the same batch of the formulated product was measured, however, at this stage it was measured in a different container. The separate measurement at 6 months was done to eliminate the effect of the measurement on the van der Waals forces of the product.

2.2.6 Particle size observation

The particle size was observed with a CLSM. Micrographs of the formulations were taken at 0, 1, 2, 3 and 6 months. Any increase or decrease in particle size that was observed visually, was documented.

2.2.7 Mass loss

Each individual container of the four batches was weighed initially. The same container was then weighed again at 1, 2, 3 and 6 months to determine if any mass loss occurred. All containers were weighed three times at each time interval to ensure accuracy.

2.2.8 Physical appearance

The stored batches were visually assessed at 0, 1, 2, 3 and 6 months. Texture, colour, odour and skin-feel were observed; and any change was noted. The colour was documented by taking photos of the formulated products and by using colour charts as indicators.

2.2.9 Skin preparation

Permeation studies were performed on abdominal caucasian female skin. The excised human skin was obtained from patients who had undergone abdominal plastic surgery (Leveque, 2004). Informed consent was given by all of the patients before surgery and the identities of all donors were kept confidential. The Research Ethics Committee of the North-West University granted ethical approval for the procurement and utilisation of the donated skin under reference number 04D08. Full-thickness skin was frozen at -20 °C within 24 h after surgery. Full thickness skin, containing all the skin layers was utilised during this study. Subcutaneous fat and connective tissue was separated from the skin by using a scalpel, while taking tremendous care not to damage or rupture the skin in any way. Skin damage or rupture can lead to incorrect results. After successful removal of fat and connective tissue the skin was punched into circles with a diameter of approximately 15 mm. Skin circles were then positioned onto Whatman® filter paper with the stratum corneum side of the skin facing upwards. It was then covered in aluminium foil sheets and sealed in plastic bags after which it was frozen at -20 °C until needed. Frozen skin circles were thawed at room temperature prior to each diffusion study (Pellet, 1999). Each skin circle was visually examined for any imperfections before it was mounted onto the diffusion apparatus.

2.2.10 Preparation of receptor solutions

The receptor solution consisted of 20:80 (v/v) methanol and PBS (pH 7.4). Inclusion of an organic solvent was to ensure the solubility of the active ingredients. PBS was prepared by weighing 13.62 g of potassium dihydrogen orthophosphate and dissolving it in 500 ml of Milli-Q®

water. Sodium hydroxide, 3.14 g, was weighed and dissolved in 786.8 ml Milli-Q® water. The two solutions were mixed and the pH was adjusted to 7.4 with 10% ortho phosphoric acid. Subsequently 20 vol of methanol was added to 80 vol of PBS. The solution was filtered through a 0.45 µm filter membrane prior to use on the HPLC.

2.2.11 Franz cell diffusion

The *in vitro* permeation studies were conducted in vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and an active diffusion area of 1.075 cm^2. A Franz diffusion cell consists of a donor and a receptor compartment, where the formulated product containing the drug and the receptor fluid are placed, respectively. Full-thickness skin circles were thawed and placed between the receptor and donor compartments of the Franz cells with the stratum corneum in contact with the donor phase. To prevent leakage, the two compartments were sealed with Dow Corning® high vacuum grease and clamped together with a metal clamp. Donor compartments were each filled with approximately 1 ml of the formulated product. To correspond to normal skin temperature, the semi-solids were preheated in a water bath to 32 °C. Subsequently, the donor compartment was covered with Parafilm® to avoid evaporation of the product. The receptor compartments were filled with 2 ml PBS (pH 7.4); taking care to prevent air bubbles beneath the skin. The diffusion cells' receptor compartments were surrounded with a water bath kept at a constant temperature of 37 °C throughout the experiment. This was done in order to control the temperature of the receptor compartments (Dias, 1999), which in effect attains the skin surface temperature of 32 °C, simulating the temperature of human skin (Leveque, 2004). The receptor medium was continuously stirred at 750 rpm with the aid of a magnetic stirring plate; moving the small magnetic stirring bar which was placed in each receptor compartment. This was done in order to maintain movement throughout the duration of the experiment. The entire content of the receptor compartments were withdrawn at predetermined intervals of 20, 40, 60, 80 and 100 min, as well as 2, 4, 6, 8, 10 and 12 h. Withdrawals were followed by the replacement with an equal amount of fresh receptor solution previously heated to 37 °C, to ensure that the skin conditions persisted throughout the duration of the experiment. The withdrawal times were determined by keeping the Pheroid™ delivery system in mind. It was expected that the Pheroid™ would promote the

permeation of acyclovir and ketoconazole, thus, the withdrawal times were started earlier. This also guaranteed that the desired amount of at least 5 data points could be achieved. Each sample was directly assayed by HPLC to determine the drug concentration of the active ingredients (acyclovir and ketoconazole) within each receptor compartment. Each permeation study was done with at least 9 Franz diffusion cells.

2.2.12 Tape stripping

Tape stripping is a technique used to remove the stratum corneum as well as the epidermis layer of the skin. The tape stripping method was used to investigate the amounts of active ingredient present in the outer layers of the skin as well as in the dermis of the skin, after application of a supersaturated solution or formulated product (Pellet et al., 1997). After the completion of the diffusion study (12 h), the donor and receptor compartments of the Franz diffusion cells were carefully dismounted. Each Franz cell's skin was pinned on a piece of Parafilm® and stapled to a solid surface. The exposed area of diffusion (≈ 1.075 cm^2) was clearly imprinted by the indentation from the diffusion cells (≈ 11.70 mm diameter). Tissue paper was used to remove the last of the semi-solid donor phase by dabbing the skin dry. Subsequently, pieces of 3M Scotch® Magic™ Tape was cut to cover the diffusional area. Pieces of tape were cut to a specific size to assure that it did not overlap the areas outside of the diffusion cell imprints. The first tape strip was discarded, because the tape may be contaminated with the drugs from the formulation, since it is seen as part of the cleaning procedure. The following 15 tape strips (referred to as the epidermis) were placed in a vial. Glistening of the viable epidermal layer indicated the complete removal of the stratum corneum. All the strips were placed in a 5 ml mixture of PBS (pH 7.4) and methanol. The strips in solution were kept overnight at 4 °C. Excess skin was cut away from the imprints of the diffusion cells and the remaining skin (referred to as dermis) was cut into smaller pieces to enlarge the surface area. It was then placed in a 2 ml mixture of PBS (pH 7.4) and methanol and kept overnight at 4 °C. The dermis samples were centrifuged the next morning at a rate of 4500 rpm and a temperature of 10 °C for 10 min. The dermis and epidermis samples were analysed with HPLC.

2.2.13 Analysis of diffusion and tape stripping samples with HPLC

HPLC analysis of acyclovir and ketoconazole was performed with a method developed and validated by Prof. J. du Preez and Ms. M.E. van der Walt at the North-West University, Potchefstroom Campus, South Africa. Samples collected from the *in vitro* experiments as well as the samples from the tape stripping experiments were analysed with HPLC. The degassed mobile phase consisted of a mixture of acetonitrile and a solution of ammonium phosphate (1.15 g/l). The mobile phase changed by linear-gradient elution over a period of time. It consisted of a mixture of 0.5 vol of acetonitrile and 9.5 vol of a solution of ammonium phosphate (1.15g/l). The mobile phase changed by linear-gradient elution after 2 min to a mixture of 8.0 vol of acetonitrile and 2.0 vol of a solution of ammonium phosphate (1.15 g/l) over 9 min. This was followed by the final elution mixture of 0.5 vol of acetonitrile and 9.5 vol of a solution of ammonium phosphate (1.15 g/l) for 5 min. A volume of 100 µl of each sample was injected. The flow rate was set to 1 ml/min and the temperature was maintained at 25 °C. The total runtime was 14 min. Detection was set at 252 nm for the first 6 min to detect acyclovir, where after ketoconazole was detected at 243 nm. The detection time of acyclovir was approximately 3.4 - 3.9 min and that of ketoconazole approximately 6.95 - 7.02 min. Ammonium PBS was prepared by weighing 1.15 g ammonium phosphate monobasic and dissolving it in 950 ml of Milli-Q® water. The pH was adjusted to 7.20 ± 0.01 with a 10% ammonium hydroxide solution and diluted to 1000 ml with Milli-Q® water. Ammonium PBS was filtered through a 0.45 µm Millipore® filter and degassed on an ultrasonic bath.

2.2.14 Transdermal and statistical data analysis

The average cumulative amount (for each Franz diffusion cell) per area (µg/cm^2) was plotted against time for acyclovir and ketoconazole for each formulation. Average flux values were obtained from the slope of the linear portion of the curve. The standard deviation (SD) of the cumulative amount diffused per time unit in the different Franz diffusion cells were indicated on the graph. The average amount of active ingredients, diffused through a minimum of 9 Franz diffusion cells was depicted as a percentage of the applied concentration (5% acyclovir and 2% ketoconazole). Statistical analysis was determined by using computer programs SAS (SAS Institute, 2005) and Statistica (Statsoft, 2008). The Kruskal-Wallis test was used to determine

the statistical significant differences between the median flux values and the tape stripping measurements of the formulae. A $p < 0.05$ was considered to be of statistically significant difference (Steyn, 1994). The pair-wise statistical comparisons of the median steady-state flux values and tape stripping measurements between formulated products were carried out in an attempt to establish where differences between the formulated products exist. Descriptive statistics were calculated and was presented as median values. Correlation between the dermis and epidermis concentrations as well as the correlation with the flux values of each formulation was determined.

3 Results and discussion

3.1 Formulation of the cream and emulgel

The formulated cream and the Pheroid™ cream applied easily, they were not too oily and had a homogenous white texture. A white emulgel and Pheroid™ emulgel were formulated which applied easily, they were not too oily or too hydrous and had a homogenous white texture. The cream and the emulgel had no odour, but the Pheroid™ cream and Pheroid™ emulgel had the characteristic "fish-oil" smell.

3.2 Assay with HPLC

In the cream formula a significant decrease in acyclovir's concentration of more than 5.00% occurred at 40 °C after 3 months on stability. Acyclovir also had a significant change in concentration in the Pheroid™ cream; at 30 °C a 6.50% decrease occurred after 6 months, and at 40 °C there was an 11.80% decrease in concentration after 3 months on stability. In the emulgel formulation a significant decrease of more than 5% in acyclovir concentration was observed; a decrease of 8.20% occurred at 30 °C after 3 months and at 40 °C a decrease of 6.23% was noted after 2 months of stability testing. The Pheroid™ emulgel depicted significant decreases at all three temperatures. At 25 °C, a decrease of 9.79% occurred after 2 months; at 30 °C a 5.67% decrease in acyclovir's concentration was noted after 1 month, and at 40 °C a 14.03% decrease occurred after 2 months of stability testing.

The concentration of the active ingredient ketoconazole depicted no significant decrease in the cream and emulgel formulations. In the Pheroid™ cream a significant decrease of 6.97% in ketoconazole concentration was seen at 30 °C after 3 months, and at 40 °C a decrease of 5.68% occurred after 1 month on stability testing. In the Pheroid™ emulgel ketoconazole's concentration decreased significantly with 7.06% and 7.54% at 25 and 30 °C, respectively after 6 months on stability. At 40 °C a 15.66% decrease in ketoconazole's concentration was observed after 3 months on stability.

Methylparaben was stable at all three temperatures, in all four formulated products, for the duration of the stability testing period.

In the Pheroid™ cream formula the concentration of propylparaben decreased significantly, with12.08% and 10.88% at 30 and 40 °C respectively, after 1 month of stability testing. A

5.63% reduction occurred in the concentration of propylparaben in the Pheroid™ emulgel at a temperature of 40 °C, after 2 months of stability testing. Although a decrease of more than 5% in the propylparaben concentrations occurred, no microbial growth was noticed during physical assessment.

In the cream formulation a 18.84% and 17.1% decrease in BHA concentration was observed after 3 months on stability at 25 and 30 °C, respectively, and a 9.06% decrease in BHA concentration was observed at 40 °C after 2 months on stability. The BHA concentration in the emulgel formulation decreased after 3 months of stability with 14.28%, 10.96% and 11.59% at 20, 30 and 40 °C, respectively. In the Pheroid™ emulgel the BHA concentration decreased with 6.98% at 20 °C after 2 months, 6.95% at 30 °C after 3 months, and 7.20% at 40 °C after 1 month of stability testing.

The BHT concentration in the Pheroid™ cream formula decreased significantly after 1 month of stability testing with 13.04%, 18.80% and 20.19% at 20, 30 and 40 °C, respectively. In the emulgel formulation the BHT concentration decreased with 9.46% at 40 °C after 2 months on stability. In the Pheroid™ emulgel the BHT concentration decreased significantly after 1 month on stability; a reduction of 19.68%, 17.46% and 26.84% occurred at 20, 30 and 40 °C, respectively.

In the Pheroid™ cream formula a significant decrease of 28.60% in tocopherol's concentration at 25 °C after 2 months was observed. The Pheroid™ cream a decrease in tocopherol of 8.22% and 14.49 % was depicted after 1 month of stability at 30 and 40 °C, respectively. Tocopherol in Pheroid™ emulgel depicted significant decreases in concentration of 14.74%, 15.68% and 27.23% after 1 month at 20, 30 and 40 °C, respectively.

The vast decrease in the anti-oxidant concentrations may be due to the fact that the anti-oxidants protected the active ingredients against oxidation.

Acyclovir and ketoconazole were stable in the cream formula at 25 and 30 °C for the duration of the stability test period. Ketoconazole was also stable at 40 °C in the cream formula. In the Pheroid™ cream formulation ketoconazole and acyclovir was stable at 25 °C, indicating a stable product. Acyclovir was stable in the emulgel formulation at 25 °C and ketoconazole was stable at all three stability temperatures for the duration of the stability program. Acyclovir and

ketoconazole were both unstable at the three temperatures in the Pheroid™ emulgel formulation, indicating an unstable product.

3.3 pH measurement

The pH of the formulated products decreased slightly at a temperature of 25 °C which indicated stable products. Although the pH decreased slightly; it was still between a pH of 5 and 6. It appears that maximum stability of all formulated products will be ensured for a longer period if it is stored at a temperature below 25 °C.

3.4 Viscosity

The viscosity of the cream and Pheroid™ cream formulation decreased slightly over the 6 month period. Comparing the cream's initial viscosity with the viscosity at 6 months, the viscosity decreased with approximately 350000 cP, which indicated a stable formula. When comparing the Pheroid™ cream's initial viscosity with the viscosity at 6 months, the viscosity decreased to about half of the initial measurement. The large decrease in viscosity was a warning sign of possible instability. The emulgel and Pheroid™ emulgel formulation showed a slight decrease in viscosity over the 6 month period. Comparing the aforementioned formulations' initial viscosity with the viscosity at 6 months, the viscosity decreased with approximately 10000 cP (emulgel) and 5000 cP (Pheroid™ emulgel). This was indicative of stable formulations. The decrease in viscosity during the first month of stability can be explained as a reaction between some of the ingredients, or it may be possible that the semi-solids were still settling before it went into the final resting stage. The decrease at month 2 and 3 may be due to the breakage of van der Waals forces between the molecules, due to previous viscosity measurements on the same product. It seems as if the emulgel and the Pheroid™ emulgel were not influenced by previous viscosity measurements on the same emulgel, but the cream and Pheroid™ cream were affected.

3.5 Particle size observation

The particle size of the cream, emulgel and Pheroid™ emulgel semi-solid products did not change significantly over the 6 month period. Acyclovir's crystal sizes increased slightly, but not significantly. The cream, emulgel and Pheroid™ emulgel formulae seemed to be stable. Particle size of the Pheroid™ cream did not change significantly over the 6 month period, but

the size of the acyclovir crystals did, however, increase. This increase was significant at 25 °C and 30 °C, but not at 40 °C. The Pheroid™ cream formulation appeared to be unstable.

3.6 Mass loss

Mass loss of the formulated products was insignificant and no significant changes occurred at any of the three temperatures. This was indicative of a stable product in a suitable container.

3.7 Physical appearance

No physical changes were noticed in the cream and emulgel formulations at the three different temperatures over the stability test period. Over the 6 months a change in colour of the Pheroid™ cream and the Pheroid™ emulgel, stored at 25 °C, 30 °C and 40 °C, occurred. In the Pheroid™ cream a slight change in colour at 25 °C was depicted, where the colour changed from white to soft yellow to light yellow. At 30 °C the colour changed from bright white to light yellow to bright yellow. At 40 °C the colour changed from white to bright yellow to a darker yellow. The Pheroid™ emulgel showed a change in colour at 25 °C and 30 °C from a cream-colour to light yellow and again to a bright yellow. At 40 °C the colour changed from a cream-colour to a bright yellow to a dark yellow. These colour changes may be due to the oxidation of BHT and tocopherol.

3.8 Franz cell diffusion

The *in vitro* permeation of acyclovir and ketoconazole with the aid of Pheroid™ technology within the formulated products were investigated. This permeation was compared to the permeation of acyclovir and ketoconazole, in the basic formula without the Pheroid™ delivery system, which served as a control.

Acyclovir showed a biphasic character in all four formulations, with a steady-state flux between 0 - 2 h (flux 1) and also between 2 - 12 h or 4 - 12 h (flux 2). Ketoconazole, on the other hand, showed no flux in the cream and the Pheroid™ cream formulae, although the Pheroid™ cream showed a diffusion of ketoconazole after 12 h. Ketoconazole in the Pheroid™ emulgel formulation showed a steady-state flux between 6 - 12 h. The emulgel formulation showed a biphasic character with a steady-state flux between 0 - 2 h and again, between 2 - 12 h.

The average and median flux results of the *in vitro* permeation studies are given in Figure 1.

Figure 1: The average flux and median flux values of **(a)** acyclovir and **(b)** ketoconazole.

For acyclovir the flux 1 values of the formulations were significantly higher than the flux 2 values, showing that there was a larger transdermal delivery of acyclovir between 0 – 2 h, in comparison to the delivery between 2 - 12 h after application of the formulated products. The flux 1 values of the cream, Pheroid™ emulgel and the emulgel were 5 times higher than flux 2 values. In the Pheroid™ cream formulation the flux 1 values were 10 times higher than the flux 2 values for the active, acyclovir.

The Pheroid™ cream formula depicted the highest flux 1 (982.89 ± 23.55) and flux 2 (113.60 ± 7.45) values for acyclovir compared to the other formulated products. On the other hand, the cream formula showed a higher flux 1 (536.54 ± 10.55) and flux 2 (79.67 ± 2.68) value for acyclovir than the Pheroid™ emulgel. Thus, the cream formulations increased transdermal delivery of acyclovir of which the Pheroid™ technology in the cream formulation enhanced the delivery of acyclovir the most.

Median and average flux values of acyclovir for flux 1 and flux 2 in each formula were closely related. The difference in the median and average flux values for acyclovir in the emulgel can be explained by the method of calculation for these values. Average flux was determined by adding the flux values and dividing it by the number of cells, where as the median flux values were determined by finding the centre of the values. The average flux was affected by outliers and significant variation in the flux values; where as the median flux was not affected by them. Therefore median flux was a more accurate approach for determining flux values (Gerber et al., 2008).

In the emulgel the flux values for ketoconazole obtained were flux 1 (6.98 ± 0.20 $\mu g/cm^2$) and flux 2 (1.14 ± 0.11 $\mu g/cm^2$). The flux 1 value was 6 times higher than the flux 2 value. The Pheroid™ emulgel showed a flux value between 6 - 12 h of 51.04 ± 25.69 $\mu g/cm^2$.

Concentrations of ketoconazole that diffused after 12 h were 38.86 $\mu g/cm^2$ (cream), 25.68 $\mu g/cm^2$ (emulgel) and 415.95 $\mu g/cm^2$ (Pheroid™ emulgel). Thus, it is clear that the emulgel formulations increased transdermal delivery of ketoconazole of which the Pheroid™ technology in the emulgel formula enhanced the delivery of acyclovir the most.

The average amount of ketoconazole that diffused through the skin over the 12 h period was 1.105%, 0.069% and 0.104% for the Pheroid™ emulgel, emulgel and the cream formulae, respectively. Median and average flux values for ketoconazole differed slightly in the Pheroid™ emulgel, the reason again being the method of calculation for these values (Gerber et al., 2008).

3.9 Tape stripping

Figure 2: The tape stripping epidermis and dermis values for **(a)** acyclovir and **(b)** ketoconazole.

There was more acyclovir delivered to the dermis of the skin than to the epidermis. This was seen in all four of the formulae. Epidermis tape stripping (µg/ml) values of acyclovir differed in all four formulations. The Pheroid™ cream (1426.51 µg/ml) depicted the largest epidermal concentration. The emulgel (106.54 µg/ml) showed a larger concentration of acyclovir in the epidermis than the Pheroid™ emulgel (85.53 µg/ml) formula. The cream formula showed the smallest delivery of acyclovir (18.10 µg/ml) to the epidermis.

Dermis tape stripping values of acyclovir were compared within the four formulations. The largest delivery of acyclovir to the dermis layer of the skin was achieved by the Pheroid™ cream (1571.96 µg/ml). The emulgel formula (262.19 µg/ml) delivered a larger concentration of acyclovir to the dermis of the skin than the Pheroid™ emulgel (126.02 µg/ml) formula. The cream formula showed the poorest delivery of acyclovir (45.56 µg/ml) to the dermal layer.

The Pheroid™ cream formulation illustrated that Pheroid™ increased delivery to the dermal layer of the skin compared to the other formulae.

Concentrations of ketoconazole delivered to the epidermis layer of the skin were closely related in the four different formulations with a delivery of between 1.41 - 2.95 µg/ml. Dermis delivery, however, varied significantly between the different formulations. Pheroid™ emulgel delivered 35 times more ketoconazole to the dermis layer of the skin than to the epidermis skin layer. The cream formulation delivered a 5 times higher concentration of ketoconazole to the dermis layer of the skin than to the epidermal layer.

The Pheroid™ emulgel formula delivered the highest concentration ketoconazole to the dermis (103.12 µg/ml) of the skin. The cream formula (13.96 µg/ml) delivered more ketoconazole to the dermal layer than the Pheroid™ cream formula (6.94 µg/ml). The Pheroid™ emulgel illustrated that the Pheroid™ increased the delivery of ketoconazole to the dermis layer of the skin compared to the emulgel (6.12 µg/ml) formula.

When comparing the flux values of acyclovir with the dermis and epidermis concentrations, it was noted that the formulation with the largest flux 1 and flux 2 values also showed the highest dermis and epidermis concentrations. Thus, the higher the flux values of acyclovir, the more acyclovir was delivered to the dermis and the epidermis layers of the skin. When comparing the 12 h concentrations of ketoconazole that diffused though the skin, with the epidermis and dermis concentrations; it was noted that the formulation with the largest 12 h concentration delivered transdermally, also depicted the largest delivery to the epidermis and dermis layers of the skin.

3.10 Statistical data

There was a statistical significant difference between the flux 1 and the flux 2 values of acyclovir in all four formulations with $p < 0.0001$. Flux 1 values of acyclovir were compared within the four formulations, where a statistical significant difference was found between the cream and Pheroid™ emulgel; the emulgel and Pheroid™ cream; and the Pheroid™ cream and Pheroid™ emulgel. The cream and the Pheroid™ cream as well as the emulgel and the Pheroid™ emulgel showed a statistical similarity between their flux 1 values with $p = 0.377$ in both instances.

When comparing the flux 2 values of acyclovir of the four formulations, a significant difference ($p < 0.05$) between the cream and the Pheroid™ emulgel; emulgel and Pheroid™ cream as well as between Pheroid™ cream and Pheroid™ emulgel was observed. As with flux 1, the cream and the Pheroid™ cream as well as the emulgel and the Pheroid™ emulgel showed a statistical similarity between their flux 1 values with $p = 0.377$ in both instances.

When the total concentration of ketoconazole diffused over 12 h, for each formula, was compared, a $p < 0.0001$ was found. This showed that there was a statistical significant difference between the cream, emulgel and Pheroid™ emulgel formulations' that diffused over

12 h. There was a statistical similarity between the flux 1 and flux 2 values of ketoconazole in the emulgel.

Acyclovir epidermis tape stripping values were compared within the four formulations. A statistical significant difference between the Pheroid™ cream and Pheroid™ emulgel; the cream and Pheroid™ cream as well as between the cream and emulgel was observed ($p < 0.05$). When comparing the total of the acyclovir dermis tape stripping values of all four formulations, a significant difference between these values ($p < 0.0001$) was found by using the Kruskal-Wallis test. Epidermis tape stripping values of acyclovir for all four formulations was compared with each other. A statistical significant difference between these values was found with $p < 0.0001$. The epidermis tape strips for acyclovir showed a statistical significant difference between the average flux 1 and the average flux 2 values, with $p < 0.0001$ in both instances. Dermal tape stripping values of acyclovir also showed a significant difference in the average flux 1 and flux 2 values with $p < 0.0001$ and $p = 0.0002$, respectively.

Ketoconazole's epidermis tape stripping values were compared within the formulated products. A statistical significant difference between the Pheroid™ cream and emulgel as well as between the emulgel and cream was obtained. The dermis tape stripping values of ketoconazole were compared within the four formulations and a statistical significant difference between the Pheroid™ cream and Pheroid™ emulgel; the emulgel and Pheroid™ emulgel as well as between the cream and emulgel, was seen.

There was no significant difference between the epidermis and dermis values ($p = 0.09$). The sum of the epidermis tape stripping values of ketoconazole in all four formulations was compared with each other using the Kruskal-Wallis test. A statistical significant difference between these values was found with $p = 0.0091$. When comparing the sum of the dermis tape stripping values of ketoconazole from all four formulations with each other a statistical significant difference between these values was found with $p < 0.0001$.

4 Conclusion

According to the chemical assay done at each test interval, not one of the four formulations was completely stable over the 6 month period. The cream formula was the most stable according to the chemical assay. There was a significant change in colour in the Pheroid™ formulae,

which was noted during the physical assessment. No significant change in either the pH or mass loss was documented. The Pheroid™ cream formulation depicted a severe decrease in viscosity, which is an indication of an unstable product. Other formulated products were stable considering their viscosity. Particle size analysis showed significant changes in the crystal size of the Pheroid™ cream formulation, which is a warning sign for instability of the product. Not one of the formulated products met the criteria for stability of the Medicines Control Council of South Africa (2006) or the ICH Tripartite Guidelines (2003). Results of the stability tests showed that the cream and emulgel formulae were more stable than the Pheroid™ formulae. When considering literature, it was expected to be possible to deliver acyclovir transdermally when looking at the relatively high aqueous solubility and low molecular weight of the drug (McEvoy, 2002; British Pharmacopoeia, 2009). *In vitro* permeation studies of acyclovir showed that acyclovir penetrated through full-thickness skin after the application of all four of the formulations. Comparing the flux values, the penetration of acyclovir was enhanced by the cream formulae in comparison with the emulgel formulae. Pheroid™ technology enhanced the delivery of acyclovir in the cream formula.

Transdermal delivery of ketoconazole was poor; this was expected when considering its low aqueous solubility and high molecular weight (McEvoy, 2002; British Pharmacopoeia, 2009). Ketoconazole penetrated through full-thickness skin in the case of the cream, emulgel and Pheroid™ emulgel formulae. Ketoconazole's concentations at 12 h and the average amount of ketoconazole diffused, illustrated that penetration of ketoconazole was enhanced by the emulgel formulae. The Pheroid™ emulgel formulation exhibited the best transdermal delivery of ketoconazole. Thus, the Pheroid™ enhanced transdermal delivery of acyclovir and ketoconazole due to the higher efficiency of delivering drugs across the stratum corneum (Grobler et al., 2008).

Acyclovir was detected in the dermis and epidermis layers of the skin after application of all four formulations. The Pheroid™ cream significantly enhanced the delivery of acyclovir to the dermis and epidermis of the skin in comparison to the cream formulation. There was detection of ketoconazole in the dermis and epidermis layers of the skin after application of all four formulations. The Pheroid™ emulgel enhanced the delivery of ketoconazole to the dermis, but

not to the epidermis compared to the emulgel. In conclusion, the Pheroid™ enhanced the delivery of acyclovir and ketoconazole to human skin.

When comparing the flux values and the 12 h concentrations of acyclovir and ketoconazole with the dermis and epidermis concentrations, it was noted that the larger the diffusion of the actives through the skin, the more active ingredients were delivered to the dermis and the epidermis. The Pheroid™ cream increased the delivery of acyclovir to its target site, the epidermis. Ketoconazole's delivery to its target site, the stratum corneum (include in the epidermis tape stripping) was increased by the Pheroid™ emulgel formulation (Pershing et al., 1994: Jiang et al., 1998). The reason for the increased delivery of acyclovir by the Pheroid™ cream and ketoconazole by the Pheroid™ emulgel, may be explained by the aqueous solubility of the drugs. Ketoconazole is more hydrophobic and acyclovir is more hydrophilic (McEvoy, 2002). The cream formula contained more oil and fatty ingredients than the emulgel, which contained more water, and less oil and fat, than the cream formula. Thus, it may be possible that acyclovir did not diffuse out of the emulgel formula, due to the fact that it had affinity for the water surroundings and that it did not effortlessly diffuse to the lipid stratum corneum (Menon, 2002). Ketoconazole may have benefited from its fatty surroundings and did not have affinity to diffuse out of the cream formula.

In conclusion, Pheroid™ technology showed to enhance dermal and transdermal delivery of acyclovir and ketoconazole to human skin, but the Pheroid™ formulations showed poor stability.

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FIGURE LEGENDS

Figure 1: The average flux and median flux values of (a) acyclovir and (b) ketoconazole

Figure 2: The tape stripping epidermis and dermis values for (a) acyclovir and (b) ketoconazole

FIGURES

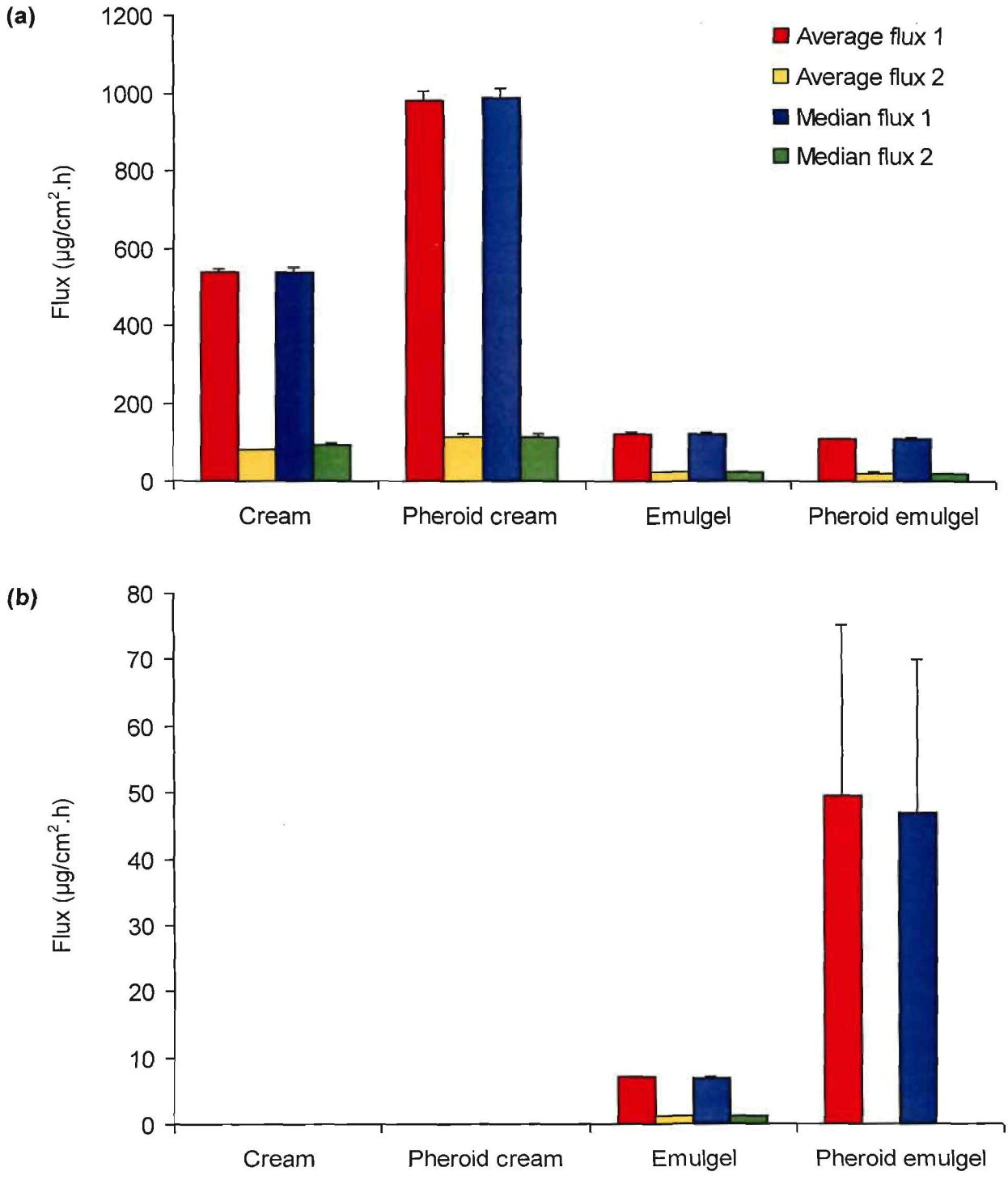


Figure 1: The average flux and median flux values of **(a)** acyclovir and **(b)** ketoconazole

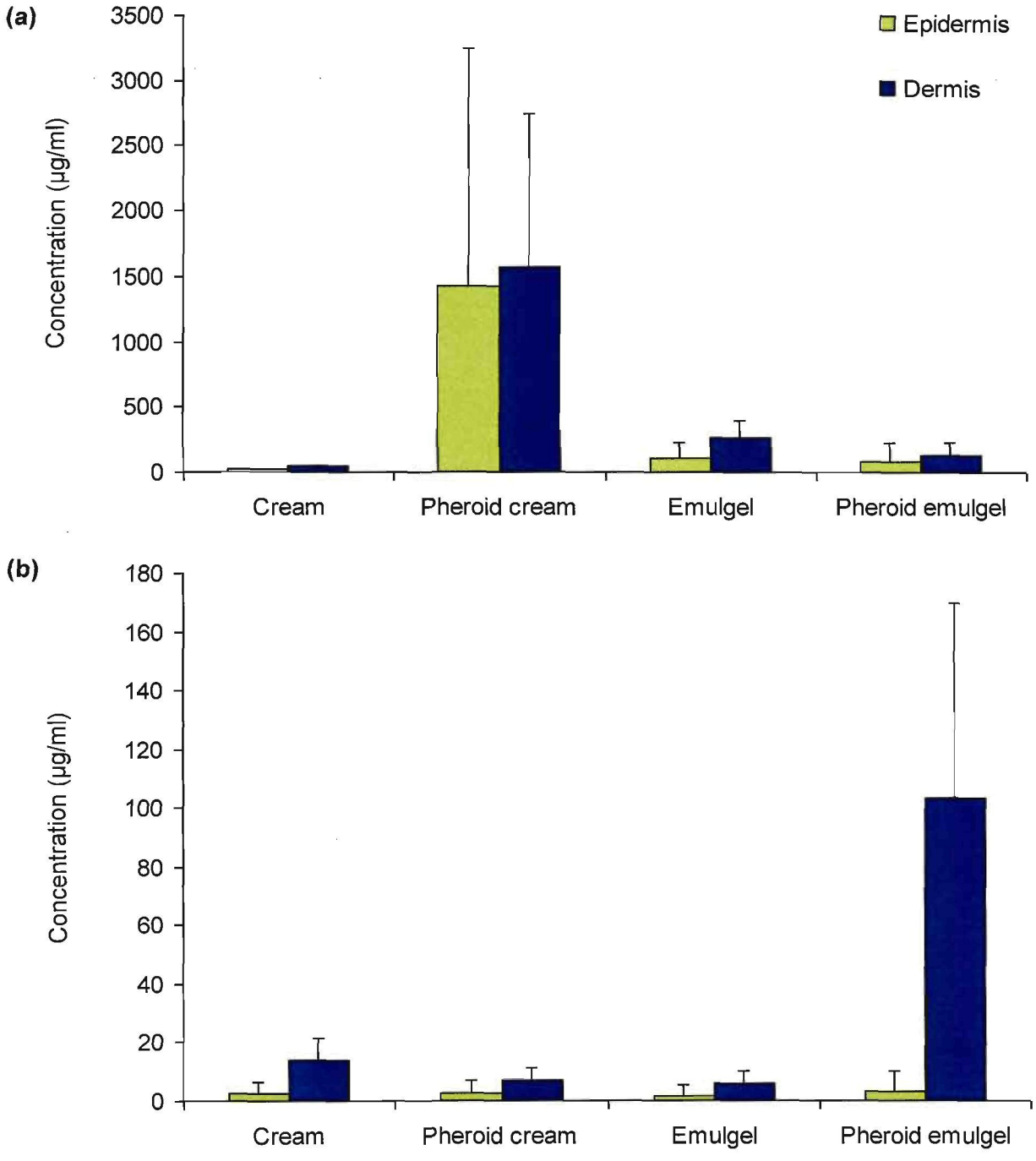


Figure 2: The tape stripping epidermis and dermis values for **(a)** acyclovir and **(b)** ketoconazole.

CHAPTER 4

FINAL CONCLUSIONS AND FUTURE PROSPECTS

The aim of this study was to formulate a product containing the active ingredients acyclovir and ketoconazole. The rationale behind formulating a product containing both acyclovir (anti-viral) and ketoconazole (anti-fungal) was due to opportunistic infections that occur in HIV/AIDS immunocompromised individuals (Ramdial, 2000:113). Viral and fungal infections are often seen in combination in these individuals.

The human skin provides a challenging barrier to dermal and transdermal delivery (Menon, 2002:4). Acyclovir and ketoconazole does not display the ideal physicochemical properties, which will allow for easy permeation across the barrier of the skin, the stratum corneum (British Pharmacopoeia, 2009). Hence, some form of penetration enhancer had to be utilised to improve their poor diffusivity. To enhance transdermal and dermal penetration, the formulations were formulated with the novel Pheroid™ delivery system.

The Pheroid™ carrier system was investigated in order to determine whether it would enhance the dermal and transdermal delivery of acyclovir and ketoconazole in a semi-solid formulation. *In vitro* studies and tape stripping were used to determine the effect of the Pheroid™ in a semi-solid formulation on skin permeation.

In vitro permeation studies were conducted using caucasian female abdominal skin as permeation membranes in vertical Franz diffusion cells over a period of 12 h. The cream and emulgel formulae containing 5% acyclovir and 2% ketoconazole in Pheroid™ vesicles were compared to the cream and emulgel formulated products containing 5% acyclovir and 2% ketoconazole. The formulae containing no Pheroid™ were used as a control against which the efficacy of the formulations containing Pheroid™ was measured. Tape stripping was conducted after the 12 h *in vitro* permeation study to determine whether the actives were present in the dermis and epidermis layers of the skin.

The formulated cream, Pheroid™ cream, emulgel and Pheroid™ emulgel formulae were tested for stability over a 6 month period. Formulated products were stored at three different temperatures and stability tests were conducted initially, and at 1, 2, 3 and 6 months. The stability program was done in accordance with the ICH Tripartite Guidelines (2003) and the MCC of South Africa (2006).

During the course of this study the following were observed:

- Stability testing conducted over 6 months indicated that none of the formulated products met the ICH Tripartite Guidelines and the MCC requirements for stability;
- The Pheroid™ delivery system was more unstable than the semi-solids containing no Pheroid™;
- *In vitro* permeation profiles of acyclovir showed a biphasic character for the formulated products with and without the Pheroid™ technology. (The first flux value was observed between 0 - 2 h and the second flux between 2 - 12 h or 4 – 12 h.);
- In comparison to the cream, emulgel and Pheroid™ emulgel formulae, the Pheroid™ cream enhanced transdermal delivery of acyclovir;
- *In vitro* diffusion of ketoconazole showed a biphasic flux in the emulgel formulation, with the first flux value between 0 - 2 h and the second flux between 2 - 12 h. The Pheroid™ emulgel showed a flux between 6 - 12h, whereas the cream formulation depicted no flux, but a total amount of ketoconazole diffused after 12 h, was obtained. There was no transdermal delivery of ketoconazole in the Pheroid™ cream formulation;
- The average amount of ketoconazole diffused over 12 h showed improved delivery of ketoconazole formulated in the emulgel formulae, with the Pheroid™ emulgel exhibiting best penetration;
- Tape stripping of the skin indicated delivery of acyclovir to the epidermis and the dermis layers of the skin. Delivery of acyclovir to the target site, the epidermis, was achieved. The dermal and epidermal delivery of acyclovir was increased by the Pheroid™ cream formulation;
- Ketoconazole penetrated into the skin. The tape stripping method of the skin circles showed that ketoconazole was delivered to the dermal and epidermal layers of the skin. The Pheroid™ emulgel depicted the best dermal and epidermal delivery of ketoconazole to the skin. The target site of ketoconazole, the stratum corneum was, thus, reached;
- The Pheroid™ drug delivery system was able to deliver acyclovir and ketoconazole in higher concentrations than the positive control. The Pheroid™ cream formula enhanced the delivery of acyclovir and the Pheroid™ emulgel formula enhanced the delivery of ketoconazole. It may be possible that acyclovir did not diffuse from the emulgel formula due to its affinity for the aqueous environment. Ketoconazole may have benefited from the fatty environment of the cream, and thus, had no affinity to diffuse from the formula; and

- In conclusion, Pheroid™ proved to be advantageous for transdermal and dermal delivery of acyclovir and ketoconazole. This was due to the fact that the Pheroid™ entraped and delivered drugs across the stratum corneum with a higher efficiency (Grober *et al.*, 2008: 296).

Future prospects for further investigation and aspects that need to be considered include the following:

- The formula of a stable Pheroid™ semi-solid formulation containing, and enhancing the dermal and transdermal delivery of both acyclovir and ketoconazole, should be perfected;
- *In vitro* efficacy testing of the semi-solid products, using reference strains of herpes viruses and *Candida* species should be conducted. Cytopathic effect inhibition (CPE-inhibition) assays and plaque inhibition assays can be done to determine whether the concentration of acyclovir correlates with the recommended concentrations required in order to produce 50% inhibition of viral cytopathic effect or plaque formation;
- *In vivo* testing of the product should be done in order to determine the possible beneficial and adverse reactions, which may be associated with the use of these semi-solid products; and
- Clinical trials on HIV/AIDS patients with mucocutaneous skin manifestations can be performed to determine the efficacy of the Pheroid™ formulations containing acyclovir and ketoconazole, as well as the duration of treatment necessary, to achieve the desired effect.

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APPENDIX A

METHOD VALIDATION FOR THE HPLC ASSAY OF SEMISOLIDS ON STABILITY TESTING

A.1 INTRODUCTION

The purpose of the validation of the high performance liquid chromatography (HPLC) method was to ensure that the analytical method was sensitive and consistent in the determination of the amount of the actives (acyclovir and ketoconazole), preservatives (methylparaben and propylparaben) and anti-oxidants in the formulated products. Anti-oxidants included butylated hytroxyanisole (BHA), butylated hydroxytoluene (BHT) and tocopherol. The method was validated for the seven analytes, which were present in the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel formulations on stability testing.

A.2 CHROMATOGRAPHIC CONDITIONS

Analytical instrument: The HPLC analysis of acyclovir, ketoconazole, methylparaben, propylparaben, BHA, BHT and tocopherol was performed by using an Agilent 1100 Series HPLC system, equipped with an Agilent 1100 pump, UV detector and autosampler. The HPLC system was interfaced with data acquisition and analysis software (Chemstation Rev. A.06.02) as configured for a Hewlett Packard® computer data acquisition and analysis software.

Column: A Luna (5 µm, 250 x 4.60 mm), C-18 column was used during the HPLC analysis for sample separation.

Mobile phase: The mobile phase consisted of 3 vol of analytical grade methanol and 7 vol of a 0.1% octanesulfonic acid solution. This changed over 8 min to 10 vol of methanol. Table A.1 illustrates the composition of the mobile phase and the timetable which was used during the gradient elution, where A indicates the percentage octanesulfonic acid solution and B indicates the percentage methanol.

Table A.1: Timetable for the composition of the mobile phase during gradient elution

Time (min)	A (%)	B (%)
1.0	70.0	30.0
8.0	0.0	100.0

The octanesulfonic acid solution was prepared by accurately weighing and dissolving 1.0 g octane-1-sulfonic acid sodium salt in 950 ml of HPLC water and adjusting pH to 3.5 ± 0.01 with a 10% orthophosphoric acid solution, made up to 1000 ml with HPLC water. The solution was filtered through a 0.45 µm Millipore® filter and degassed on an ultrasonic bath.

Flow rate: The flow rate was set to 1.0 ml/min.

Injection volume: The injection volume was 5 µl.

Detection: The UV detector was set at 220 nm for the detection of acyclovir, ketoconazole, BHA, BHT and tocopherol; and was set at 254 nm to detect methylparaben and propylparaben.

Run time: The run time was set to 30 min.

Retention time: The retention times of the analyses are shown in Table A.2.

Table A.2: The retention time of each analyte

Analyte	Time (min)
Acyclovir	3.6 - 3.7
Ketoconazole	9.6 - 9.7
Methylparaben	8.6 - 8.7
Propylparaben	10.1 - 10.3
BHA	10.8 - 10.9
BHT	12.5 - 12.6
Tocopherol	19.0 - 19.2

Solvent: Methanol was used as solvent for all sample and standard preparations.

A.3 STANDARD PREPARATION

The standard solution consisted of a solution of all analytes in methanol. Table A.3 indicates the amount of each analyte that was accurately weighed.

Table A.3: The amount (mg) used for standard preparation of each analyte

Acyclovir	50 mg
Ketoconazole	20 mg
BHA	0.2 mg
BHT	2.0 mg
Methylparaben	4.0 mg
Propylparaben	0.8 mg
Tocopherol	2.0 mg

The analytes were transferred to a 100 ml volumetric flask and made up to volume with methanol. To ensure that all the analytes were completely dissolved, the standard was placed in an ultrasonic bath for approximately 5 min. The standard was filtered through a 0.45 µm syringe filter. 1 ml of the standard solution was transferred into a HPLC vial and analysed using the HPLC method described in Section A.2.

A.4 SAMPLE PREPARATION

Approximately 1 g of each formulation was weighed into a 100 ml volumetric flask, using a syringe with a tube attached to the tip. Methanol was added and the samples were sonificated for approximately 20 min, until completely dispersed. The samples were also shaken repeatedly by hand during this period to ensure that the base was completely dispersed in the methanol. The samples were allowed to cool to room temperature and subsequently filled to volume in the volumetric flask. Samples were filtered with a 0.45 µm syringe filter, transferred to a HPLC vial and analysed by HPLC.

A.5 VALIDATION PARAMETERS

A.5.1 Linearity

The linearity of an analytical method is its capability (within a given range) to obtain test results, which are directly proportional to the amount (concentration) of analytes in the sample. Linearity of the analytes was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml). The standards were prepared as described in the standard preparation (A.3).

Table A.4 to Table A.10 and Figure A.1 to Figure A.7 illustrate the linear regression curves obtained for all the analytes. The acceptance criteria for linear regression analysis should yield a regression coefficient (r^2) of ≥ 0.99.

Table A.4: The mean peak area ratio values of acyclovir

Concentration (µg/ml)	Peak area ratio
0.0	0.00
390.0	1463.15
455.0	1679.35
500.5	1801.90
552.5	1902.35
617.5	2072.60

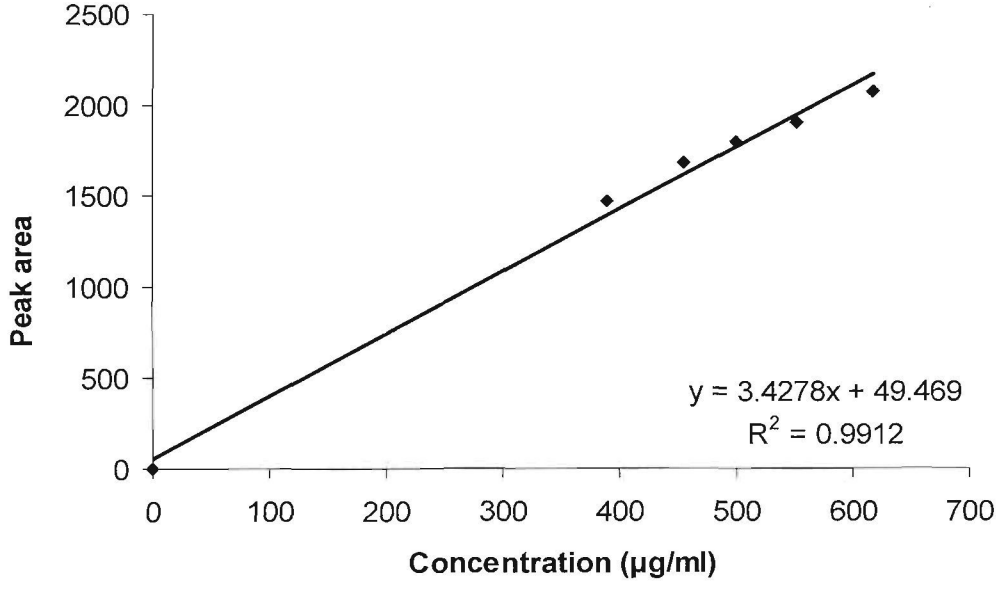


Figure A.1: Linear regression curve of acyclovir

The method was linear for acyclovir over the concentration range 390 - 620 µg/ml. The regression value ($r^2 = 0.9912$) obtained for acyclovir indicated a high degree of linearity and, therefore, demonstrated good stability of the analysis system.

Table A.5: The mean peak area ratio values of ketoconazole

Concentration (µg/ml)	Peak area ratio
0.0	0.00
156.0	1948.15
182.0	2278.95
200.2	2486.80
221.0	2716.75
247.0	3001.30

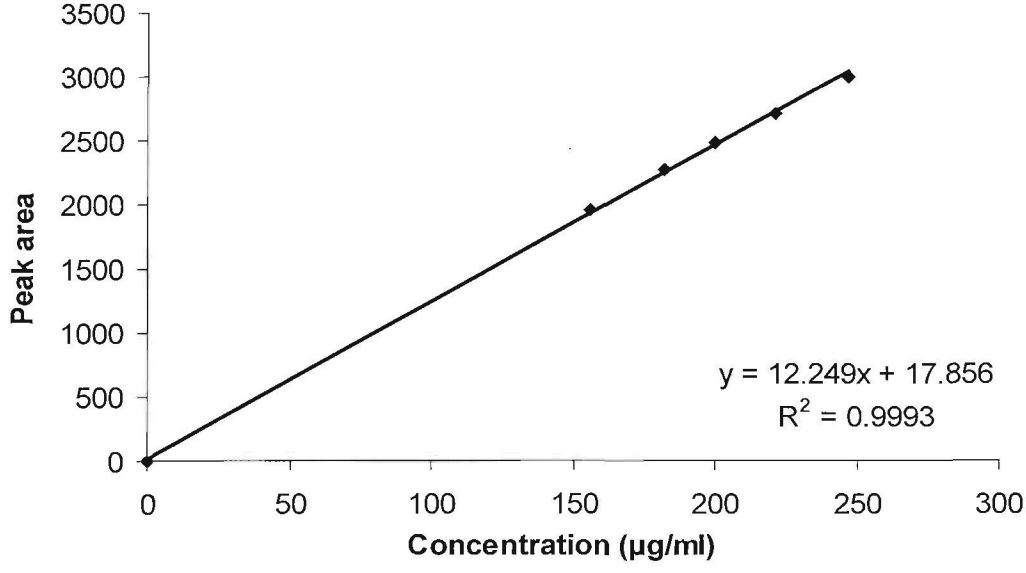


Figure A.2: Linear regression curve of ketoconazole

The method was linear for ketoconazole over the concentration range 150 - 250 µg/ml. The regression value ($r^2 = 0.9993$) obtained for ketoconazole indicated a high degree of linearity and, therefore, demonstrated good stability of the analysis system.

Table A.6: The mean peak area ratio values of methylparaben

Concentration (µg/ml)	Peak area ratio
0.0	0.00
31.2	408.75
36.4	460.00
40.0	490.55
44.2	548.40
49.4	601.35

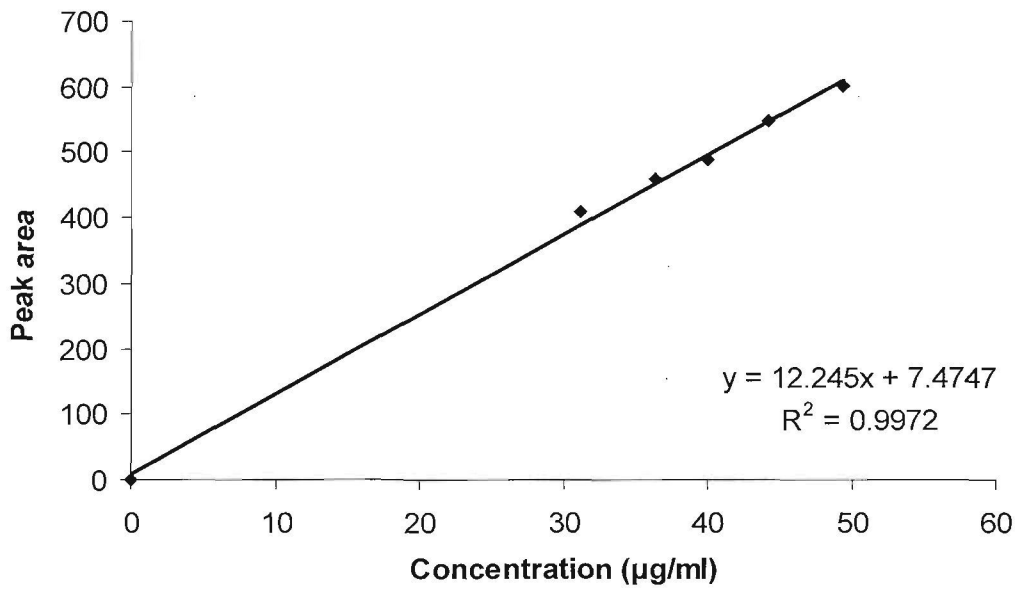


Figure A.3: Linear regression curve of methylparaben

The method was linear for methylparaben over the concentration range 30 - 50 µg/ml. The regression value ($r^2 = 0.9972$) obtained for methylparaben indicated a high degree of linearity and, therefore, demonstrated good stability of the analysis system.

Table A.7: The mean peak area ratio values of propylparaben

Concentration (µg/ml)	Peak area ratio
0.0	0.00
6.24	79.20
7.28	89.75
8.00	97.25
8.84	104.60
9.88	113.50

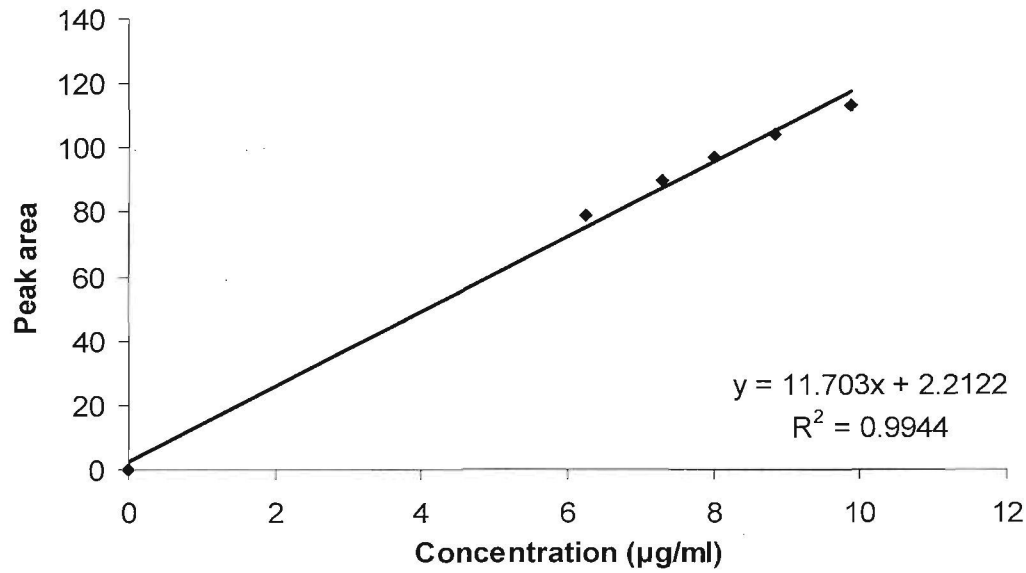


Figure A.4: Linear regression curve of propylparaben

The method was linear for propylparaben over the concentration range 6 - 10 µg/ml. The regression value ($r^2 = 0.9944$) obtained for propylparaben indicated a high degree of linearity and, therefore, demonstrated good stability of the analysis system.

Table A.8: The mean peak area ratio values of BHA

Concentration (µg/ml)	Peak area ratio
0.0	0.00
1.56	20.55
1.82	24.10
2.00	26.03
2.21	30.49
2.47	33.70

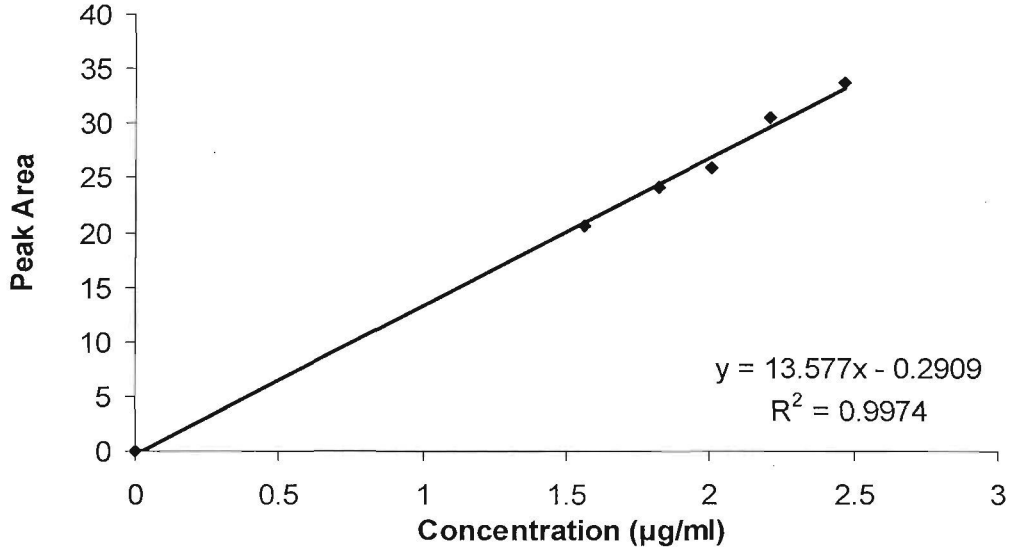


Figure A.5: Linear regression curve of BHA

The method was linear for BHA over the concentration range 1 - 3 µg/ml. The regression value (r^2 = 0.9974) obtained for BHA indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.9: The mean peak area ratio values of BHT

Concentration (µg/ml)	Peak area ratio
0.0	0.00
15.60	227.55
18.20	279.10
20.02	304.60
22.10	333.25
24.70	372.84

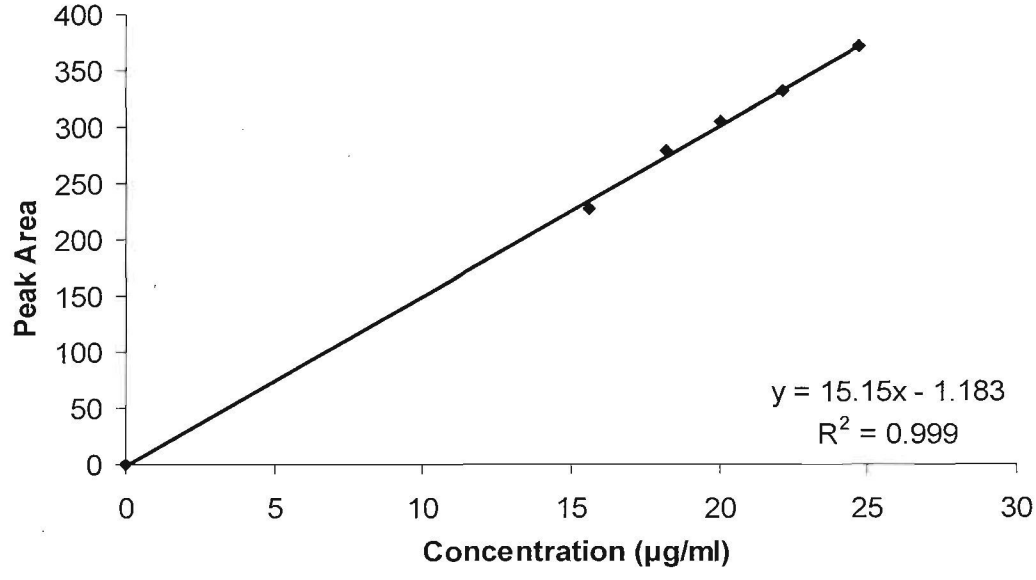


Figure A.6: Linear regression curve of BHT

The method was linear for BHT over the concentration range 15 - 25 µg/ml. The regression value ($r^2 = 0.999$) obtained for BHT indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.10: The mean peak area ratio values of tocopherol

Concentration (µg/ml)	Peak area ratio
0.0	0.00
15.60	104.00
18.20	123.75
20.02	130.90
22.10	146.80
24.70	162.15

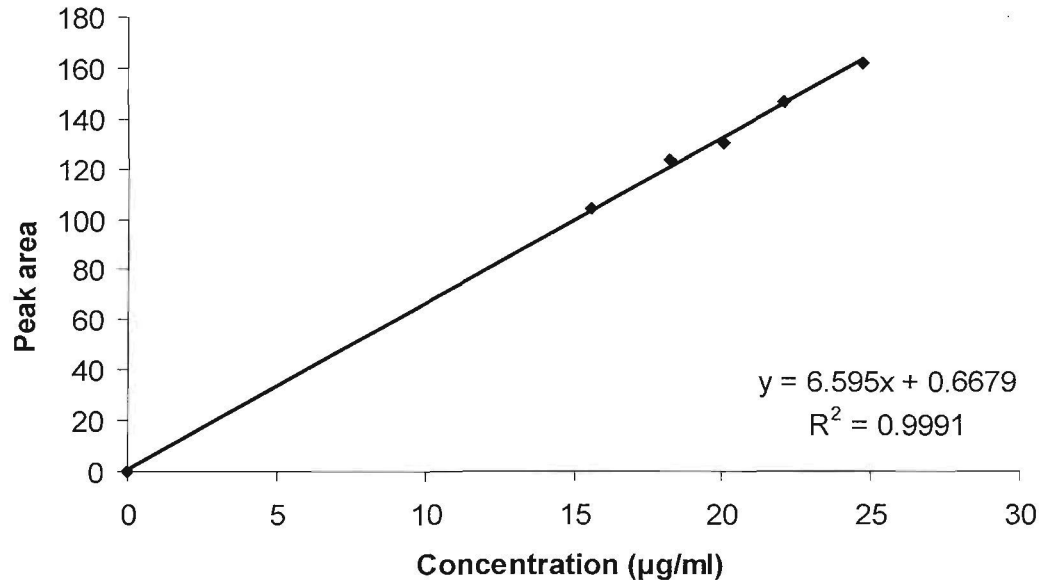


Figure A.7: Linear regression curve of tocopherol

The method was linear for tocopherol over the concentration range 15 - 25 µg/ml. The regression value ($r^2 = 0.9991$) obtained for tocopherol indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

A.5.2 Accuracy

The accuracy of an analytical procedure represents the closeness of the value found to the true value. Accuracy was assessed by weighing the following amounts of placebo cream or emulgel: 3 x 0.8 g, 3 x 1.0 g and 3 x 1.2 g into 100 ml volumetric flasks. The placebo's were spiked with known amounts of standard solution at concentrations of approximately 80, 100 and 120% of the expected sample concentration. Samples were filtered with syringe filters and placed in HPLC vials. The vials were placed into an autosampler for analyses and it was injected in duplicate. Results are shown in Table A.11 to Table A.17. The percentage recovery is an indication of the accuracy of the system. According to the acceptance criteria the recovery should be between 98 - 102%.

Table A.11: Accuracy of acyclovir

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	400.0	1428.9	1363.2	1396.1	420.5	105.1
80.0	400.0	1389.7	1397.9	1393.8	419.9	104.9
80.0	400.0	1392.3	1384.7	1388.5	418.3	104.5
100.0	500.0	1641.7	1639.7	1640.7	494.3	98.8
100.0	500.0	1641.7	1639.7	1640.7	494.2	98.8
100.0	500.0	1651.9	1615.3	1633.6	492.1	98.4
120.0	600.0	1891.4	1897.3	1894.3	570.7	95.1
120.0	600.0	1799.1	1819.6	1809.4	545.1	90.8
120.0	600.0	1737.1	1741.5	1739.3	524.0	87.3

Statistical analysis	
Mean	98.2
Standard deviation (SD)	0.2
% Relative standard deviation (RSD)	0.2

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 98% for acyclovir.

Table A.12: Accuracy of ketoconazole

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	160.6	1892.1	1816.1	1854.1	155.4	96.7
80.0	160.6	1867.5	1886.4	1877.0	157.3	97.9
80.0	160.6	1896.5	1871.8	1884.2	157.9	98.3
100.0	201.0	2318.1	2370.4	2344.3	196.4	97.7
100.0	201.0	2378.6	2382.0	2380.3	199.4	99.2
100.0	201.0	2398.5	2338.4	2368.5	198.4	98.7
120.0	241.0	2910.4	2903.5	2907.0	243.6	101.1
120.0	241.0	2914.8	2919.2	2917.o	244.4	101.4
120.0	241.0	2917.7	2922.4	2920.1	244.7	101.5

Statistical analysis	
Mean	99.2
SD	0.6
% RSD	0.6

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 99.19% for ketoconazole.

Table A.13: Accuracy of methylparaben

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	32.3	333.1	335.1	334.1	33.3	103.2
80.0	32.3	330.1	330.7	330.4	33.0	102.1
80.0	32.3	331.1	340.9	336.2	33.5	103.9
100.0	40.4	408.9	402.7	405.8	40.5	100.1
100.0	40.4	410.1	409.2	409.7	40.9	101.1
100.0	40.4	412.8	410.7	411.8	41.1	101.6
120.0	48.5	491.1	490.1	490.6	48.9	101.0
120.0	48.5	487.4	497.9	492.7	49.1	101.4
120.0	48.5	497.7	497.8	497.8	49.7	102.5

Statistical analysis	
Mean	101.9
SD	0.6
% RSD	0.6

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 101.89% for methylparaben.

Table A.14: Accuracy of propylparaben

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	6.4	53.7	53.4	53.5	6.6	102.9
80.0	6.4	53.7	53.1	53.4	6.6	102.7
80.0	6.4	47.6	53.4	50.5	6.2	97.1
100.0	8.3	65.9	67.4	66.7	8.2	98.4
100.0	8.3	66.3	68.4	67.4	8.3	99.5
100.0	8.3	65.3	65.2	65.3	8.0	96.4
120.0	10.0	83.6	87.1	85.4	10.5	105.3
120.0	10.0	84.6	83.5	84.1	10.3	103.7
120.0	10.0	82.6	83.6	83.1	10.2	102.5

Statistical analysis	
Mean	100.9
SD	1.3
% RSD	1.3

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 100.93% for propylparaben.

Table A.15: Accuracy of BHA

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	1.7	14.6	14.9	14.8	1.7	103.6
80.0	1.7	14.2	14.1	14.2	1.7	99.4
80.0	1.7	14.5	14.6	14.6	1.7	102.2
100.0	2.1	18.9	18.4	18.7	2.2	104.7
100.0	2.1	17.9	18.0	18.0	2.1	100.8
100.0	2.1	18.4	19.3	18.9	2.2	105.8
120.0	2.5	21.9	20.1	21.0	2.5	98.2
120.0	2.5	21.3	20.8	21.1	2.5	98.4
120.0	2.5	22.1	21.1	21.6	2.5	101.0

Statistical analysis	
Mean	101.6
SD	2.2
% RSD	2.1

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 101.56% for BHA.

Table A.16: Accuracy of BHT

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	16.0	133.0	132.8	132.9	15.9	99.5
80.0	16.0	134.7	134.8	134.8	16.1	100.8
80.0	16.0	133.5	132.6	133.1	15.9	99.8
100.0	20.0	166.7	166.4	166.6	19.9	99.6
100.0	20.0	168.3	168.9	168.6	20.2	100.8
100.0	20.0	167.2	165.7	166.5	19.9	99.5
120.0	24.6	197.2	200.1	198.7	23.8	96.9
120.0	24.6	197.4	197.7	197.5	23.6	96.3
120.0	24.6	198.5	197.9	198.2	23.7	96.6

Statistical analysis	
Mean	98.9
SD	0.6
% RSD	0.6

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 98.86% for BHT.

Table A.17: Accuracy of tocopherol

Concentration spiked					Recovery	
%	µg/ml	Area	Area	Mean	µg/ml	%
80.0	16.0	91.3	91.8	91.6	15.38	96.39
80.0	16.0	89.1	91.7	90.4	15.19	95.17
80.0	16.0	90.2	91.5	90.9	15.26	95.64
100.0	20.0	119.7	118.6	119.2	20.02	100.00
100.0	20.0	119.?	118.1	118.6	19.92	99.50
100.0	20.0	117.5	119.3	118.4	19.89	99.37
120.0	24.6	145.9	143.8	144.9	24.34	99.14
120.0	24.6	144.3	144.8	144.6	24.29	98.93
120.0	24.6	145.2	145.2	145.2	24.40	99.38

Statistical analysis	
Mean	98.2
SD	0.3
% RSD	0.3

Over the range of 80 - 120% of the sample concentration, the method yielded an acceptable mean recovery of 98.17% for tocopherol.

A.5.3 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous substance, under the prescribed conditions. Precision was investigated in terms of intra-day (repeatability) variation and inter-day (reproducibility) variation, under normal operating conditions.

A.5.3.1 Intra-day precision (within-day variation)

Intra-day precision was determined by accurately weighing 3 x 0.8 g, 3 x 1.0 g and 3 x 1.2 g of sample (cream or emulgel) into nine 100 ml volumetric flasks and filling it to volume with methanol. A 100% standard solution was prepared as described in Section A.4. The samples and the standard was then injected into the HPLC, in duplicate for analysis.

The results can be seen in Table A.18 to Table A.24, with all the results complying with acceptable pharmaceutical standards and meeting the requirements of the current United States Pharmacopoeia (USP). The acceptance criterion for intra-day precision was repeatability more than 2%.

Table A.18: Intra-day precision of acyclovir

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	1501.89	1503.03	1502.50	443.62	430.00	103.17
0.43	1502.65	1503.41	1503.00	443.79	430.00	103.21
0.42	1505.24	1516.90	1511.10	446.16	420.00	106.23
0.51	1788.7	1816.20	1802.50	532.20	510.00	104.35
0.53	1884.4	1923.10	1903.80	562.11	530.00	106.06
0.52	1821.7	1829.70	1825.70	539.06	520.00	103.67
0.62	2118.84	2090.50	2104.70	621.43	620.00	100.23
0.61	2133.8	2102.40	2118.10	625.40	610.00	102.52
0.61	2101.1	2126.50	2113.80	624.123	610.00	102.32
					Mean	103.53
					SD	1.76
					%RSD	**1.70**

Precision of acyclovir was satisfactory with a RSD of 1.70%.

Table A.19: Intra-day precision of ketoconazole

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	1960.60	2007.10	1983.90	158.56	172.00	92.19
0.43	2002.50	2000.20	2001.40	159.96	172.00	93.00
0.42	1948.30	1909.10	1928.70	154.15	168.00	91.76
0.51	2294.22	2340.25	2317.20	185.21	204.00	90.79
0.53	2414.30	2465.30	2439.80	195.00	212.00	91.98
0.52	2339.90	2333.30	2336.60	186.76	208.00	89.79
0.62	2784.23	2777.50	2780.90	222.26	248.00	89.62
0.61	2784.34	2766.90	2775.60	221.85	244.00	90.92
0.61	2747.30	2780.90	2764.10	220.92	244.00	90.54
					Mean	91.18
					SD	1.07
					%RSD	**1.17**

Precision of ketoconazole was satisfactory with a RSD of 1.17%.

Table A.20: Intra-day precision of methylparaben

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	381.04	380.09	380.60	31.49	34.40	91.54
0.43	374.50	387.10	380.80	31.51	34.40	91.60
0.42	376.60	369.40	373.00	30.86	33.60	91.86
0.51	443.80	448.20	446.00	36.91	40.80	90.45
0.53	461.70	472.80	467.30	38.66	42.40	91.19
0.52	461.70	458.00	459.90	38.05	41.60	91.47
0.62	541.05	505.10	523.10	43.28	49.60	87.26
0.61	532.10	517.10	524.60	43.41	48.80	88.95
0.61	519.10	522.90	521.00	43.11	48.80	88.34
					Mean	90.30
					SD	1.59
					%RSD	**1.76**

Precision of acyclovir was satisfactory with a RSD of 1.76%.

Table A.21: Intra-day precision of propylparaben

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	66.80	66.50	66.70	5.66	6.88	82.22
0.43	66.90	66.20	66.60	5.65	6.88	82.09
0.42	64.90	65.20	65.10	5.52	6.72	82.15
0.51	80.49	80.55	80.50	6.83	8.16	83.74
0.53	82.60	83.20	82.90	7.04	8.48	82.97
0.52	80.90	81.10	81.00	6.88	8.32	82.62
0.62	98.20	99.89	99.00	8.41	9.92	84.73
0.61	98.50	97.50	98.00	8.32	9.76	85.21
0.61	99.20	98.88	99.00	8.41	9.76	86.12
					Mean	83.54
					SD	1.41
					%RSD	**1.69**

Precision of propylparaben was satisfactory with a RSD of 1.69%.

Table A.22: Intra-day precision of BHA

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	20.94	20.10	20.50	1.42	1.72	82.71
0.43	20.80	20.90	20.90	1.45	1.72	84.04
0.42	20.60	20.30	20.50	1.42	1.68	84.39
0.51	24.30	24.90	24.60	1.71	2.04	83.60
0.53	24.60	24.10	24.40	1.69	2.12	79.62
0.52	24.60	24.20	24.40	1.70	2.08	81.32
0.62	29.33	29.10	29.20	2.03	2.48	81.67
0.61	29.30	29.40	29.40	2.03	2.44	83.39
0.61	29.04	29.30	29.20	2.02	2.44	82.88
					Mean	82.62
					SD	1.43
					%RSD	**1.73**

Precision of BHA was satisfactory with a RSD of 1.73%.

Table A.23: Intra-day precision of BHT

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	82.50	80.20	81.40	7.59	17.20	44.13
0.43	81.50	80.20	80.90	7.54	17.20	43.85
0.42	78.20	79.20	78.70	7.34	16.80	43.70
0.51	95.50	95.80	95.70	8.92	20.40	43.74
0.53	98.10	97.50	97.80	9.12	21.20	43.04
0.52	97.20	97.30	97.30	9.07	20.80	43.62
0.62	113.50	113.80	113.70	10.60	24.80	42.75
0.61	111.15	112.10	111.60	10.41	24.40	42.68
0.61	111.90	111.60	111.80	10.43	24.40	42.73
					Mean	43.36
					SD	0.53
					%RSD	**1.21**

Precision of BHT was satisfactory with a RSD of 1.21%.

Table A.24: Intra-day precision of tocopherol

Mass	Area	Area	Mean	µg/ml	Injected [ ]	%
0.43	108.98	100.85	104.90	1.74	1.72	101.07
0.43	102.23	102.01	102.10	1.69	1.72	98.38
0.42	104.89	103.52	104.20	1.73	1.68	102.78
0.51	123.90	121.20	122.60	2.03	2.04	99.54
0.53	125.79	126.41	126.10	2.09	2.12	98.60
0.52	125.70	124.30	125.00	2.07	2.08	99.58
0.62	149.80	149.80	149.80	2.48	2.48	100.09
0.61	149.40	146.70	148.10	2.45	2.44	100.54
0.61	147.30	149.60	148.50	2.46	2.44	100.81
					Mean	100.15
					SD	1.28
					%RSD	**1.28**

Precision of tocopherol was satisfactory with a RSD of 1.28%.

A.5.3.2 Inter-day precision (inter laboratory variation)

The same homogenous samples were analysed in triplicate as described above to determine intra-day precision (at 100% of the sample concentration), and on two more occasions (day 2 and day 3) to determine the between-day variability of the method. On one occasion (day 3) a different analyst performed the analysis on a different set of equipment. Inter-day precision

must be more than 5%. The results can be seen in Table A.25 to Table A.31, with all the results complying with acceptable pharmaceutical standards, meeting the requirements of the USP.

Table A.25: Inter-day precision of acyclovir

Acyclovir	Day 1	Day 2	Day 3	Between days
	104.35	112.37	114.30	
	106.06	114.51	113.84	
	103.67	112.52	112.13	
Mean	104.69	113.10	113.40	110.42
SD	1.01	1.00	0.90	4.05
%RSD	0.96	0.90	0.80	**3.67**

The inter-day precision was acceptable at 3.67%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

Table A.26: Inter-day precision of ketoconazole

Ketoconazole	Day 1	Day 2	Day 3	Between days
	90.79	87.83	88.93	
	91.98	89.68	90.97	
	89.79	86.41	86.71	
Mean	90.42	88.00	88.90	89.09
SD	2.89	1.30	1.70	1.01
%RDS	1.17	1.50	2.00	**1.14**

The inter-day precision was acceptable at 1.14%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

Table A.27: Inter-day precision of methylparaben

Methylparaben	Day 1	Day 2	Day 3	Between days
	90.45	94.33	94.37	
	91.19	86.28	92.92	
	91.47	90.27	87.99	
Mean	91.04	90.30	91.80	91.03
SD	0.43	3.30	2.70	0.60
%RSD	0.47	3.60	3.00	**0.66**

The inter-day precision was acceptable at 0.66%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

Table A.28: Inter-day precision of propylparaben

Propylparaben	Day 1	Day 2	Day 3	Between days
	83.74	83.26	82.96	
	82.97	84.57	83.44	
	82.62	84.11	84.11	
Mean	83.11	83.98	83.50	83.53
SD	0.47	0.55	0.47	0.36
%RSD	0.56	0.65	0.56	**0.43**

The inter-day precision was acceptable at 0.43%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

Table A.29: Inter-day precision of BHA

BHA	Day 1	Day 2	Day 3	Between days
	83.60	82.41	82.92	
	79.63	80.61	78.97	
	81.32	79.66	80.82	
Mean	81.52	80.89	80.90	81.10
SD	1.63	1.14	1.61	0.29
%RSD	2.00	1.41	1.99	**0.36**

The inter-day precision was acceptable at 0.36%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

Table A.30: Inter-day precision of BHT

BHT	Day 1	Day 2	Day 3	Between days
	43.74	44.18	44.05	
	43.04	43.84	44.36	
	43.62	45.07	44.53	
Mean	43.47	44.36	44.32	44.05
SD	0.31	0.52	0.20	0.41
%RSD	0.71	1.16	0.45	**0.93**

The inter-day precision was acceptable at 0.93%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

Table A.31: Inter-day precision of tocopherol

Tocopherol	Day 1	Day 2	Day 3	Between days
	99.54	99.01	99.26	
	98.56	97.35	95.24	
	99.58	98.27	97.23	
Mean	99.23	98.21	97.24	98.23
SD	0.47	0.68	1.64	0.81
%RSD	0.48	0.69	1.69	**0.83**

The inter-day precision was acceptable at 0.83%. Repeatability was within acceptable limits and the assay should perform well, even when carried out by other personnel in a different laboratory.

A.5.4 Ruggedness

A.5.4.1 Stability of sample solutions

A sample was prepared as described in Section A.4. The sample was injected into the HPLC. The sample was left in the autosampler tray and reanalysed over a period of 12 h to determine the stability of the sample. Sample solutions should not be used for a period longer than it takes to degrade by 2%, and in the case of degradation, special precautions should be followed to compensate for the loss. Stability data can be observed in Table A.32 to A.38.

Table A.32: The stability of acyclovir

Time (h)	Peak area acyclovir	% Remaining
0	1870.0	100.000
1	1829.7	97.844
2	1861.4	99.540
3	1864.7	99.716
4	1867.1	99.844
5	1868.2	99.903
6	1870.5	100.026
7	1868.6	99.925
8	1870.6	100.032
9	1866.2	99.796
10	1869.3	99.962
11	1870.7	100.037
12	1863.1	99.631
Mean	1864.6	**99.712**
SD	10.479	0.560
%RSD	0.561	0.561

Acyclovir stability proved acceptable for the medium used during the analysis with an average degradation of 0.28%.

Table A.33: The stability of ketoconazole

Time (h)	Peak area ketoconazole	% Remaining
0	2320.1	100.000
1	2279.0	98.228
2	2299.6	99.116
3	2306.2	99.400
4	2312.9	99.689
5	2318	99.909
6	2321.2	100.047
7	2321.5	100.060
8	2325.9	100.250
9	2326.6	100.280
10	2327.5	100.319
11	2329.6	100.409
12	2330.4	100.443
Mean	2316.808	**99.858**
SD	14.000	0.603
%RSD	0.604	0.604

The stability of ketoconazole proved acceptable for the medium used during the analysis with an average degradation of 0.142%.

Table A.34: The stability of methylparaben

Time (h)	Peak area methylparaben	% Remaining
0	444.8	100.000
1	444.6	99.955
2	447.8	100.674
3	450.2	101.214
4	463.4	104.181
5	442.6	99.505
6	455.3	102.360
7	438.1	98.493
8	450.8	101.348
9	444.0	99.820
10	450.9	101.371
11	451.4	101.483
12	438.4	98.561
Mean	447.869	**100.69**
SD	6.679	1.501
%RSD	1.491	1.491

The stability of methylparaben proved acceptable for the medium used during the analysis.

Table A.35: The stability of propylparaben

Time (h)	Peak area propylparaben	% Remaining
0	177.8	100.000
1	175.2	98.538
2	175.5	98.706
3	178.5	100.394
4	176.8	99.438
5	181.3	101.969
6	177.2	99.663
7	179.0	100.675
8	179.4	100.900
9	178.4	100.337
10	175.4	98.650
11	174.3	98.031
12	174.3	98.031
Mean	177.162	**99.641**
SD	2.066	1.162
%RSD	1.166	1.166

The stability of propylparaben proved acceptable for the medium used during the analysis with an average degradation of 0.359%.

Table A.36: The stability of BHA

Time (h)	Peak area BHA	% Remaining
0	28.5	100.000
1	30.0	105.263
2	29.1	102.105
3	28.7	100.702
4	29.5	103.509
5	29.7	104.211
6	29.3	102.807
7	29.9	104.912
8	28.2	98.947
9	28.1	98.596
10	30.4	106.667
11	29.3	102.807
12	28.4	99.649
Mean	29.162	**102.321**
SD	0.708	2.483
%RSD	2.427	2.427

The stability of BHA proved acceptable for the medium used during the analysis.

Table A.37: The stability of BHT

Time (h)	Peak area BHT	% Remaining
0	86.1	100.000
1	90.6	105.226
2	92.1	106.969
3	92.4	107.317
4	93.0	108.014
5	92.3	107.201
6	93.4	108.479
7	92.5	107.433
8	93.0	108.014
9	92.5	107.433
10	92.8	107.782
11	93.3	108.362
12	91.8	106.620
Mean	91.985	**106.835**
SD	1.837	2.133
%RSD	1.997	1.997

The stability of BHT proved acceptable for the medium used during the analysis.

Table A.38: The stability of tocopherol

Time (h)	Peak Area tocopherol	% Remaining
0	118.4	100.000
1	116.7	98.564
2	116.9	98.733
3	118.6	100.169
4	116.9	98.733
5	116.8	98.649
6	117.6	99.324
7	117.6	99.324
8	117.2	98.986
9	117.2	98.986
10	118.0	99.662
11	119.9	101.267
12	120.3	101.605
Mean	117.854	**99.539**
SD	1.119	0.946
%RSD	0.950	0.950

The stability of tocopherol proved acceptable for the medium used during the analysis, with an average degradation of 0.461%.

A.5.4.2 System repeatability

In an attempt to evaluate the repeatability of the peak area and retention time, a sample was injected six times. Table A.39 to Table A.45 shows the variations in response (% RSD) of the detection system when six determinations were made on the same day, and under the same conditions. The peak area and retention times should have an RSD of 2% or less.

Table A.39: System repeatability of acyclovir

Injection	Peak area of acyclovir	Retention time (min)
1	1467.700	3.691
2	1445.500	3.691
3	1449.600	3.682
4	1450.800	3.684
5	1453.900	3.663
6	1452.700	3.663
Mean	1453.367	3.676
SD	6.938	0.011
%RSD	**0.477**	**0.312**

System performance proved acceptable with a RSD value of 0.477% for peak area and 0.312% for retention time, respectively.

Table A.40: System repeatability of ketoconazole

Injection	Peak area of ketoconazole	Retention time (min)
1	2593.800	9.657
2	2574.300	9.652
3	2575.300	9.653
4	2584.300	9.649
5	2586.700	9.650
6	2582.600	9.649
Mean	2582.833	9.652
SD	6.671	0.003
%RSD	**0.258**	**0.029**

System performance proved acceptable with a RSD value of 0.258% for peak area and 0.029% for retention time, respectively.

Table A.41: System repeatability of methylparaben

Injection	Peak area of methylparaben	Retention time (min)
1	468.300	8.715
2	473.700	8.702
3	465.100	8.704
4	464.600	8.607
5	472.400	8.694
6	461.200	8.688
Mean	467.550	8.685
SD	4.415	0.036
%RSD	**0.944**	**0.413**

System performance proved acceptable with a RSD value of 0.944% for peak area and 0.413% for retention time, respectively.

Table A.42: System repeatability of propylparaben

Injection	Peak area of propylparaben	Retention time (min)
1	108.800	10.215
2	104.100	10.204
3	104.200	10.204
4	105.000	10.198
5	101.800	10.199
6	104.700	10.198
Mean	104.767	10.203
SD	2.077	0.006
%RSD	**1.983**	**0.058**

System performance proved acceptable with a RSD value of 1.983% for peak area and 0.058% for retention time, respectively.

Table A.43: System repeatability of BHA

Injection	Peak area of BHA	Retention time (min)
1	53.200	10.835
2	50.400	10.825
3	51.800	10.824
4	51.400	10.820
5	51.300	10.823
6	51.400	10.820
Mean	51.583	10.825
SD	0.837	0.005
%RSD	**1.624**	**0.047**

System performance proved acceptable with a RSD value of 1.624% for peak area and 0.047% for retention time, respectively.

Table A.44: System repeatability of BHT

Injection	Peak area of BHT	Retention time (min)
1	199.100	12.517
2	203.400	12.506
3	204.900	12.508
4	204.200	12.507
5	207.900	12.498
6	206.800	12.504
Mean	204.383	12.507
SD	2.810	0.006
%RSD	**1.375**	**0.045**

System performance proved acceptable with a RSD value of 1.375% for peak area and 0.045% for retention time, respectively.

Table A.45: System repeatability of tocopherol

Injection	Peak area of tocopherol	Retention time (min)
1	150.700	19.136
2	150.900	19.096
3	153.800	19.086
4	156.200	19.082
5	157.200	19.045
6	157.500	19.050
Mean	154.383	19.083
SD	2.798	0.030
%RSD	**1.813**	**0.159**

System performance proved acceptable with a RSD value of 1.813% for peak area and 0.159% for retention time, respectively.

A.5.6 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may interfere with analyte detection. The method is selective when no interfering peaks with the same retention time as the actives are detected. A blank injection of methanol was injected and illustrated no interference.

A standard solution was prepared, where after 1 ml of the standard solution was placed in 4 test tubes. The standard solution was diluted 1:1 with Milli-Q® water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide, respectively. These solutions were stored

overnight in closed test tubes at room temperature to degrade. The next day the samples were injected in duplicate into the HPLC and examined to determine whether any additional peaks were formed.

The samples did not contain any peaks that would interfere with the determination of the analytes. Extra peaks that formed in the stressed samples were visible from the active components.

A.6 CONCLUSION

The HPLC method developed has been found to be reliable and sensitive enough for the determination of the concentration of acyclovir, ketoconazole, methylparaben, propylparaben, BHA, BHT and tocopherol.

The method performed well and should be appropriate to analyse all the analytes in the formulations for stability testing, quality control and batch release purposes. No interference was encountered from stressed samples or known related substances, thus, the method can be considered as being stability indicating.

APPENDIX B

FORMULATION OF A COSMECEUTICAL SEMISOLID FOR TRANSDERMAL DELIVERY

B.1 INTRODUCTION

Cosmeceuticals are used regularly by an increasing number of people. The main purpose of cosmeceuticals is to treat and prevent illnesses whilst improving self-esteem. It is there to help people enjoy a fuller and more rewarding life (Mitsui, 1997:3). Topical products are divided into two groups namely cosmetics and pharmaceuticals (Kligman, 2002:5).

A cosmetic is any article intended to be used by means of rubbing, sprinkling or by similar application to the human body. The action of the product on the body has to be mild. Cosmetics are used by healthy people to maintain personal hygiene and/or appearance. It has no side effects and is safe to use on a daily basis (Mitsui, 1997:3). In contrast, pharmaceutical drugs are used for the treatment and prevention of illness of the human body. These products are only used for a short period of time to treat medical conditions. Minor side effects can sometimes occur with the use of these drugs (Mitsui, 1997:4).

A third category namely "cosmeceuticals" also exists. The Japanese call it "quasi-drugs". The term "cosmeceuticals" was originally used approximately 20 years ago by Albert Kligman, a well-known dermatologist (Umbach, 2002:10). Cosmeceuticals is a term that has been created by combining the words "cosmetic" and "pharmaceutical" to assign active cosmetics (Khaiat, 2002:8). Cosmeceuticals can also be defined as non-prescription, topical treatment products (Kligman, 2002:5). During this study cosmeceutical products were formulated.

B.2 DEVELOPMENT PROCESS OF A PRODUCT

The development process of a product consists of a preformulation, early formulation and final formulation phase. These three phases are discussed in the following section.

B.2.1 Preformulation

Preformulation includes those studies that should be carried out before the commencement of formulation development. The primary goal of the preformulation phase is to allow the rational development of safe, stable, efficacious dosage forms; and it is mainly concerned with the physicochemical properties of the drug substance. The only important aspect of preformulation, that is specific to dermatological and transdermal formulation, involves drug delivery characteristics (Walters & Brain, 2002:321). In this study the preformulation phase consisted of a literature study. All the required information was available.

B.2.2 Early formulation

A trial and error approach was used during the early formulation stage. Acyclovir and ketoconazole was incorporated into existing formulae obtained in the literature. These formulae were then altered as necessary. During this phase an assay method was developed to analyse the main ingredients of each formulation (see Appendix A). The problems that occurred during the early formulation phase are discussed in Section B.2.2.2.

B.2.2.1 Encountered problems

During the early formulation phase there was difficulty to dissolve 5% acyclovir in the available water to produce a 100% formulation. According to McEvoy (2002:11) acyclovir's solubility in water is 2.5 mg/ml at a temperature of 25 °C. The solubility of acyclovir was tested in ingredients suitable for topical use. Solubility tests were done in glycerine, silicone, boric acid, oleic acid, ethanol, PEG 400 and liquid Paraffin™ , only to name a few. Water showed the best solubility.

A Confocal Laser Scanning Microscope (CLSM) Nikon PCM 2000 with digital camera (DMX 1200) with a He/Ne laser (543 nm) and an Argon ion laser (457 - 517 nm) was used to inspect two acyclovir formulations available on the South African market. The oil used on the confocal was Nikon Immersion Oil for microscopy (50cc, Type A nd = 1.515) (23 °C). An objective of 60 x was used throughout. The two acyclovir formulations were inspected for acyclovir crystals and uniformity. In Figure B.1 the two acyclovir creams are shown.

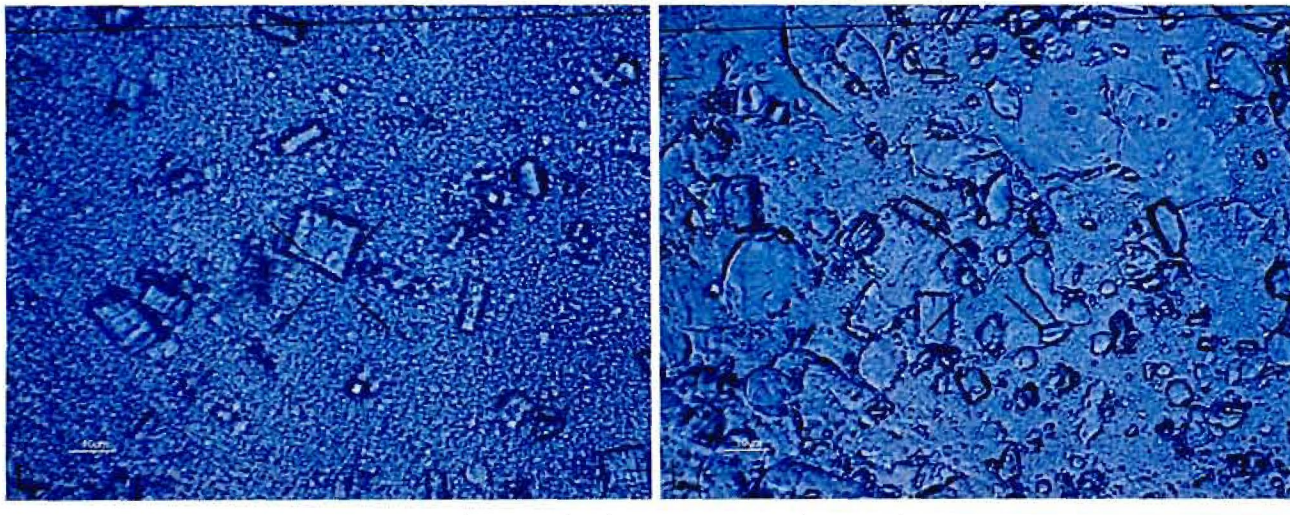


Figure B.1: Acyclovir crystals in acyclovir creams on the market

Crystals were found in the products that are currently available on the market. It was decided to decrease acyclovir's particle size by grinding the active ingredient. The smaller particle size can possibly contribute to increase acyclovir's solubility and penetration through the skin. The acyclovir powder was grinded with a Retsck K – ZM1 type mill. An average particle size of 10 µm or smaller was needed for the Pheroid™ to be able to entrap the acyclovir crystals.

the products which can lead to bad smells (Mitsui, 1997:199). Contamination by micro-organisms arising during production, or during the usage of the product by the consumer is called primary contamination and secondary contamination, respectively (Mitsui, 1997:200).

The ideal preservative should have the following characteristics: (1) effective against many species of micro-organisms; (2) soluble in the respective solvent or cosmetic ingredients; (3) high safety with no irritation; (4) neutral with no effect on product pH; (5) no reduction of the active ingredient's effectiveness; (6) no adverse effects on product appearance; (7) stabile over a wide temperature and pH range; (8) readily available; and (9) cost effective and economical to use (Mitsui, 1997:202).

In this study methylparaben and propylparaben were used as preservatives. Methylparaben and propylparaben are effective anti-bacterial and anti-fungal agents, which are commonly used as preservatives in foods, beverages, cosmetics and pharmaceuticals. A combination of methylparaben and propylparaben are used, because they have a synergistic effect (Hajkova *et al.*, 2002:91). The chemical structures of methylparaben and propylparaben are shown in Figure B.2.

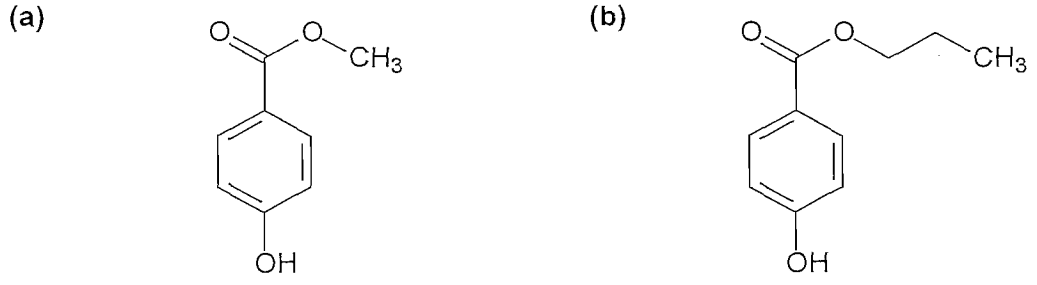


Figure B.2: Structures of (a) methylparaben and (b) propylparaben (Kollmorgen & Kraut, 1997)

Pharmaceutical preparations can deteriorate during storage, because some components oxidise when oxygen is present. This decomposition can be particularly troublesome in emulsions, because emulsification may introduce air into the product. Another reason for decomposition in emulsions is due to the high interfacial contact between the two phases of an emulsion (Barry, 2002:532).

The ideal antioxidant should posses the following characteristics: (1) effective at low concentrations; (2) non-toxic, non-irritant, non-sensitising, odourless and colourless; (3) stable and effective over a wide pH range; (4) neutral, should not chemically react with other ingredients; and (5) should be non-volatile (Barry, 2002:532).

BHA and the related compound BHT were used as antioxidants in this study. The chemical structures of BHA and BHT are shown in Figure B.3.

Particle size distributions of the original and grinded acyclovir were measured with a Malvern Mastersizer fitted with a Hydro 2000SM dispersion unit. Ethanol was used to disperse the acyclovir particles.

B.2.2.2 Results and discussion

The particle sizes obtained was significantly smaller. Table B.1 summarises the results of the samples' particle size distribution.

Table B.1: A summary of the particle size results of acyclovir prior to grinding. Values in brackets represent % RSD

	D(0.1) (µm)	D(0.5) (µm)	D(0.9) (µm)
Prior to grinding	10.93 (20.00)	27.12 (12.95)	57.56 (7.37)
After grinding	3.07 (12.66)	13.00 (2.15)	29.38 (1.92)

The average particle size prior to grinding was 31.92 µm. Acyclovir particles depicted a particle distribution where 10% of the particles were smaller than 10.93 µm, 50% of the particles were smaller than 27.12 µm and 90% of the particles depicted a particle size of less than 57.56 µm.

After grinding the acyclovir with the mill, the particle size decreased significantly. The average particle size obtained was 15.17 µm. Acyclovir particles depicted a particle distribution where 10% of the particles were smaller than 3.07 µm, 50% of the particles were smaller than 13.00 µm and 90% of the particles depicted a particle size of less than 29.38 µm.

These results show that the average particle size of acyclovir decreased with 16.75 µm. It will, therefore, be possible for Pheroid™ to encapsulate approximately 38% of the particles.

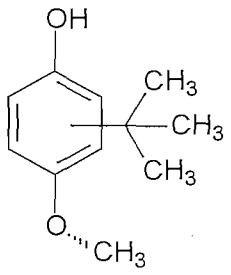
B.2.3 Final formulation

The products were prepared in bulk for storing and testing purposes after the final formulae were perfected. A stability program that complied with the guidelines of the ICH (2003:8) and the MCC (2006) was followed. The formulated products were stored at three storage conditions for a period 6 months.

B.3 PRESERVATION OF SEMISOLID FORMULATIONS

It is important to add preservatives and antioxidants to cosmeceuticals for the protection of the formulated products against microbial contamination. Microbial contamination can occur during prolonged periods of storage and usage of the products. Preservatives and antioxidants are, thus, added for the long term protection of cosmeceuticals against contamination. Primary or secondary contamination with bacterial, fungal and yeasts species can cause deterioration of

(a)



(b)

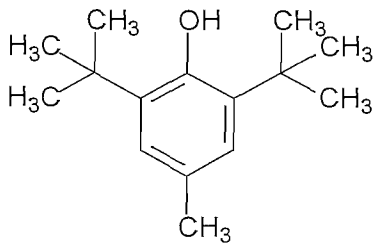


Figure B.3: Structures of antioxidants (a) BHA and (b) BHT (Helmenstine, 2009)

B.4 FORMULATION OF A CREAM

Creams are very important skin care cosmetics, because of its wide stability range over a number of different conditions. Creams are a type of emulsion in which two immiscible liquids, such as water and oil, are formulated into a stable dispersion. A dispersion are prepared where one of the liquids (the dispersion phase) is dispersed in the other liquid (the continuous phase) (Mitsui, 1997:341).

B.4.1 The purpose and function of a cream

The main functions of creams are to maintain the moisture balance, and keep the skin moist and supple. This is achieved through the supply of water, humectants and oils. Additional functions of creams are to stimulate circulation, cleanse the skin and to remove makeup.

Creams are easy to use and anyone can apply it. They also include a wide range of possible formulations. The formulator can formulate a cream to feel light with use or somewhat oily, soft or hard, give a moist feeling, penetrate well or be easy or difficult to wipe off. It is simple to vary the amount of water, and the amount and type of humectants and oil in the formulation. This can be done to fit different purposes of use, in order to cater to different skin types, skin conditions, cosmetic routines and preferences (which vary with the season, age and living environment) of the user (Mitsui, 1997:342).

B.4.2 Main ingredients of a cream

The main ingredients of creams are aqueous ingredients, oily ingredients, surfactants, preservatives, antioxidants, chelating agents, perfumes and pharmaceutical agents.

Creams are divided into oil-in-water (O/W) or water-in-oil (W/O) emulsions. In the case of O/W emulsions; hydrophilic surfactants are used. The oily ingredients used can vary widely from those with no polarity to those with very high polarity. For the W/O emulsion cream; a lipophilic surfactant is mainly used. The oily ingredients used are mainly non-polar. In order to raise stability, it is important to prevent sedimentation in the internal phase (water phase). To achive this it is necessary to select a combination of oils with care.

In the case of creams which have a large concentration of oil, the W/O emulsion has been used to increase the oily nature. O/W type is used when a light feeling is desired. The emulsion type may be freely selected when a large concentration of oil is used. As a result, almost all creams containing a little oil component have been limited to the O/W emulsion (Mitsui, 1997:343). An O/W cream was formulated during this study.

B.4.3 Cream formula

The final formula for acyclovir and ketoconazole cream is given in Table B.2

Table B.2: Formula of acyclovir and ketoconazole cream

Raw material	% m/m	Activity
Acyclovir	5.00%	Active ingredient
Ketoconazole	2.00%	Active ingredient
Liquid Paraffin™	20.00%	Oil phase of emulsion
Cetyl alcohol	10.00%	Thickening agent
Glycerin	10.00%	Emulsifier and co-solvent for acyclovir
PEG 400	5.50%	Co-solvent for ketoconazole
Tween 80	4.50%	Emulsifier and thickening agent
Xanthan gum	0.50%	Thickening agent
Span 60	0.50%	Emulsifier
Methylparaben	0.40%	Preservative
Propylparaben	0.08%	Preservative
BHT	0.20%	Antioxidant
BHA	0.02%	Antioxidant
H_2O	qs	Solvent

B.4.3.1 Procedure to prepare the cream formula

- Heat approximately 50 ml H_2O to ± 40 °C.
- Weigh the xanthan gum.
- Add xanthan gum slowly to the heated H_2O, while homogenising at 200 rpm until dissolved *and homogenous.*
- Weigh the PEG 400.
- Weigh ketoconazole and add to PEG 400.
- Heat at 80 °C until dissolved.
- Weigh the acyclovir.
- Weigh the glycerine.

- Add a little glycerine to the acyclovir to make a paste.
- Then add the rest of the glycerine and mix well.
- Add the rest of the water to the acyclovir and glycerine mixture.
- Weigh the following ingredients and heat to 80 °C: Cetyl alcohol, liquid paraffin™, span 60, tween 80, methylparaben, propylparaben, BHT and BHA.
- Add the acyclovir mixture to the xanthan gum and water.
- Heat the water mixture to 80 °C.
- Immediately add the oil phase to the water phase while homogenising at 13500 rpm.
- Homogenise until a temperature of exactly 40 °C is reached.
- Cool with ice water.
- Stir at 200 rpm until room temperature is reached.
- Leave overnight.

B.4.3.2 Results

The cream applied easily and it was not too oily. It had a homogenous white texture with no odour.

B.4.4 Pheroid™ cream formula

The same basic cream formula was used for the Pheroid™ cream. Pheroid™ ingredients for example tocopherol were added to the oil phase in order to form acyclovir and ketoconazole vesicles in cream.

B.4.4.1 Results

The cream applied easily and it was not too oily. It had a homogenous white texture with a characteristic "fish oil" Pheriod™ smell.

B.5 FORMULATION OF EMULGEL

B.5.1 Purpose and function of a gel

Gels are two-component semi-solid systems, which are rich in liquid. Their one characteristic feature is the presence of a continuous structure providing solid-like properties (Barry, 2002:528). The system may be transparent, semi-transparent or opaque due to either the

gelling agent that does not fully dissolve, or because it forms aggregates, which disperse the light (Barry, 1983:300).

Aqueous gels contain a high concentration of moisture, thus, are used to supply moisture to the skin and have a cooling effect. Gels can also be used as the base of a cleaner for light makeup. Aqueous gels are used for oily skin types in the summer time. Oily gels supply oil to the skin and are used in the winter for a dry skin due to its moisturising properties (Mitsui, 1997:351).

B.5.2 Main ingredients of a gel

In a typical polar gel, a natural or synthetic polymer builds a three-dimensional matrix throughout a hydrophilic liquid. Typical polymers used, include the natural gums tragacanth, carrageenan, pectin, agar and alginic acid, semi-synthetic materials (such as methylcellulose and carboxymethylcellulose) as well as the synthetic polymer carbapol. Certain clays for example veegum and laponite may also be used (Barry, 2002:529). Gel products consist of a gel base, humectants, surfactants, preservatives, pharmaceutical agents, colouring agents and perfumes (Mitsui, 1997:351).

Oil containing emulsion-type aqueous gels are also available. They are transparent as well as semi-transparent or opaque. These gels are prepared by adding a small amount of oil and a surfactant to an aqueous gel (Mitsui, 1997:352).

B.5.3 Emulgel

An emulgel can be described as a gelified O/W emulsion. It is a two phase system consisting of large organic molecules interpenetrated by water and a small fraction of emulsified lipids. Emulgels are used for the treatment of acne, allergic skin conditions and acute eczema. It is an effective formulation for normal to oily skin types and inflamed skin areas (Radulescu *et al.*, 2009). In this study an emulgel was formulated. An O/W emulsion was gelified and thickened with xanthan, a natural gum.

B.5.4 Emulgel formula

The final formula for acyclovir and ketoconazole emulgel is given in Table B.3.

Table B.3: Formula of acyclovir and ketoconazole emulgel

Raw material	% m/m	Activity
Acyclovir	5.00	Active ingredient
Ketoconazole	2.00	Active ingredient
Liquid Paraffin™	20.00	Oil phase of emulsion
Glycerin	10.00	Emulsifier and co-solvent for acyclovir
PEG 400	5.50	Co-solvent for ketoconazole
Tween 80	4.50	Emulsifier and thickening agent
Xanthan gum	1.50	Thickening agent (gelified)
Span 60	0.50	Emulsifier
Methylparaben	0.40	Preservative
Propylparaben	0.08	Preservative
BHT	0.20	Antioxidant
BHA	0.02	Antioxidant
H_2O	qs	Solvent

B.5.4.1 Procedure to prepare the emulgel formula

- Heat approximately 60 ml H_2O to 40 °C.
- Weigh 30.3 g of H_2O.
- Weigh xanthan gum.
- Add xanthan gum slowly to 30.3 g heated H_2O while homogenising at a rate of 400 rpm until dissolved and homogenous.
- Weigh PEG 400.
- Weigh ketoconazole and add to PEG 400.
- Heat ketoconazole at 80 °C until dissolved.
- Weigh acyclovir and glycerine.
- Add a little glycerine to the acyclovir and make a paste. Then add the rest of the glycerine to the paste and mix well.
- Add 20 g of water to the acyclovir and glycerine mixture.
- Weigh the following ingredients and heat together (oil phase): Liquid Paraffin™, span 60, tween 80, methylparaben, propylparaben, BHT and BHA.
- Add ketoconazole to the oil phase.
- Heat to 80 °C.

- Add the acyclovir mixture to the xanthan gum and mix well (water phase).
- Heat the xanthan mixture to 80 °C;
- Immediately add the oil phase to the water phase while homogenising at a rate of 13500 rpm.
- Homogenise until a temperature of exactly 40 °C is reached.
- Cool with ice water.
- Stir at 200 rpm until room temperature is reached.
- Leave overnight.

B.5.4.2 Results

A white emulgel was obtained. It applied easily, and it was not too oily or too hydrous. It had a homogenous white texture with no odour.

B.5.5 Pheroid™ emulgel formula

The same basic emulgel formula was used for the Pheroid™ emulgel. Pheroid™ ingredients for example tocopherol were added to the oil phase to form acyclovir and ketoconazole vesicles in the emulgel.

B.4.5.1 Results

A white emulgel was obtained. It applied easily, and it was not too oily or too hydrous. It had a homogenous white texture with a characteristic "fish oil" Pheriod™ smell.

B.6 MATERIALS USED IN THE FORMULATIONS

The supplier and batch numbers of the materials used in the formulations of the creams and emulgels in this study are listed in Table B.4.

Table B.4: Ingredients used in formulations

Ingredients	Supplier	Batch number
Acyclovir	DB Fine Chemicals (Pty) Ltd.	090314
Ketoconazole	DB Fine Chemicals (Pty) Ltd.	SBML/KTN/811203
Liquid Paraffin™	Merck Chemicals	1032980
PEG 400	Merck Chemicals	102787
Glycerin	Sigma-Aldrich	7206/433P
Span 60	Fluka Analytical	423065/141002
Tween 80	Merck Chemicals	1032991
Xanthan gum	Warren Chem Specialities	445090279C
Cetyl alcohol	Merck Chemicals	S 518 3304 904
Methylparaben	Merck Chemicals	GBGA 0001371
Propylparaben	Merck Chemicals	GBGA 032949
BHA	Merck Chemicals	115K0028
BHT	Sigma-Aldrich	04416 KD-076
Tocopherol	Chempure	UT 0805099

B.7 CONCLUSION

The final formulations were prepared in sufficient quantities for stability studies. Each formulation was inspected for appearance and texture. All the formulations were found acceptable before storage at the three different temperatures used for stability testing. In Appendix C, the stability test evaluations done on the formulations and the results obtained, will be discussed.

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APPENDIX C

STABILITY OF COSMECEUTICALS

C.1 INTRODUCTION

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental conditions such as temperature, humidity and light (ICH Tripartite Guidelines, 2003:5). The formulation and manufacturing of a new product are not complete without an evaluation of its stability. The MCC of South Africa requires a written testing program designed to assess stability characteristics of dosage forms.

C.2 GUIDELINES FOR STABILITY TESTING

C.2.1 Selection of batches

Stability information from accelerated and long term testing is to be provided on at least two batches of the same formulation and dosage form, of the same manufacturing process. One of the two batches should be a pilot scale batch, whereas the second batch may be smaller (MCC, 2006:12). For this study, smaller batches of approximately 2000 ml were manufactured for each formulation.

C.2.2 Packaging material

The containers used in stability testing studies should be the same as, or simulate the packaging proposed for storage, distribution and marketing (ICH Tripartite Guidelines, 2003:6; MCC, 2006:12). In this study small plastic containers were used.

C.2.3 Specifications

Stability tests should be done on all attributes that can influence the quality, safety and efficacy of the product, which are likely to change during storage. Stability testing should cover physical, chemical, biological and microbiological attributes, preservative content and functionality tests (ICH Tripartite Guidelines, 2003:11). Analytical procedures should be fully validated and stability indicating (ICH Tripartite Guidelines, 2003:11).

The assay method used for the stability testing of the products in this study, was fully validated. The validation of the analytical procedure was discussed in Appendix A.

C.2.4 Testing frequency

- Long term study - the frequency of testing should be sufficient to establish the stability profile of the product. Products with a proposed shelf-life of 12 months should be tested every 3 months for the first year, every 6 months for the second year and annually thereafter (ICH Tripartite Guidelines, 2003:12).
- Intermediate study - a minimum of four time points, including the initial and final time is recommended (ICH Tripartite Guidelines, 2003:12).
- Accelerated study - a minimum of three time points, including the initial and final time is recommended (ICH Tripartite Guidelines, 2003:12).

C.2.5 Storage conditions

- Long term study - testing is done at a temperature of 25 ± 2 °C and a relative humidity (RH) of 60 ± 5% for a minimum time period of 6 months (MCC, 2006:19).
- Intermediate study - testing is done at a temperature of 30 ± 2 °C and a RH of 65 ± 5% for a minimum time period of 6 months (MCC, 2006:19).
- Accelerated study - testing is done at a temperature of 40 ± 2 °C and a RH of 75 ± 5% for a minimum time period of 6 months (MCC, 2006:19).

Exceptions that exceed these ranges for more than 24 hours should be described in the study report and their impact should be assessed. Short-term spikes due to the opening of doors of the storage facility are accepted as unavoidable (MCC, 2006:19).

Long term, intermediate and accelerated stability testing were done at time intervals of 0, 1, 2, 3 and 6 months. According to the MCC (2006:13) testing at an intermediate condition is only required where "significant change" occurs due to accelerated testing. The formulated products were stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH in different Labcon® humidity chambers. In Figure C.1 a Labcon® humidity chamber is shown.



Figure C.1: Labcon® humidity chamber

C.2.6 Guidelines for stability

According to the MCC (2006:13) "significant change" at accelerated conditions is defined as:

- 5% potency loss from the initial assay value of the active ingredient;
- Any specified degradant exceeding its specification limit;
- The product exceeding its pH limits; and
- Failure to meet specifications for appearance and physical properties for example colour, odour and phase separation.

Products containing preservatives to control microbial contamination, should have the preservative content determined initially and at intervals throughout the stability testing period of the product. This may be accomplished by performing microbial challenge tests and by performing chemical assays on the preservatives. When the minimum quantity of preservative to achieve effective microbial control has been determined for products, the chemical assays for the full period of the shelf life may be adequate to determine stability (MCC, 2009:22).

C.2.7 Expiry date

The expiry date of a product is the time during which a batch of the product is expected to remain within the approved shelf life specifications, if stored at defined conditions. It can also be defined as the date after which it should not be used (MCC, 2009:16). Long term testing should be continued at appropriate test periods for a sufficient time beyond 12 months to determine the shelf life of the product (MCC, 2006:13).

These MCC requirements were followed in the manufacturing and testing of the new formulations developed in this study. Long term, intermediate and accelerated stability studies

were done at time intervals of 0, 1, 2, 3 and 6 months. Tests are still continuing and will be done again at 12 months (long term).

C.2.8 Stability program

The following tests must be included in application for registration of new products for all dosage forms: (1) appearance; (2) assay of all actives; and (3) degradation (MCC, 2006:20).

The MCC (2006:22) also requires the following tests to be done on creams and ointments: homogeneity, pH, rheological properties, particle size and mass loss in plastic containers. Emulsions and suspensions should be tested for appearance, odour, pH and viscosity, resuspendability, particle size and the preserving ability and preservative content. The stability tests conducted on all the formulations in this study are shown in Table C.1.

Table C.1: Stability tests conducted on the four formulations

Test	Test Intervals				
	Initial	1 Month	2 Months	3 Months	6 Months
Acyclovir assay	x	x	x	x	x
Ketoconazole assay	x	x	x	x	x
Preservative assay	x	x	x	x	x
Anti-oxidant assay	x	x	x	x	x
Tocopherol assay	x	x	x	x	x
Physical assessment	x	x	x	x	x
pH	x	x	x	x	x
Viscosity	x	x	x	x	x
Mass loss	x	x	x	x	x
Particle size analysis	x	x	x	x	x

C.3 TEST METHODS

In order to ensure the accuracy of the test results, all tests were done under Good Laboratory Practice (GLP) conditions. The test methods used for the stability testing are discussed hereafter.

C.3.1 Assay with HPLC

HPLC analysis was done to determine the concentrations of acyclovir, ketoconazole, methylparaben, propylparaben, BHA, BHT and tocopherol in all the formulated products. The HPLC analysis was performed by using an Agilent 1100 Series HPLC system, equipped with an Agilent 1100 pump, UV detector and auto sampler. The HPLC system was interfaced with data acquisition and analysis software (Chemstation Rev. A.06.02) as configured for a Hewlett

Packard® computer data acquisition and analysis software. In Figure C.2 the HPLC system is shown.



Figure C.2: Agilent 1100 Series HPLC system

The full validation of the assay method is described in Annexure A. A standard solution was used as a control for the analysis. A new standard was made each month and analysed before the samples. The standard solution consisted of a solution of all analytes in methanol. In Table C.2 the amounts of each analyte that were accurately weighed are given.

Table C.2: The amount (mg) of each analyte weighed for standard preparation

Acyclovir	50.0 mg
Ketoconazole	20.0 mg
BHA	0.2 mg
BHT	2.0 mg
Methylparaben	4.0 mg
Propylparaben	0.8 mg
Tocopherol	2.0 mg

Analytes were transferred to a 100 ml volumetric flask and filled to volume with methanol. To ensure that all the analytes were completely dissolved, the standard was ultrasonified for approximately 5 min. It was filtered with a 0.45 µm syringe filter where after 1 ml of the standard solution was transferred into a HPLC vial and analysed.

The samples were prepared by weighing approximately 1 g of each formulation into a 100 ml volumetric flask, using a syringe with a tube attached to the tip. Methanol was added, and the samples were sonicated for approximately 20 min, until completely dispersed. The samples were also shaken repeatedly by hand during this period to ensure that the base was completely dispersed in the methanol. All samples were allowed to cool to room temperature and filled to

volume in the volumetric flask. It was filtered with a 0.45 µm syringe filter and transferred to a HPLC vial where after it was injected into the HPLC.

C.3.2 Physical assessment

Each stored trail batch was visually assessed initially, and at 1, 2, 3 and 6 months. Texture, colour, odour and skin feel were observed; and any change was noted. The colour was documented by taking photos and using colour charts as indicators.

C.3.3 pH

A Mettler Toledo Seven Multi pH meter, with an In Lab 410 NTC electrode 9823, was used to determine the pH of each formulation. The pH meter was calibrated with Mettler Toledo pH buffer solutions of pH 4.01, 7.00 and 10.10. Calibration was done directly prior to measurements of trail batches. Three measurements were taken on each trial batch at different temperatures and time intervals to ensure accuracy. The pH meter is shown in Figure C.3.



Figure C.3: Mettler Toledo Seven Multi pH meter

C.3.4 Viscosity

Viscosity of a fluid or semisolid may be described as its resistance to flow or movement. Rheology may be defined as the science of the flow of matter, which in pharmacy is a means of classification of fluids and semisolids (Marriott, 2002:41).

The viscosity of the different formulations was determined on a Brookfield Model DV - II+ viscometer. The direct sample temperature was controlled by a Brookfield circulating water bath with a temperature controller. A Helipath spindle set (T-bar spindle and chuck) was used to measure viscosity. A Helipath D20733 was used to move the viscometer up and down in the cream or emulgel. The spindle and temperature used for each formulation is shown in Table C.3.

Table C.3: Viscosity parameters

Formula	Spindle	Entry code	Rpm	Temperature
Cream	T-F	96	0.3 - 0.6	35 °C
Pheroid™ cream	T-F	96	0.3 - 0.6	25 °C
Emulgel	T-F	96	1.5	25 °C
Pheroid™ emulgel	T-F	96	1.5	25 °C

Viscosity of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel were determined initially, at 1, 2, 3 and 6 months in glass containers, containing 100 ml of the cream or emulgel. The viscosity was only determined on the batches stored at a stability temperature of 25 °C/60% RH. Viscosity readings were measured for 5 min, during which time a total of 32 viscosity readings were taken every 10 s for each formulation. For measurement at 0, 1, 2 and 3 months, the same formula was measured. At 6 months the same formula was measured, but in another container. The Brookfield Model DV - II+ viscometer, Brookfield circulating water bath with a temperature controller and the Helipath D20733 are shown in Figure C.4.



Figure C.4: The Brookfield Model DV - II+ viscometer, Brookfield circulating water bath with temperature controller, Helipath D20733 and the Helipath spindle set

C.3.5 Mass loss

The trial batches in their individual containers were weighed initially. The same containers were weighed again at 1, 2, 3 and 6 months to determine if a loss in mass occurred. All containers were weighed three times at each time interval to ensure accuracy. The scale, a Shimadzu AUW 120 D, was calibrated on a daily basis and is shown in Figure C.5.

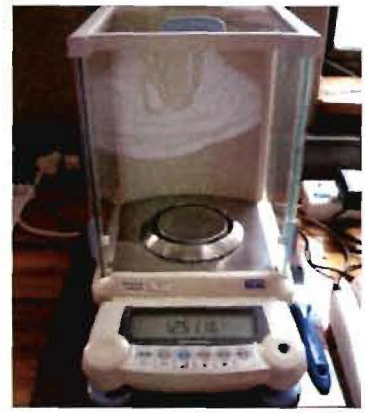


Figure C.5: The Shimadzu AUW 120 D scale

C.3.6 Particle size observation

The particle size was observed with a CLSM Nikon PCM 2000 with digital camera DMX 1200, with a He/Ne laser-543 nm and a Argon ion laser, with 457 - 517 nm as well as an 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 with 457 - 514 nm. Micrographs of the formulations were taken at 0, 1, 2, 3 and 6 months. The two CLSM's are shown in Figure C.6.

(a) 

(b) 

Figure C.6: The CLSM (a) Nikon D-eclipse C1 si and (b) Nikon PCM 2000

C.4 RESULTS AND DISCUSSION

The outcomes of the test procedures, described in Section C.3, are shown and discussed in the following section.

C.4.1 Assay with HPLC

The concentrations of acyclovir, ketoconazole, methylparaben, propylparaben, BHA, BHT and tocopherol in all the formulated products were determined with HPLC analysis. The assay was

done initially, and at 1, 2, 3 and 6 months. In this study microbial challenge tests were not done to determine the minimum amount of preservative needed. The assay of the preservatives was done, but according to the MCC (2009:22) this is not sufficient to determine stability.

C.4.1.1 Cream

The assay values of the analytes in cream are given in Table C.4.

Table C.4: The % values of the analytes in cream

Ingredient	Temperature	Month				
Values given are %		0	1	2	3	6
Acyclovir	25 °C	108.96	108.88	106.71	104.85	103.96
	30 °C	-	105.52	105.80	105.54	105.10
	40 °C	-	104.62	104.93	102.84	102.09
Ketoconazole	25 °C	102.87	102.18	102.29	102.54	102.60
	30 °C	-	102.39	102.46	102.84	102.68
	40 °C	-	102.07	102.01	102.75	102.16
Methylparaben	25 °C	108.93	106.72	105.49	106.84	105.59
	30 °C	-	108.40	107.44	107.27	106.94
	40 °C	-	108.30	108.18	107.70	107.46
Propylparaben	25 °C	102.21	101.39	100.55	98.06	99.41
	30 °C	-	101.29	100.96	99.99	99.37
	40 °C	-	101.24	101.45	99.27	99.17
BHA	25 °C	109.28	108.22	105.39	90.44	55.56
	30 °C	-	105.45	102.56	92.18	54.89
	40 °C	-	103.07	100.22	69.58	53.86
BHT	25 °C	99.67	98.35	97.59	97.29	96.31
	30 °C	-	98.35	97.49	97.13	96.50
	40 °C	-	97.16	97.47	97.36	95.98

The concentration of the active ingredient, acyclovir, in the cream formulation decreased with less than 5.00% at 25 and 30 °C. A significant change was seen at a temperature of 40 °C, with a decrease in concentration of 6.87%. The concentration of the active ingredient, ketoconazole, in the cream formulation decreased with less than 5.00% at 25, 30 and 40 °C. Preservatives and BHT depicted a decline in concentration of less than 5.00% over the 6 month period. BHA concentration decreased significantly. A 53.72, 54.39 and 55.42% decrease in BHA's initial concentration was detected for 25, 30 and 40 °C, respectively. This significant decrease in the anti-oxidant was due to its protection of the active ingredients against oxidation. A significant change in acyclovir concentration at 40 °C was observed and this was an indication of instability of the cream.

C.4.1.2 Pheroid™ Cream

The assay values of the analytes in the Pheroid™ cream are given in Table C.5.

Table C.5: The % values of the analytes in Pheroid™ cream

Ingredient	Temperature	Month				
Values given are %		0	1	2	3	6
Acyclovir	25 °C	109.33	109.41	107.82	107.08	106.52
	30 °C	-	108.67	107.07	103.81	102.83
	40 °C	-	108.00	105.37	97.53	95.90
Ketoconazole	25 °C	106.54	103.99	103.76	102.98	102.47
	30 °C	-	102.50	102.76	99.57	98.86
	40 °C	-	100.86	100.34	98.02	93.11
Methylparaben	25 °C	109.12	108.41	106.92	106.72	106.41
	30 °C	-	106.13	105.74	105.43	103.03
	40 °C	-	105.22	104.59	104.60	104.39
Propylparaben	25 °C	110.25	109.34	108.47	107.97	107.29
	30 °C	-	98.17	98.57	95.37	95.20
	40 °C	-	99.37	99.96	98.16	98.03
BHA	25°C	107.89	107.62	104.49	105.9	103.29
	30°C	-	106.41	103.27	102.75	102.65
	40°C	-	106.18	105.38	104.21	103.39
BHT	25 °C	87.68	74.64	68.50	58.83	57.25
	30 °C	-	68.88	58.21	56.74	54.96
	40 °C	-	67.49	66.05	60.11	57.06
Tocopherol	25 °C	100.84	97.99	78.04	68.99	64.39
	30 °C	-	92.62	70.88	60.55	59.09
	40 °C	-	86.35	66.38	55.73	34.60

Acyclovir was stable in the Pheroid™ cream at 25 °C with a decrease in concentration of less than 5.00%. A significant change in the acyclovir concentration occurred at 30 and 40 °C, with a decrease in concentration of 6.50 and 13.43%, respectively. A significant change in ketoconazole concentration occurred at a temperature of 30 and 40 °C, with a decrease of more than 5.00%. The concentration of ketoconazole decreased with less than 5.00% at 25 °C.

The preservative, methylparaben, and antioxidant, BHT, depicted a slight decrease in concentration over the 6 month period, with a less than 5.00% decrease at 25, 30 and 40 °C. The concentration of propylparaben decreased significantly at 30 and 40 °C, but was less than 5.00% at 25 °C. Although a decrease of more than 5.00% in the preservative concentration occurred, no microbial growth was noticed during physical assessment. A significant decrease

of more than 5.00% in BHA and tocopherol's concentrations over the 6 months period was observed at 25, 30 and 40 °C. The decrease in the concentrations may be due to the anti-oxidants protection of the active ingredients against oxidation. A significant change in acyclovir and ketoconazole concentration at 30 and 40 °C occurred, this was an indication of instability in the Pheroid™ cream.

C.4.1.3 Emulgel

The assay values of the analytes in emulgel are given in Table C.6.

Table C.6: The % values of the analytes in emulgel

Ingredient	Temperature	Month				
Values given are %		0	1	2	3	6
Acyclovir	25 °C	107.94	107.01	107.04	105.23	104.20
	30 °C	-	106.79	105.95	99.74	98.31
	40 °C	-	102.40	101.71	86.32	81.63
Ketoconazole	25 °C	106.10	105.87	105.39	105.15	104.48
	30 °C	-	105.23	106.28	105.28	104.99
	40 °C	-	106.71	104.44	103.43	102.12
Methylparaben	25 °C	106.88	106.20	106.80	105.65	104.17
	30 °C	-	105.23	104.57	104.13	103.68
	40 °C	-	105.87	104.81	103.66	103.21
Propylparaben	25 °C	106.05	105.29	104.45	104.35	103.90
	30 °C	-	105.16	103.27	102.96	102.07
	40 °C	-	103.50	101.60	101.44	101.30
BHA	25 °C	106.35	106.19	105.20	92.07	80.88
	30 °C	-	105.38	103.06	95.39	78.28
	40 °C	-	104.00	100.96	94.76	80.59
BHT	25 °C	103.46	103.18	103.05	102.84	102.16
	30 °C	-	102.14	101.11	100.93	100.01
	40 °C	-	99.42	94.00	93.30	91.14

Acyclovir was stable in the emulgel at 25 °C with a decrease in concentration of less than 5.00%. However a significant change in acyclovir concentration was observed at higher storage temperatures after 2 months of stability testing. At storage temperatures of 30 and 40 °C, acyclovir concentration decreased with 9.63% and 26.31%, respectively over the 6 month period. No significant change in ketoconazole concentration occurred. The preservatives depicted no significant change in concentration during stability testing. Antioxidant, BHT depicted a slight decrease in concentration over the 6 month period with a less than 5.00% decrease at 25 and 30 °C. At 40 °C, a decrease in BHT concentration of 15.21% was

observed. BHA's concentration decreased significantly at 25, 30 and 40 °C. The decrease in the anti-oxidants concentrations may be due to their protection of the active ingredients against oxidation. The significant change in acyclovir concentration is an indication of an unstable product.

C.4.1.4 Pheroid™ Emulgel

The assay values of the analytes in Pheroid™ emulgel are presented in Table C.7.

Table C.7: The assay of the analytes in Pheroid™ emulgel

Ingredient	Temperature	Month				
Values given are %		0	1	2	3	6
Acyclovir	25 °C	100.62	98.79	90.83	82.16	81.15
	30 °C	-	94.95	91.39	77.63	75.18
	40 °C	-	95.58	86.59	59.17	59.03
Ketoconazole	25 °C	102.77	101.14	101.86	98.88	95.71
	30 °C	-	101.34	102.22	98.11	95.23
	40 °C	-	100.28	99.94	87.11	96.35
Methylparaben	25 °C	104.45	103.26	102.94	102.36	101.70
	30 °C	-	102.61	102.21	101.89	101.78
	40 °C	-	101.79	104.28	103.86	102.38
Propylparaben	25 °C	102.01	101.32	101.32	101.29	101.15
	30 °C	-	100.99	99.57	98.34	98.21
	40 °C	-	99.91	96.38	95.98	95.41
BHA	25 °C	108.31	107.85	101.33	101.25	101.18
	30 °C	-	108.14	102.19	101.36	100.21
	40 °C	-	101.11	97.94	81.93	78.61
BHT	25 °C	96.58	76.90	75.00	73.27	70.47
	30 °C	-	79.12	76.89	70.89	66.78
	40 °C	-	69.74	67.07	61.55	56.42
Tocopherol	25 °C	98.15	83.41	65.54	36.12	31.72
	30 °C	-	82.54	57.31	46.77	26.86
	40 °C	-	70.92	55.67	42.86	15.78

The concentration of acyclovir decreased significantly in the Pheroid™ emulgel formulation at 25, 30 and 40 °C over the period of stability testing. A significant change in ketoconazole concentration was observed over the 6 month period of stability testing at 25, 30 and 40 °C. Acyclovir and ketoconazole were both unstable in the Pheroid™ emulgel formulation. The preservatives, methylparaben and propylparaben depicted no significant change in concentration during the stability testing at 25 and 30 °C. A significant change occurred in the

propylparaben concentration at a temperature of 40 °C. Although a decrease of more than 5.00% in the propylparaben concentration occurred, no microbial growth was observed during physical assessment. The antioxidants; BHT, BHA and tocopherol depicted a significant decrease in concentration over the 6 month period, with a decrease of 5.00% or more at 25, 30 and 40 °C. The decrease in the concentrations may be due to the anti-oxidants protection of the active ingredients against oxidation. The significant change in concentration of acyclovir and ketoconazole is an indication of an unstable product.

C.4.2 Physical assessment

The physical assessments conducted on the different formulations are shown in Table C.8, Table C.9, Table C.10 and Table C.11.

C.4.2.1 Cream

The physical assessment of the cream formula is shown in Table C.8. A slight change in colour of the cream stored at 40 °C occurred after the first month of storage. The change in colour at 40 °C is shown in Figure C.7.

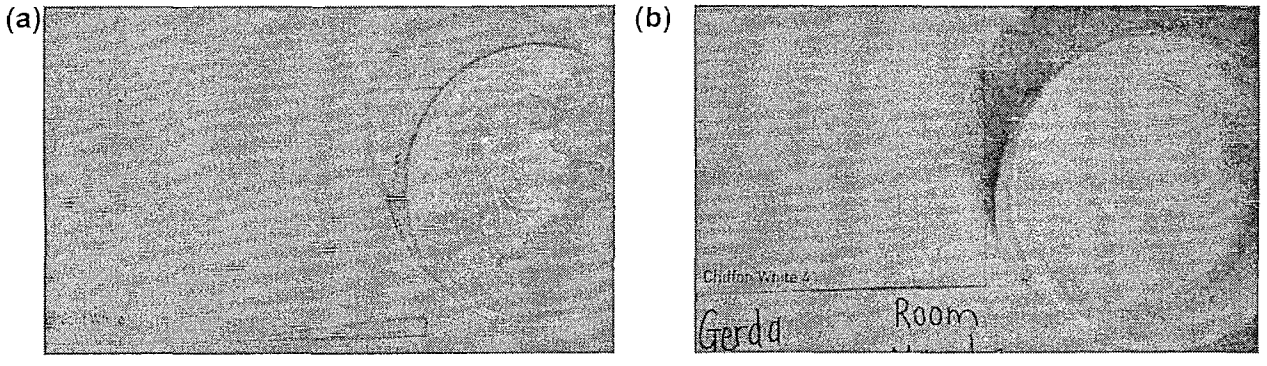


Figure C.7: Colour change of cream formula from (a) 0 months to (b) 1 month at 40 °C

Table C.8: Physical assessment of the cream formula

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Colour: Brilliant white	No change	No change	No change	No change
	Odour: odourless	No change	No change	No change	No change
	Fluent and smooth	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
	Moisturizing	No change	No change	No change	No change
30 ± 2 °C 60 ± 5% RH	Colour: Brilliant white	No change	No change	No change	No change
	Odour: odourless	No change	No change	No change	No change
	Fluent and smooth	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
	Moisturizing	No change	No change	No change	No change
40 ± 2 °C 75 ± 5% RH	Colour: Brilliant white	Colour change to chiffon white	No change	No change	No change
	Odour: odourless	No change	No change	No change	No change
	Fluent and smooth	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
	Moisturizing	No change	No change	No change	No change

No other changes than that of 0 - 1 months at 40 °C were observed with the physical assessment of the cream formula.

C.4.2.2 Pheroid™ cream

A slight change in colour of the Pheroid™ cream stored at 25 °C occurred after the first month of storage and also during the first and the second month. The colour changed from white to soft yellow, to light yellow over the aforementioned stability period as can be seen in Figure C.8. Figure C.9 illustrates a more defined colour change in the Pheroid™ cream stored at 30 °C. The colour changed from bright white to light yellow to bright yellow over the 6 month period. The Pheroid™ cream stored at 40 °C also depicted a change in colour, from white to bright yellow to a darker yellow, which can be observed in Figure C.10. This significant colour change may be due to oxidation of tocopherol and BHT. No other changes were noticed.

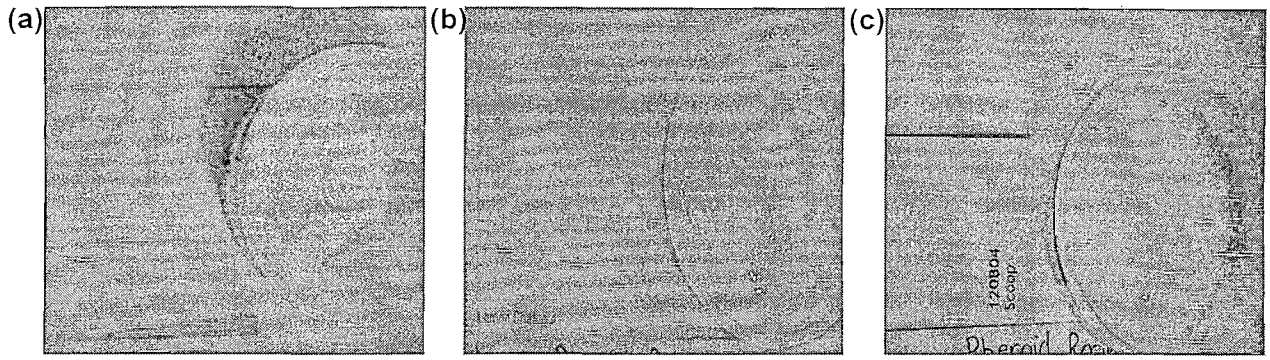


Figure C.8: Colour change of Pheroid™ cream formula from (a) 0 months, (b) 1 month and (c) 2 months at 25 °C

Table C.9: Physical assessment of the Pheroid™ cream formula

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Colour: Brilliant white	Colour change to a soft yellow, Lemmon chiffon	Light yellow, Scoop	No change	No change
	Odour: Distinctive "fish-oil" Pheroid™ smell,	No change	No change	No change	No change
	Fluent and smooth	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
	Moisturizing	No change	No change	No change	No change
30 ± 2 °C 60 ± 5% RH	Colour: Brilliant white	Colour: light yellow, Scoop	Colour: yellow, Miranda's call	No change	No change
	Odour: Distinctive "fish-oil" Pheroid™ smell,	No change	No change	No change	No change
	Fluent and smooth	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
	Moisturizing	No change	No change	No change	No change
40 ± 2 °C 75 ± 5% RH	Colour: Brilliant white	Colour: bright yellow, Yellow Jubilee	No change	No change	Colour: dark yellow, Full moon
	Odour: Distinctive "fish-oil" Pheroid™ smell,	No change	No change	No change	No change
	Fluent and Smooth	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
	Moisturizing	No change	No change	No change	No change

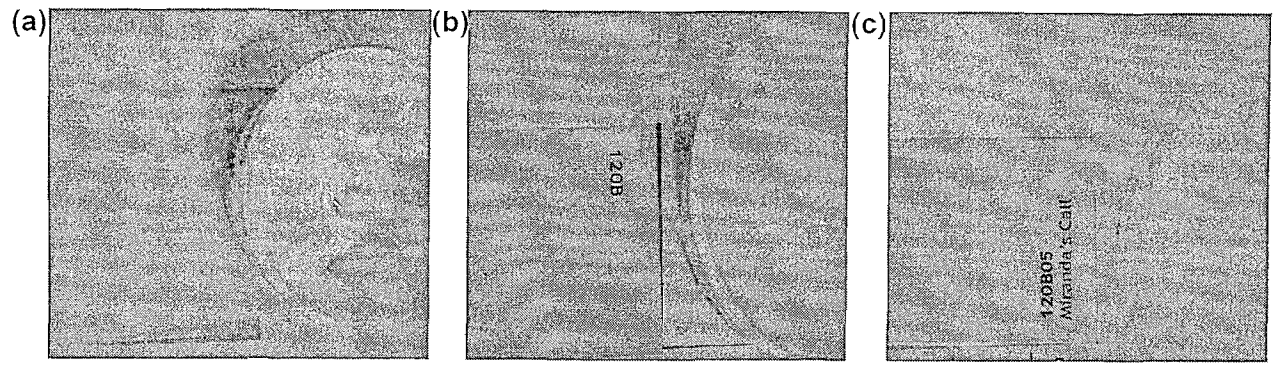


Figure C.9: Colour change of Pheroid™ cream formula from (a) 0 months, (b) 1 month and (c) 2 months at 30 °C

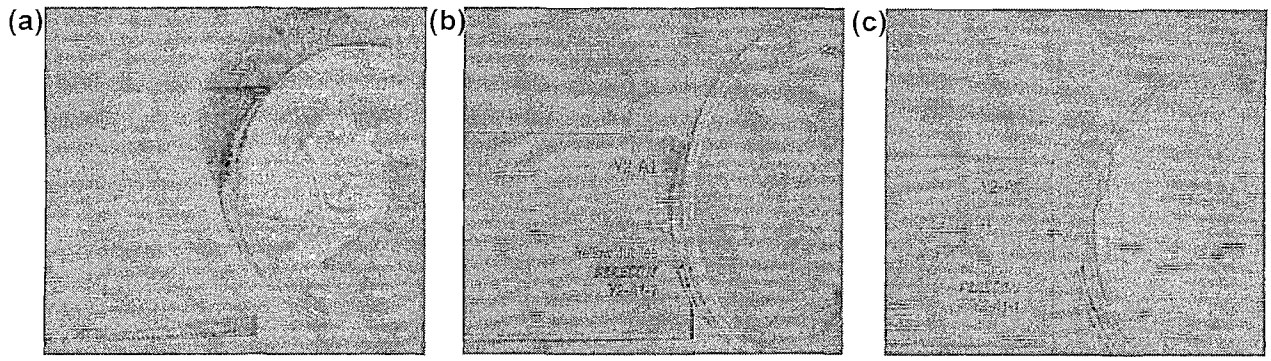


Figure C.10: Colour change of Pheroid™ cream formula from (a) 0 months, (b) 1 month and (c) 2 months at 40 °C

C.4.2.3 Emulgel

Table C.10: Physical assessment of the emulgel formula

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Colour: Luxurious silk timeless	No change	No change	No change	No change
	Odour: Odourless	No change	No change	No change	No change
	Fluent, smooth and cool	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too hydrous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
30 ± 2 °C 60 ± 5% RH	Colour: Luxurious silk timeless	No change	No change	No change	No change
	Odour: Odourless	No change	No change	No change	No change
	Fluent, smooth and cool	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too hydrous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
40 ± 2 °C 75 ± 5% RH	Colour: Luxurious silk timeless	No change	No change	No change	No change
	Odour: Odourless	No change	No change	No change	No change
	Fluent, smooth and cool	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too hydrous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change

No physical changes were noticed in any of the emulgel formulae at 25, 30 and 40 °C over the stability test period.

C.4.2.4 Pheroid™ emulgel

A change in colour of the Pheroid™ emulgel stored at 25, 30 and 40 °C occurred. At 25 and 30 °C the colour changed from a cream-colour to light yellow and again to a bright yellow as illustrated in Figure C.11. The colour change in the Pheroid™ emulgel stored at 40 °C was from a cream-colour to a bright yellow to a dark yellow as observed in Figure C.12. These colour changes may be due to the oxidation of BHT and tocopherol. No other changes were observed.

Table C.11: Physical assessment of the Pheroid™ emulgel formula

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Colour: Luxurious silk timeless	Colour change to light yellow, Scoop	Colour change to yellow, Miranda's call	No change	No change
	Odour: Distinctive "fish-oil" Pheroid™ smell,	No change	No change	No change	No change
	Fluent, smooth and cool	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too hydrous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change
30 ± 2 °C 60 ± 5% RH	Colour: Luxurious silk timeless	Colour change to light yellow, Scoop	Colour change to yellow, Miranda's call	No change	No change
	Odour: Distinctive "fish-oil" Pheroid™ smell,	No change	No change	No change	No change
	Fluent, smooth and cool	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too hydrous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	
40 ± 2 °C 75 ± 5% RH	Colour: Luxurious silk timeless	Colour yellow, Miranda's call	Colour dark yellow, Full Moon	No change	No change
	Odour: Distinctive "fish-oil" Pheroid™ smell,	No change	No change	No change	No change
	Fluent, smooth and cool	No change	No change	No change	No change
	Applies easily	No change	No change	No change	No change
	Homogenous	No change	No change	No change	No change
	Not too hydrous	No change	No change	No change	No change
	Not too oily	No change	No change	No change	No change

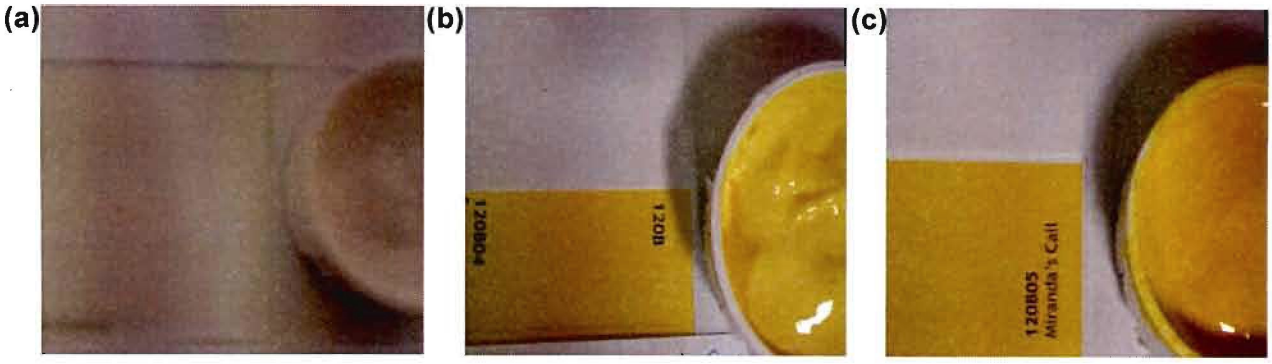


Figure C.11: Representative colour change at 25 and 30 °C of the Pheroid™ emulgel formula from (a) 0 months, (b) 1 month and (c) 2 months

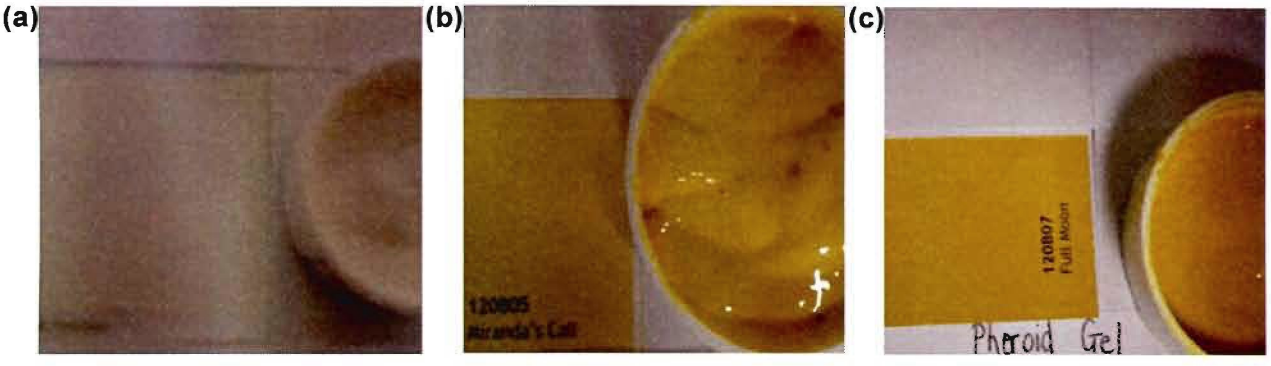


Figure C.12: Colour change of Pheroid™ emulgel formula from (a) 0 months, (b) 1 month and (c) 2 months at 40 °C

C.4.3 pH

The pH of a formulation may influence the stability of the actives in the formulation. Acyclovir is practically stable at a neutral pH and very stable in alkaline conditions. According to Skiba *et al.* (2000:1) ketoconazole was least stable at pH of 1 and most stable at a pH of 9. Human skin is also sensitive to extreme pH ranges. The stratum corneum is extremely resistant to changes in pH, tolerating a pH range of 3 - 9 (Barry, 2002:511). The pH of all formulations were between 5 and 6.

C.4.3.1 Cream

The pH at a temperature of 25 °C decreased slightly with ± 0.4 for the cream formulation, which is again an indication of excellent stability. Although the pH decreased at higher temperatures, the pH range was still between 5 and 6. A decrease in pH is indicative of decrease in the stability of acyclovir and ketoconazole. According to this data maximum stability of the cream will be ensured for a longer period if it is stored at a temperature below 25 °C. The results of pH measurement of the cream formulae are given in Table C.12.

Table C.12: The pH of the cream formulation measured over 6 months

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	6.58	6.25	6.28	6.18	6.15
30 ± 2 °C 60 ± 5% RH	–	6.27	6.09	6.00	5.89
40 ± 2 °C 75 ± 5% RH	–	5.90	5.64	5.49	5.32

C.4.3.2 Pheroid™ cream

The results of the pH measurement of the Pheroid™ cream formulation are given in Table C.13.

Table C.13: The pH of the Pheroid™ cream formulation measured over 6 months

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	6.65	6.77	6.64	6.35	6.18
30 ± 2 °C 60 ± 5% RH	–	6.82	6.45	6.18	5.80
40 ± 2 °C 75 ± 5% RH	–	6.13	5.80	5.53	5.21

The pH at a temperature of 25 °C decreased slightly with ± 0.4 for the Pheroid™ cream formulation; this shows excellent stability. The pH showed a decrease at higher temperatures, but the pH range was still between 5 and 6. A decrease in pH is indicative of a decrease in the stability of acyclovir and ketoconazole. It appears that maximum stability of the Pheroid™ cream will be guaranteed for a longer period if it is stored at a temperature below 25 °C.

C.4.3.3 Emulgel

The pH of the emulgel formulation decreased by approximately 0.7, at a temperature of 25 °C over a period of 6 months. The slight decrease is an illustration of good stability of the emulgel. Although the pH decreased at higher temperatures, the pH range was still between 5 and 6. A decrease in pH also decreases the stability of acyclovir and ketoconazole. According to this data maximum stability of the emulgel will be guaranteed for a longer period if it is stored at a temperature below 25 °C. The results of the pH measurement of the emulgel formulation are given in Table C.14.

Table C.14: The pH of the emulgel formulation measured over 6 months

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	6.65	6.22	6.07	5.97	5.91
30 ± 2 °C 60 ± 5% RH	–	6.23	5.90	5.80	5.70
40 ± 2 °C 75 ± 5% RH	–	5.96	5.57	5.40	5.27

C.4.3.4 Pheroid™ emulgel

The results of the pH measurement of the Pheroid™ emulgel formulation are given in Table C.15.

Table C.15: The pH of the Pheroid™ emulgel measured over 6 months

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	6.80	6.69	6.44	6.29	5.84
30 ± 2 °C 60 ± 5% RH	–	6.56	6.32	5.96	5.43
40 ± 2 °C 75 ± 5% RH	–	6.27	5.53	5.36	5.08

The pH at a temperature of 25 °C decreased with ± 0.9 over a period of 6 months for the Pheroid™ emulgel formulation. Change in the pH was more significant at the higher temperatures of 30 and 40 °C, but the pH range was still between 5 and 6. A decrease in pH also decreases the stability of acyclovir and ketoconazole. According to this data maximum stability of the Pheroid™ emulgel will be guaranteed for a longer period if it is stored at a temperature below 25 °C.

C.4.4 Viscosity

Viscosity of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel were determined initially, at 1, 2, 3 and 6 months. The same container of each formulation was used for the measurements at 0, 1, 2 and 3 months. Another container with the same formulated batches was used for the viscosity measurement at 6 months. The results are given in Table C.16 to Table C.19.

C.4.4.1 Cream

The results of the viscosity measurement of the cream formulation measured over a period of 6 months are given in Table C.16.

Table C.16: The viscosity measurements (cP) of the cream

Storage temperature		Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Mean	1644292.0	914697.4	511646.8	456862.7	1298218.3
	SD	175228.5	119040.3	132220.3	61303.4	112525.4
	%RSD	10.7	13.0	25.8	13.4	8.7

Viscosity of the cream formulation decreased slightly over the 6 months. Comparing the initial viscosity to that at 6 months, the viscosity decreased with approximately 350000 cP, which is an indication of a stable formula. The decrease in viscosity may be explained as a reaction between some ingredients, or it may be due to the fact that the cream was still settling before it went into the final resting stage. The viscosity at month 1, 2 and 3 were significantly lower than at 6 months. This immense decrease may be because of broken van der Waals forces between the molecules that occurred during the previous viscosity measurements on the same cream.

C.4.4.2 Pheroid™ cream

The results of the viscosity measurement of the Pheroid™ cream formulation measured over 6 months are given in Table C.17.

Table C.17: The viscosity measurements (cP) of the Pheroid™ cream

Storage temperature		Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Mean	829595.7	452026.9	189751.2	209580.3	430533.1
	SD	119890.7	71582.7	24367.6	34983.3	76353.1
	%RSD	14.5	15.8	12.8	16.7	17.7

The viscosity of the Pheroid™ cream formulation decreased slightly over the 6 month period. Comparing the initial viscosity with that at 6 months, the viscosity decreased to about half of the initial measurement. The decrease in viscosity can be explained as a reaction between some ingredients, or it may be due to the fact that the cream was still settling before it went into the final resting stage. Viscosity at month 2 and 3 was significantly lower than the viscosity at 6 months. This immense decrease may be due to broken van der Waals forces between the molecules that occurred during the previous viscosity measurements on the same cream. The large decrease in viscosity is an indication of possible instability.

C.4.4.3 Emulgel

The results of the viscosity measurement of the emulgel formulation over the 6 month period are given in Table C.18.

Table C.18: The viscosity measurements (cP) of the emulgel

Storage temperature		Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Mean	102985.8	47831.6	99080.4	94159.6	89902.7
	SD	9870.7	3673.1	11767.1	9382.7	6281.3
	%RSD	9.6	7.7	11.9	10.0	7.0

The emulgel formulation depicted a slight decrease in viscosity over the 6 month peroid. Comparing the initial viscosity with that at 6 months, the viscosity decreased with approximately 10000 cP, which is an indication of a stable emulgel formula. The decrease in viscosity in the first month can be explained as a reaction between some ingredients, or it may be due to the fact that the emulgel was still settling before it went into the final resting stage. At month 2 and 3 an increase in viscosity was observed. It seems that the emulgel was not influenced by previous viscosity measurements on the same emulgel.

C.4.4.4 Pheroid™ emulgel

The results of the viscosity measurement of the Pheroid™ emulgel formulation over the 6 month period are given in Table C.19.

Table C.19: The viscosity measurements (cP) of the Pheroid™ emulgel

Storage temperature		Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	Mean	119896.3	58298.1	118705.1	112065.9	114253.0
	SD	12307.2	6644.7	17053.7	12622.3	8562.2
	%RSD	10.3	11.4	14.4	11.3	7.5

As with the emulgel formulation, the Pheroid™ emulgel depicted a slight decrease in viscosity over the 6 month period. Comparing the initial viscosity with the viscosity at 6 months, the viscosity decreased with approximately 5000 cP, which is an indication of a very stable formula. The decrease in viscosity in the first month can be explained as a reaction between some ingredients, or it may be that the emulgel was still settling before it went into the final resting stage. The viscosity at month 2 and 3 increased again. It seems that the Pheroid™ emulgel was not influenced by previous viscosity measurements on the same emulgel.

C.4.5 Mass loss

The mass loss of a formula is an indication of water evaporating during the continued exposure of the formula to high temperatures. Mass loss measurements of the formulated products are given in Table C.20 to Table C.23.

C.4.5.1 Cream

Table C.20: The mass loss of the cream formulation

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	31.00 g	30.99 g	30.98 g	30.98 g	30.95 g
30 ± 2 °C 60 ± 5% RH	31.03 g	30.99 g	30.94 g	30.92 g	30.81 g
40 ± 2 °C 75 ± 5% RH	30.95 g	30.90 g	30.87 g	30.85 g	30.80 g

The mass loss of the cream formula was small and no significant changes occurred at 25, 30 and 40 °C; this is indicative of a stable product and a suitable container.

C.4.5.2 Pheroid™ cream

Table C.21: The mass loss of the Pheroid™ cream formulation

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	30.91 g	30.93 g	30.84 g	30.80 g	30.63 g
30 ± 2 °C 60 ± 5% RH	30.95 g	30.93 g	30.90 g	30.89 g	30.82 g
40 ± 2 °C 75 ± 5% RH	30.89 g	30.86 g	30.85 g	30.84g	30.76 g

The mass loss of the Pheroid™ cream formula was small and no significant changes occurred at 25, 30 and 40 °C; this is indicative of a stable product and a suitable container.

C.4.5.3 Emulgel

Table C.22: The mass loss of the emulgel formulation

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	31.00 g	30.99 g	30.96 g	30.95 g	30.91 g
30 ± 2 °C 60 ± 5% RH	30.99 g	30.95 g	30.91 g	30.89 g	30.77 g
40 ± 2 °C 75 ± 5% RH	30.98 g	30.83 g	30.76 g	30.71 g	30.57 g

The mass loss of the emulgel formula was small and no significant changes occurred at 25, 30 and 40 °C; this is indicative of a stable product and a suitable container.

C.4.5.4 Pheroid™ emulgel

Table C.23: The mass loss of the Pheroid™ emulgel formulation

Storage temperature	Initial	1 Month	2 Months	3 Months	6 Months
25 ± 2 °C 60 ± 5% RH	30.88 g	30.87 g	30.86 g	30.85 g	30.82 g
30 ± 2 °C 60 ± 5% RH	30.97 g	30.93 g	30.88 g	30.83 g	30.85 g
40 ± 2 °C 75 ± 5% RH	31.18 g	31.13 g	31.12 g	31.11 g	31.07 g

The mass loss of the Pheroid™ emulgel formula was small and no significant changes occurred at 25, 30 and 40 °C; this is indicative of a stable product and a suitable container.

C.4.6 Particle size observation

The particle size was observed by taking micrographs of the formulations at 0, 1, 2, 3 and 6 months. Exact sizes could not be determined, but by using the scale, an increase or decrease in particle size could be observed. A representation of the particle size of each formula is shown in Figure C.13 to Figure C.16. The CLSM micrographs shown in Figure C.13 to C.16 were taken initially and at 6 months.

C.4.6.1 Cream

(a) (b)

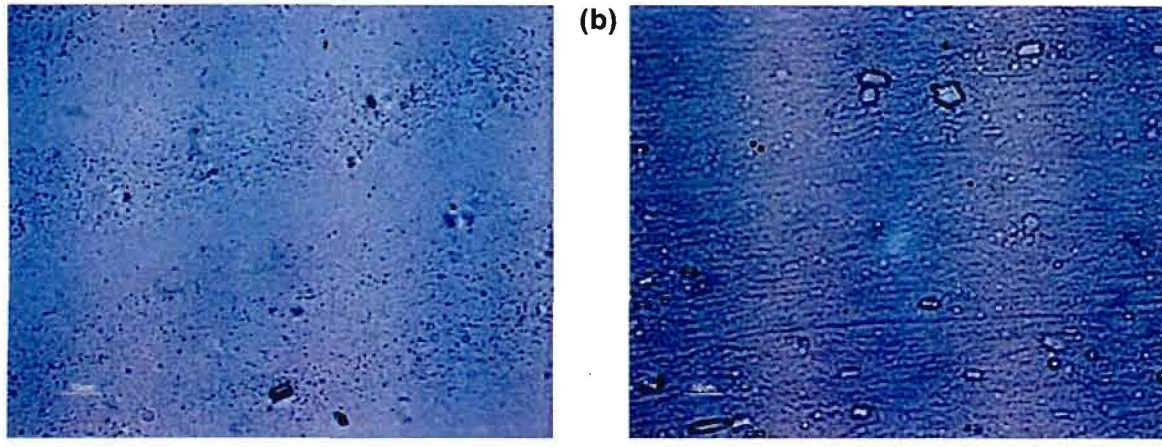


Figure C.13: A representation of the particle size at all three temperatures of the cream formula at (a) 0 months and (b) 6 months

The particle size of the cream did not change significantly over the 6 month period. The size of the acyclovir crystals increased, but not significantly. When observing the particle and crystal size, the cream formulation seemed to be stable.

C.4.6.2 Pheroid™ cream

(a) (b)

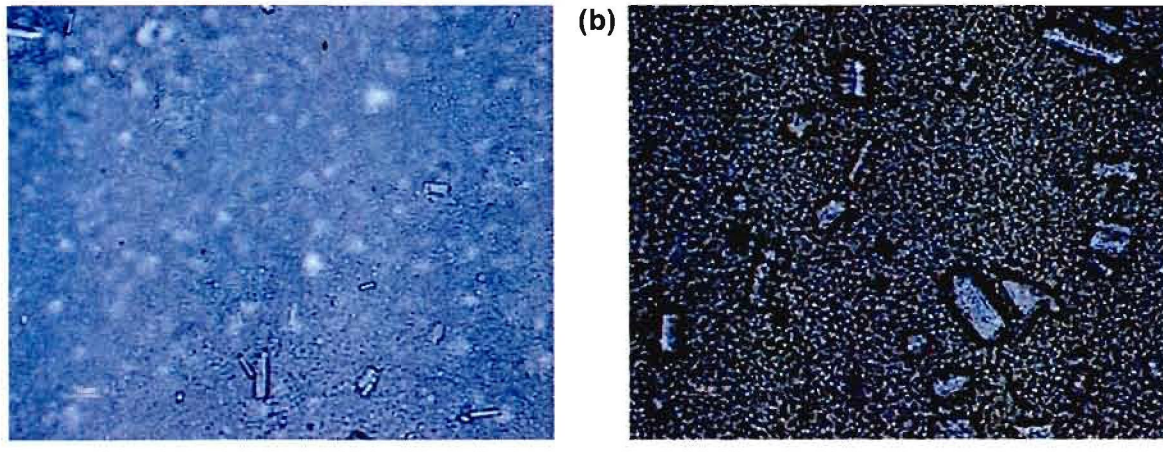


Figure C.14: A representation of the particle size at all three temperatures of the Pheroid™ cream formula at (a) 0 months and (b) 6 months

The particle size of the Pheroid™ cream did not change significantly over the 6 month period. The size of the acyclovir crystals, however, increased and this increase was significant at 25 and 30 °C, but not at 40 °C. Considering the particle and crystal size at 25, 30 and 40 °C, the Pheroid™ cream formulation appeared to be unstable.

C.4.6.3 Emulgel

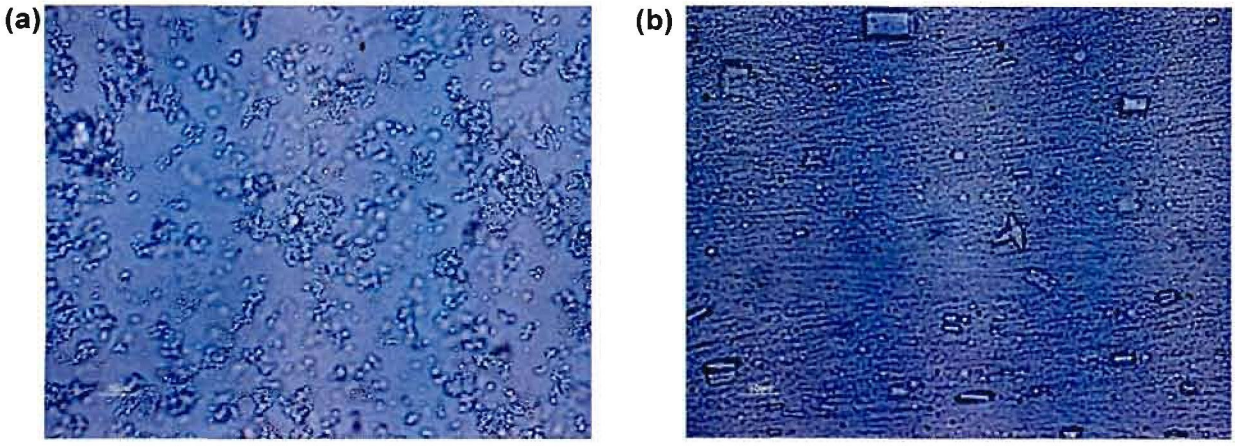


Figure C.15: A representation of the particle size at all three temperatures of the emulgel formula at (a) 0 months and (b) 6 months

The particle size of the emulgel did not change significantly over the 6 month period. The size of the acyclovir crystals increased, but not drastically. When observing the micrographs of the particle and crystal size, the emulgel formulation appeared to be stable.

C.4.6.4 Pheroid™ emulgel

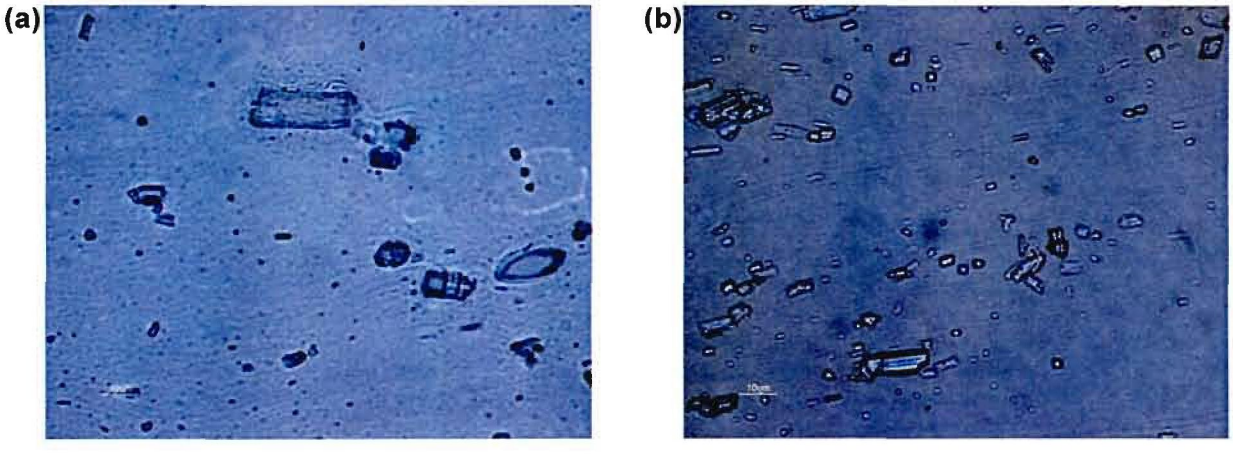


Figure C.16: A representation of the particle size at all three temperatures of the Pheroid™ emulgel formula at (a) 0 months and (b) 6 months

The particle size of the Pheroid™ emulgel did not change significantly over the period of 6 months. The size of the acyclovir crystals increased, but this was not significant. Considering the particle size, the Pheroid™ emulgel formulation appeared to be stable.

C.5 CONCLUSION

The formulated products were tested for stability over a period of 6 months. The outcomes of the stability tests showed that the cream and emulgel formulae were more stable than the Pheroid™ formulae. None of the four formulations were completely stable for all the active ingredients over the 6 months. According to the chemical assay the cream formula was the most stable formula. Significant changes in the colour of the Pheroid™ formulae were noted with the physical assessment. No significant changes in pH or mass loss were documented. The Pheroid™ cream formulation depicted a severe decrease in viscosity, which was indicative of an unstable product. Particle size analysis showed significant changes in the crystal size of the Pheroid™ cream formulation, which again was a warning sign for instability of the product. None of the formulations met the MCC's criteria for stability.

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APPENDIX D

DIFFUSION STUDIES

D.1 INTRODUCTION

Skin penetration studies play an essential role in the optimisation of drug and formulation design in dermal and transdermal delivery. *In vitro* permeation studies (used to indicate the transdermal delivery of active ingredient) and tape stripping techniques (used to determine the dermal delivery of the active) are, therefore, highly important (Leveque, 2004:323). The Franz type diffusion cell is widely used, because of its reproducibility and the fact that it is less time consuming than other methods. This method has also been used to assess drug release from semi-solid dosage forms (Chattaraj, 1995:120). The use of *in vitro* methods to determine if the active ingredient is released from a semi-solid, is increasingly gaining attention from the pharmaceutical industry and regulatory authorities. No compendium method exists for determining the *in vitro* release of a drug from topical products such as creams, gels and ointments (Chattaraj, 1995:119). In this study, the Franz cell diffusion and tape stripping methods were used to determine the release, transdermal and dermal delivery of the active ingredients.

D.2 METHODS

The methods used during the diffusion studies of acyclovir and ketoconazole will be discussed in this section.

D.2.1 Skin preparation

In order to decrease the variability in skin permeability properties, the permeation studies were exclusively performed on abdominal caucasian female skin. The excised human skin was obtained from patients who had undergone abdominal plastic surgery (Leveque, 2004:234). Informed consent was given by all of the patients before surgery and the identities of all donors were kept confidential. The Research Ethics Committee of the North-West University granted ethical approval for the procurement and utilisation of the donated skin under reference number 04D08.

Full-thickness skin was frozen at - 20 °C within 24 h after surgery. During this study full thickness skin, containing all the skin layers, was utilised. Subcutaneous fat and connective tissue were separated from the skin by using a scalpel, taking care not to damage or rupture the skin in any way as this could lead to incorrect results. After successful removal, the skin was

punched into circles with a diameter of approximately 15 mm. In Figure D.1 the punch and hammer used for the cutting of the skin in circles are shown.



Figure D.1: The punch and hammer

The skin circles were positioned onto Whatman® filter paper with the stratum corneum side of the skin facing upwards. The skin circles on filter paper were covered in aluminium foil sheets and sealed in plastic bags. They were subsequently frozen at - 20 °C until used. Prior to the diffusion study, the frozen skin circles were thawed at room temperature (Pellet, 1999:29). Each skin circle was examined for any imperfections before it was mounted onto the diffusion apparatus.

D.2.2 Preparation of receptor solutions

The receptor solution consisted of 20:80 (v/v) methanol and PBS (pH 7.4). The inclusion of an organic solvent was to ensure solubility of the active ingredients. PBS was prepared by accurately weighing 13.62 g potassium dihydrogen ortho-phosphate and dissolving it in 500 ml of Milli-Q® water. Subsequently 3.14 g sodium hydroxide was weighed and dissolved in 786.8 ml Milli-Q® water. The two solutions were mixed together and the pH adjusted to 7.4 with 10% H_3PO_4. Finally, 20 vol methanol were added to 80 vol PBS. The solution was vacuum filtered through a 0.45 µm filter membrane prior to use.

D.2.3 Franz cell diffusion

The *in vitro* permeation studies were conducted in vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and an active diffusion area of about 1.075 cm^2. The Franz diffusion cells consist of a donor (top) and a receptor (bottom) compartment into which the formulation containing the drug and the receptor fluid (PBS, pH 7.4) were placed, respectively. In Figure D.2 the Franz diffusion cell is shown, with the donor and receptor compartments.

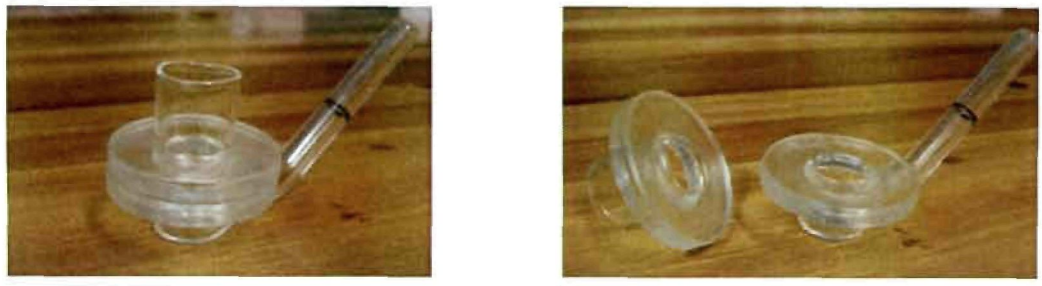


Figure D.2: The donor and receptor compartments of the Franz diffusion cell

Full-thickness skin circles were thawed and placed between the receptor and donor compartments with the stratum corneum in contact with the donor phase (upwards). The two compartments of the Franz cell were sealed with Dow Corning® high vacuum grease and clamped together with a metal horseshoe clamp to prevent any leakage. In Figure D.3 and Figure D.4 the vacuum grease and the metal clamps are shown.



Figure D.3: Dow Corning® high vacuum grease



Figure D.4: Metal clamps

A small magnetic stirrer bar was placed within each receptor compartment. The donor compartments were filled with 1 ml of the formulated product (cream or emulgel) preheated in a water bath to 32 °C, corresponding to normal skin temperature. The receptor compartment was covered with Parafilm® to avoid evaporation of the product for the whole duration of the experiment. Receptor compartments were filled with 2 ml receptor solution consisting of 20:80 (v/v) methanol and PBS (pH 7.4), taking care to prevent air bubbles beneath the skin. An assembled Franz diffusion cell is shown in Figure D.5.



Figure D.5: Assembled Franz diffusion cell

The receptor compartments of the diffusion cells were surrounded with a water bath held constant at a temperature of 37 °C throughout the experiment, in order to control the temperature of the receptor compartments (Dias, 1999:42). This attains the skin surface temperature of 32 °C, simulating the temperature of human skin (Leveque, 2004:324). The water bath is shown in Figure D.6.

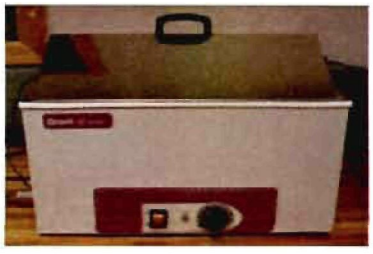


Figure D.6: Grant water bath

The receptor medium was continuously stirred at 750 rpm with the aid of a Variomag® magnetic stirring plate moving the small magnetic stirring bar (which was placed in each receptor compartment) in order to maintain movement throughout the entire experiment. The magnetic stirring plate is shown in Figure D.7.



Figure D.7: Variomag® magnetic stirring plate

The entire content of the receptor compartments were withdrawn at predetermined intervals (20; 40, 60, 80, and 100 min, as well as 2, 4, 6, 8, 10 and 12 h), followed by the replacement with an equal amount of fresh receptor solution; previously heated to 37 °C. This was done to ensure that the skin conditions persisted during the entire experiment. The withdrawal times were determined with the Pheroid™ delivery system in mind. It was expected that the Pheroid™ would promote the permeation of acyclovir and ketoconazole, thus, the withdrawal times were

started earlier. This also ensured that the desired amount of at least 5 data points would be achieved. Each sample was directly assayed by HPLC to determine the drug concentrations of the active ingredients (acyclovir and ketoconazole) within the receptor compartment.

D.2.4 Tape stripping

Tape stripping is a technique used to remove the outermost layers of the skin, the stratum corneum as well as the epidermis. The tape stripping method was used to investigate the amounts of active ingredient present in the outer layers of the skin, as well as in the dermis of the skin after application of a supersaturated solution or formulated product (Pellet *et al.*, 1997:92).

At 12 h, after the completion of the diffusion study, the donor and receptor compartments of the Franz diffusion cells were carefully dismounted. Each piece of skin was pinned on a piece of Parafilm® and stapled to a solid surface. The exposed area of diffusion ($\approx 1.075\ cm^2$) was clearly imprinted by the indentation from the diffusion cells ($\approx$ 11.7 mm diameter). The skin was dabbed dry with tissue paper to remove the last of the semi-solid donor phase. Pieces of 3M Scotch® Magic™ Tape were cut to cover the diffusional area. Pieces of tape were cut to a size assuring that it did not overlap the areas outside of the diffusion cell imprints. The first tape strip was discarded because the tape mighy have been contaminated with the drugs from the formulation, and it was seen as part of the cleaning procedure. The following 15 tape strips (referred to as the epidermis) were placed in a vial. The viable epidermal layer that glistened indicated the complete removal of the stratum corneum.

All the strips were placed in a 5 ml mixture of PBS (pH 7.4) and methanol. These were kept overnight at 4 °C. The excess skin was cut away from the imprints of the diffusion cells and the remaining skin (dermis) was cut into pieces to enlarge surface area. It was placed in a 2 ml mixture of PBS (pH 7.4) and methanol, and was kept overnight at 4 °C. The next morning, the dermis samples were centrifuged with an Eppendorf centrifuge 5804R at a rate of 4500 rpm and at a temperature of 10 °C for 10 min. The dermis and epidermis samples were then analysed with the HPLC. In Figure D.8 the centrifuge used is shown.

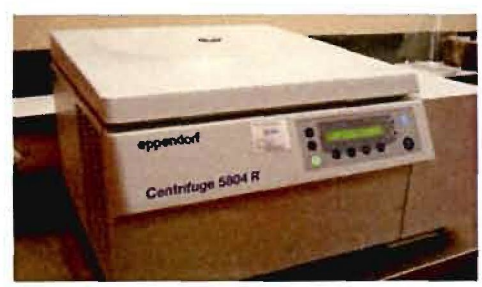


Figure D.8: Eppendorf centrifuge 5804R

D.2.5 Analysis of samples with HPLC

The HPLC analysis was performed by employing a method developed and validated by *Prof. J. du Preez and Ms. M.E. van der Walt at the North-West University*, Potchefstroom Campus, South Africa. This method was used to analyse acyclovir and ketoconazole.

Samples collected from the receptor compartments during the permeation experiments were analysed using an Agilent 1200 Series HPLC, equipped with an Agilent 1200 pump, auto sampler, UV detector and Chemstation Rev. A.06.02 data acquisition. An Agela Venusil XBP C-18, 4.6 x 150 mm, 5 µm column was used during the HPLC analysis.

The degassed mobile phase consisted of a mixture of 0.50 vol acetonitrile and 9.50 vol of a 1.15 g/l ammonium phosphate solution. The mobile phase changed by linear-gradient elution after 2 min to a mixture of 8.00 vol acetonitrile and 2.00 vol of a 1.15 g/l ammonium phosphate solution over 9 min, followed by the final elution mixture (0.50 vol acetonitrile and 9.50 vol of a 1.15 g/l ammonium phosphate solution) for 5 min. Table D.1 illustrates the gradient elution method for the mobile phase composition and adjustment, where A indicates the percentage ammonium PBS and B indicates the percentage acetonitrile.

Table D.1: The gradient elution method for the mobile phase composition

Time (min)	A (%)	B (%)
2.00	95.0	5.0
4.00	20.0	80.0
9.00	20.0	80.0
9.20	95.0	5.0

A volume of 100 µl of each sample was injected. The flow rate was set to 1 ml/min and the temperature was maintained at 25 °C. Total runtime was 14 min. Detection was set at 252 nm for the first 6 min to detect acyclovir, where after it was set to 243 nm for the detection of ketoconazole. Detection time of acyclovir was approximately 3.40 - 3.90 min and that of ketoconazole approximately 6.95 - 7.02 min.

Ammonium PBS was prepared by accurately weighing and dissolving 1.15 g ammonium phosphate monobasic in 950 ml Milli-Q® water and adjusting the pH to 7.20 ± 0.01 with a 10% ammonium hydroxide solution, diluted to 1000 ml with Milli-Q® water. It was filtered through a 0.45 µm Millipore® filter and degassed on an ultrasonic bath.

D.2.6 Transdermal and statistical data analysis

The average cumulative amount (for each Franz diffusion cell) per area (μg/cm^2) was plotted against time for acyclovir and ketoconazole in each formulation. Average flux values were obtained from the slope of the linear portion of the curve. The standard deviation (SD) of the cumulative amount diffused per time unit in the different Franz diffusion cells was indicated on the graph.

The average amount of active ingredients that diffused through the 10 Franz diffusion cells were depicted as a percentage of the applied concentration (5% acyclovir and 2% ketoconazole).

Statistical analysis was determined by using computer programs SAS (SAS Institute, 2005) and Statistica (Statsoft, 2008). The Kruskal-Wallis test was used to determine the statistically significant differences between the medians of the flux values and tape stripping measurements of the formulas. A $p < 0.05$ was considered to be of statistical significant difference (Steyn, 1994:604-606).

The pair-wise statistical comparisons of the median steady-state flux values and tape stripping measurements between formulated products were carried out in an attempt to establish where differences between the formulated products existed. Descriptive statistics were calculated and presented as median values. The correlation between the dermis and epidermis concentrations of each formulation was determined as well as the correlation with flux values.

D.2.7 Permeation experiments

Four sets of permeation studies were performed. A permeation study for each formulated product namely the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel was performed. All the formulated products contained 5% acyclovir and 2% ketoconazole. Each permeation study was done with 10 Franz diffusion cells.

D.3 RESULTS

Acyclovir showed a biphasic character with a steady-state flux between 0 - 2 h and between 2 - 12 h or 4 – 12 h in all four formulations. Ketoconazole, on the other hand showed no steady-state flux in the cream and the Pheroid™ cream; although the Pheroid™ cream depicted diffusion of ketoconazole over 12 h. The Pheroid™ emulgel formulation showed a flux between 6 - 12 h. The emulgel formulation showed a biphasic character with a steady-state flux between 0 - 2 h and between 2 - 12 h. Results of each formulation are given in the following section.

D.3.1 Cream

D.3.1.1 Acyclovir

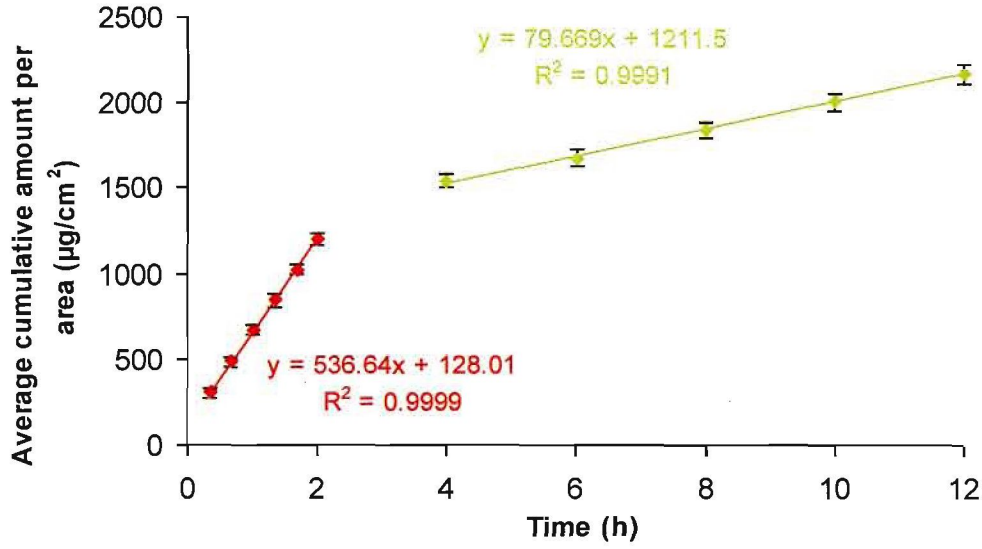


Figure D.9: Average cumulative amount that diffused through the skin as a function of time to illustrate the average values for flux 1 (0 - 2 h) and flux 2 (4 - 12 h) of acyclovir in cream

D.3.1.2 Ketoconazole

Ketoconazole diffused through the skin when the cream formulation was applied but no flux was observed. An average cumulative concentration of 38.86 µg/cm^2 ketoconazole was observed which diffused through the skin over 12 h.

D.3.2 Pheroid™ cream

D.3.2.1 Acyclovir

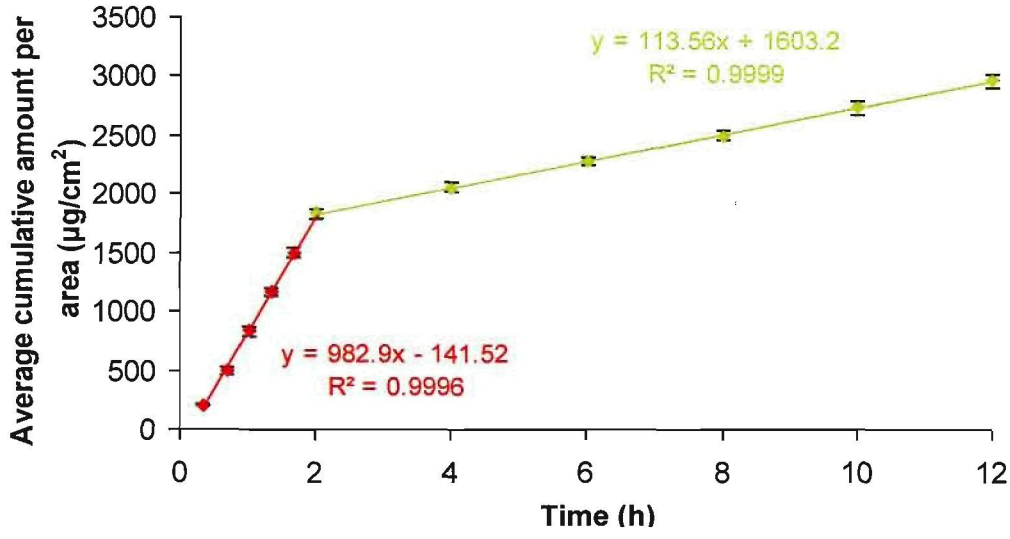


Figure D.10: Average cumulative amount that diffused through the skin as a function of time to illustrate the average values for flux 1 (0 - 2 h) and flux 2 (2 - 12 h) of acyclovir in Pheroid™ cream

D.3.2.2 Ketoconazole

Ketoconazole did not diffuse through the skin when the Pheroid™ cream formula was applied.

D.3.3 Emulgel

D.3.3.1 Acyclovir

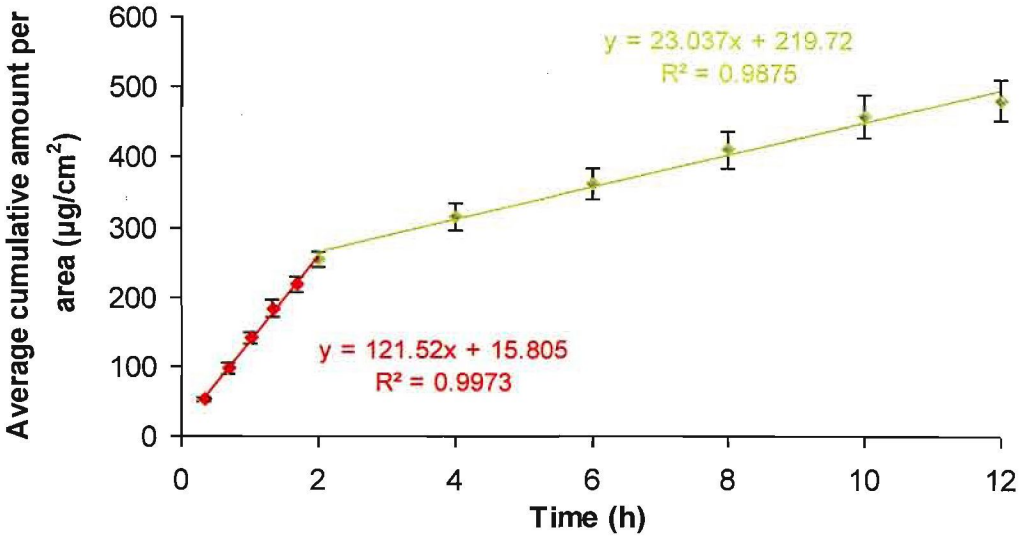


Figure D.11: Average cumulative amount that diffused through the skin as a function of time to illustrate the average values for flux 1 (0 - 2 h) and flux 2 (2 - 12 h) of acyclovir in emulgel

D.3.3.2 Ketoconazole

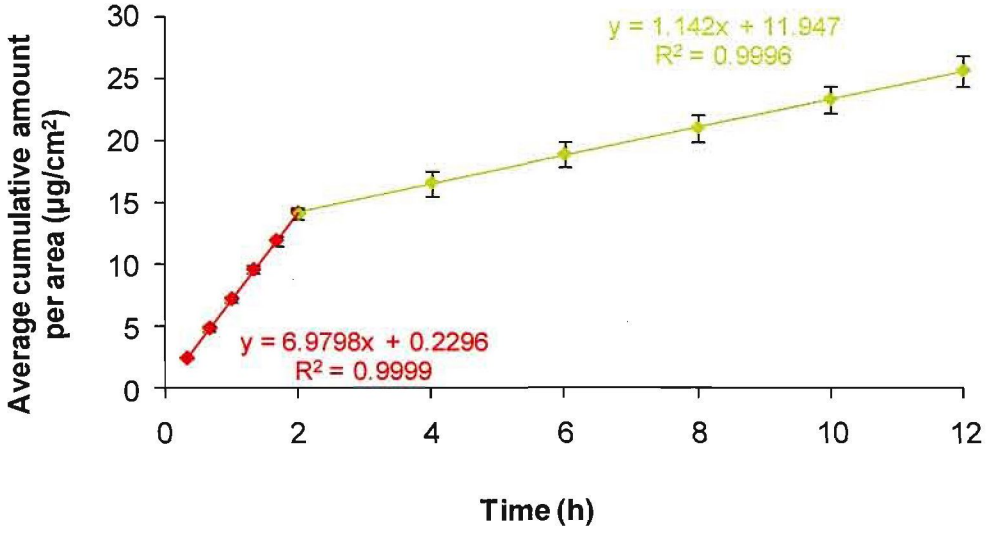


Figure D.12: Average cumulative amount that diffused through the skin as a function of time to illustrate the average values for flux 1 (0 - 2 h) and flux 2 (2 - 12 h) of ketoconazole in emulgel

D.3.4 Pheroid™ emulgel

D.3.4.1 Acyclovir

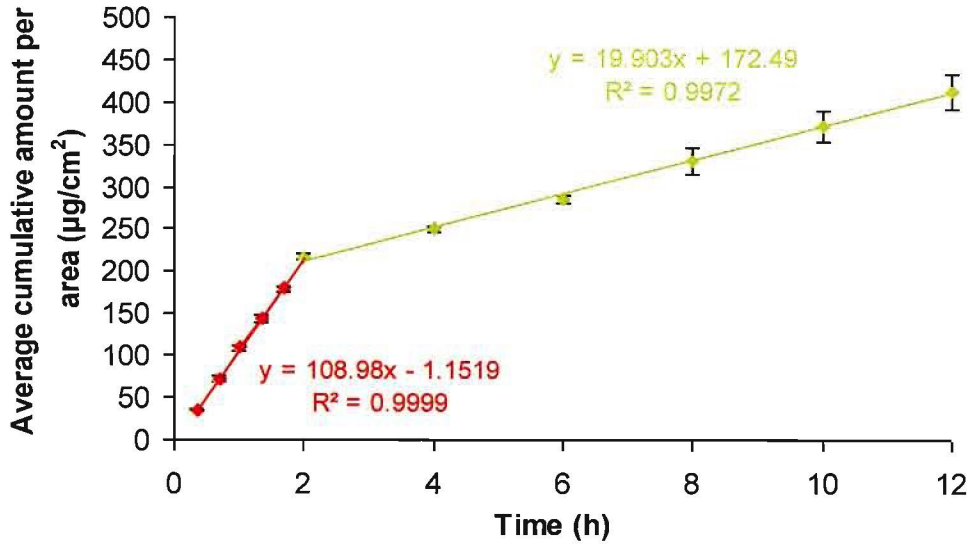


Figure D.13: Average cumulative amount that diffused through the skin as a function of time to illustrate the average values for flux 1 (0 - 2 h) and flux 2 (2 - 12 h) of acyclovir in Pheroid™ emulgel

D.3.4.2 Ketoconazole

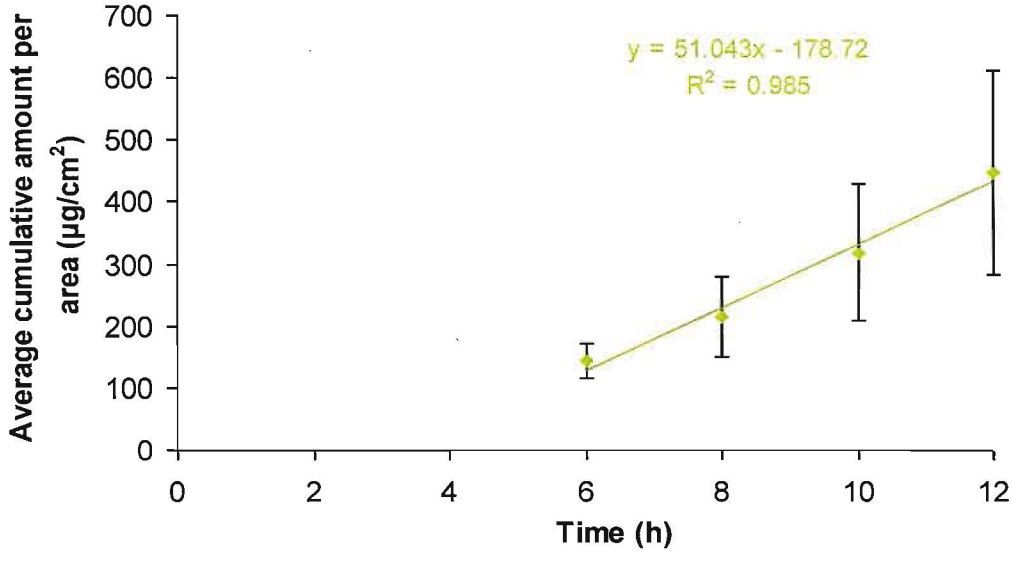


Figure D.14: Average cumulative amount that diffused through the skin as a function of time to illustrate the average values for flux (6 - 12 h) of acyclovir in Pheroid™ emulgel

D.3.5 Summary of results

A summary of the vertical Franz cell diffusion studies and the tape stripping results are given in Table D.2 and Table D.3. Box-plots of the flux values to illustrate the median flux and the average flux of the formulations are illustrated by Figure D.15, Figure D.16 and Figure D.17.

Table D.2: The average and median flux values and average tape stripping values of acyclovir

	Average flux 1 ($\mu g/cm^2$)	Average flux 2 ($\mu g/cm^2$)	Median flux 1 ($\mu g/cm^2$)	Median flux 2 ($\mu g/cm^2$)	Average epidermis ($\mu g/ml$)	Average dermis ($\mu g/ml$)
Cream	536.64 ± 10.56	79.67 ± 2.68	538.00	92.19	18.10	45.56
Pheroid™ cream	982.90 ± 23.55	113.56 ± 7.45	989.78	111.76	1426.51	1571.96
Emulgel	121.52 ± 4.96	23.04 ± 2.11	120.26	23.02	106.54	262.19
Pheroid™ emulgel	108.98 ± 2.02	19.90 ± 4.16	109.31	19.29	85.53	126.02

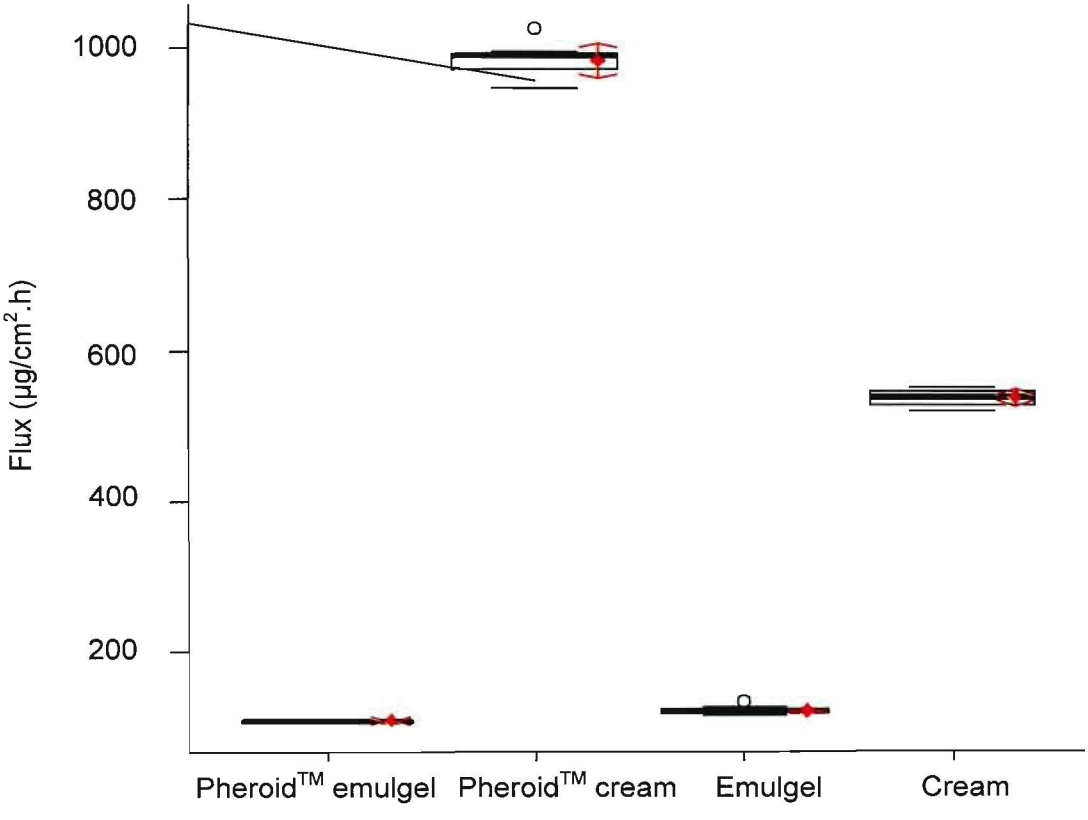


Figure D.15: Box-plots of the flux 1 values to illustrate the median flux 1 and red lines to illustrate the average flux 1 of Pheroid™ emulgel, emulgel, Pheroid™ cream and cream at time 0 - 2 h.

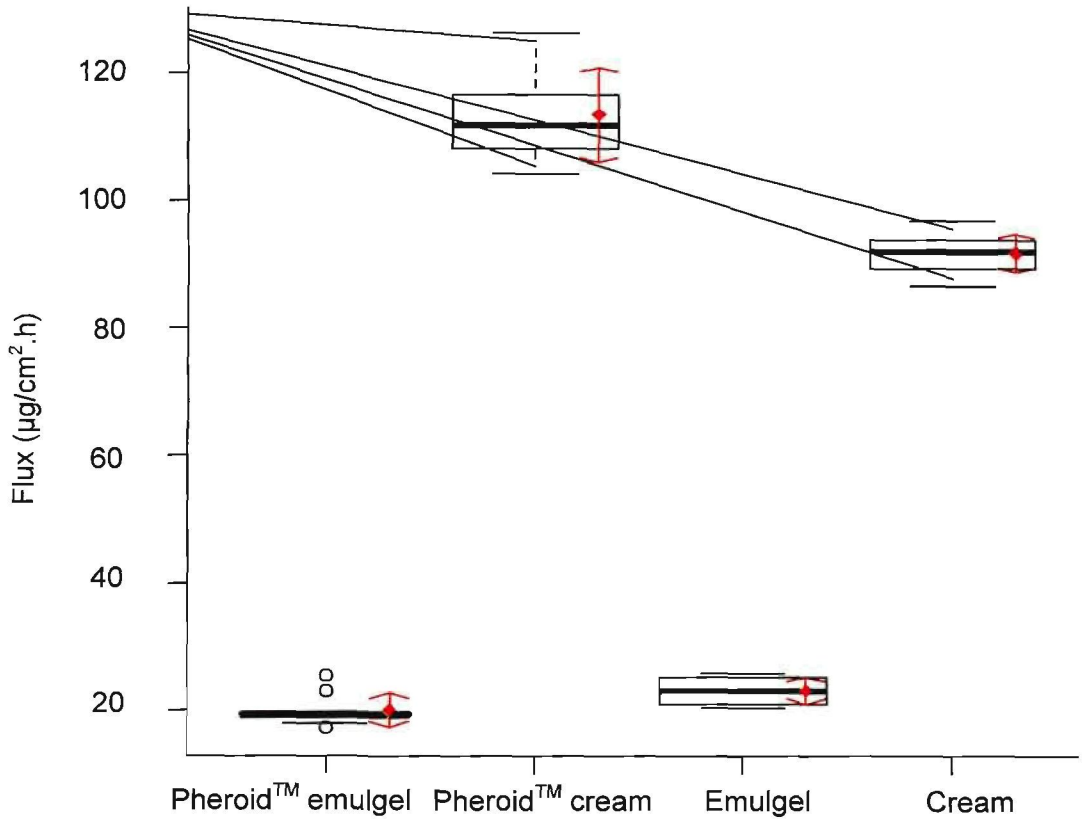


Figure D.16: Box-plots of the flux 2 values to illustrate the median flux 2 and red lines to illustrate the average flux 2 of Pheroid™ emulgel, emulgel, Pheroid™ cream and cream at time 2 - 12 h.

Table D.3: The average and median flux values and average tape stripping values of ketoconazole

	Average flux 1 (µg/cm²)	Average flux 2 (µg/cm²)	Median flux 1 (µg/cm²)	Median flux 2 (µg/cm²)	Average epidermis (µg/ml)	Average dermis (µg/ml)
Cream	-	-	-	-	2.71	13.96
Pheroid™ cream	-	-	-	-	2.81	6.94
Emulgel	6.98 ± 0.20	1.14 ± 0.11	6.92	1.13	1.41	6.12
Pheroid™ emulgel	-	51.04 ± 25.74	-	46.85	2.93	103.12

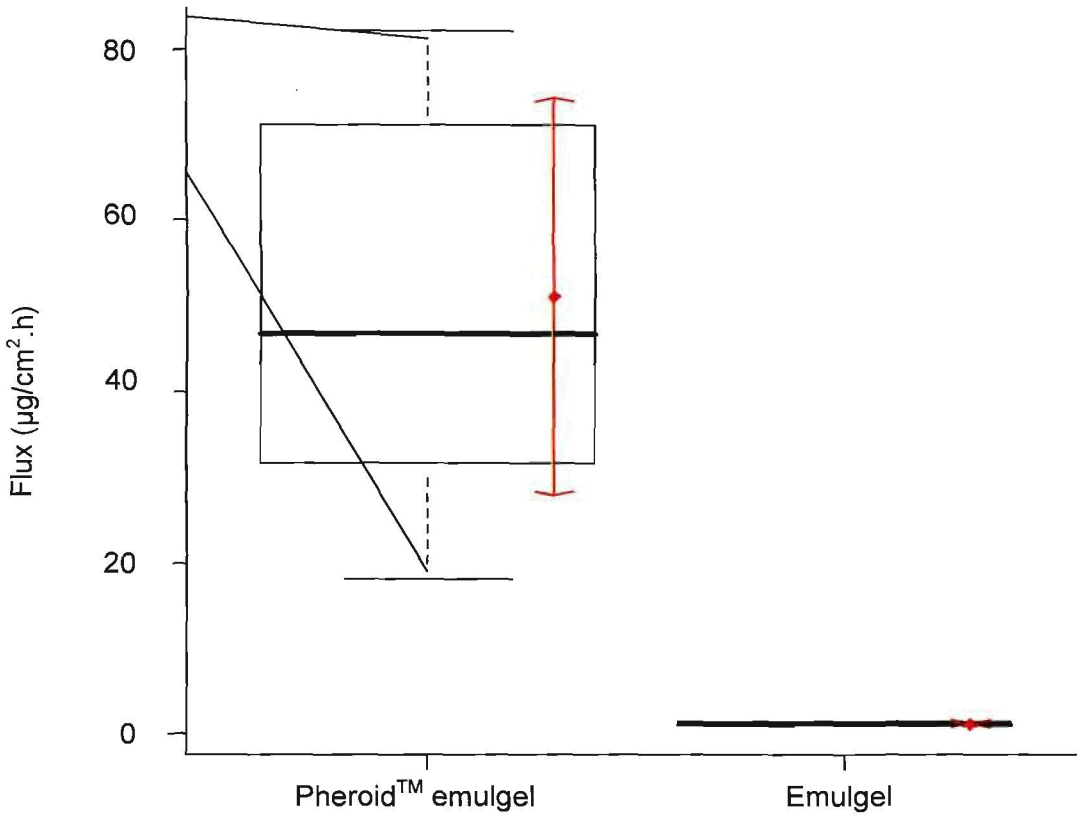


Figure D.17: Box-plots of the flux values to illustrate the median flux and red lines to illustrate the average flux of Pheroid™ emulgel and emulgel at time 0 - 2 h.

D.4 DISCUSSION

The average and median flux values of the active ingredients of each formulation were compared. The dermis and epidermis tape stripping data of each formulation was compared. Tape stripping values of the four formations were also compared. Subsequently, the tape stripping data was compared to the flux values of the formulated products. Comparisons were done separately for acyclovir and ketoconazole. The comparisons of the results are given in this section.

D.4.1 Acyclovir

D.4.1.1 Comparison of acyclovir's flux values

The Pheroid™ cream formula depicted the highest flux 1 and flux 2 values compared to the other formulated products. The cream formula depicted a significantly higher flux 1 and flux 2 value than the Pheroid™ emulgel. The emulgel and the Pheroid™ emulgel showed no significant difference in flux values. The cream formulae, thus, enhanced the transdermal delivery of acyclovir in comparison to the emulgel and Pheroid™ emulgel formulae. The Pheroid™ enhanced acyclovir's diffusion through the skin in the cream formulation, but not in the emulgel formulation. Median and average flux values for flux 1 and flux 2 in each formula

were closely related. The difference in the median and average flux values in the emulgel can be explained by the method of calculation of these values. The average flux was determined by adding the flux values and dividing it by the number of cells, whereas the median flux values were determined by finding the centre of the values. The median flux was not affected by outliers.

There was a significant difference between the flux 1 and the flux 2 values of acyclovir with $p < 0.0001$.

Flux 1 values of acyclovir were compared within the four formulations. There was a significant difference between the cream and Pheroid™ emulgel with $p = 0.0008$. The emulgel and Pheroid™ cream also showed a significant difference with $p = 0.0008$. A significant difference between the Pheroid cream and Pheroid™ emulgel was seen, with $p < 0.0001$. There was, thus, a statistical similarity between the flux 1 values of the cream and the Pheroid™ cream as well as between flux 1 values of the emulgel and the Pheroid™ emulgel formulation.

Flux 2 values of acyclovir were compared within the four formulations. There was a significant difference between the cream and the Pheroid™ emulgel with $p = 0.0026$; the emulgel and Pheroid™ cream showed a statistical significant difference with $p = 0.00026$. A significant difference between the Pheroid™ cream and Pheroid™ emulgel was seen, with $p < 0.0001$. As with flux 1, there was a statistical similarity between the flux 2 values of the cream and the Pheroid™ cream as well as between the emulgel and the Pheroid™ emulgel.

D.4.1.2 Comparison of acyclovir's tape striping data

The epidermis tape stripping (µg/ml) values of acyclovir differed in all four formulations. The Pheroid™ cream depicted the highest value of 1426.51 µg/ml. The emulgel (106.54 µg/ml) showed a larger concentration of acyclovir in the epidermis than the Pheroid™ emulgel (85.53 µg/ml) formula. On the other hand, the cream formula showed the poorest delivery of acyclovir (18.10 µg/ml) to the epidermis. The Pheroid™ cream formulation illustrated that Pheroid™ increased delivery to the epidermal layer of the skin.

The dermis tape stripping values of acyclovir were compared within the four formulations. There was more acyclovir delivered to the dermis of the skin than to the epidermal layer of the skin and this which was observed in all four formulations. The Pheroid™ cream (1571.96 µg/ml) depicted the most significant delivery of acyclovir to the dermis layer of the skin. The emulgel formula (262.19 µg/ml) delivered a larger concentration of acyclovir to the dermis than the Pheroid™ emulgel (126.02 µg/ml) formula. The cream formula showed the poorest delivery of acyclovir (45.56 µg/ml) to the dermal layer. Pheroid™ did not enhance delivery to the dermal layer of the skin in the emulgel formulation when compared with the emulgel containing no

Pheroid™. The Pheroid™ cream formulation illustrated that Pheroid™ increased delivery to the dermal layer of the skin in comparison with the cream and emulgel formulae.

There was a significant difference between the Pheroid™ cream and Pheroid™ emulgel with $p = 0.0024$; the cream and Pheroid™ cream showed a difference of $p < 0.0001$. A significant difference between the cream and emulgel was seen with $p = 0.0011$.

Acyclovir epidermis tape stripping values were compared within the four formulations. A significant difference between the Pheroid™ cream and Pheroid™ emulgel with $p = 0.0101$ was observed. The cream and Pheroid™ cream also showed a difference with $p < 0.0001$. A significant difference between the cream and emulgel was seen with $p = 0.0047$. There was a statistical similarity between the emulgel and the Pheroid™ emulgel formulations. When comparing the total of the dermis tape stripping values of all four formulations with each other using the Kruskal-Wallis test, a significant difference ($p < 0.0001$) between these values was found. The sum of the epidermis tape stripping values of all four formulations was compared using the Kruskal-Wallis test. A significant difference between these values was found with $p < 0.0001$.

D.4.1.3 Statistical comparison of flux and tape stripping values of acyclovir

The epidermis tape strips depicted a significant difference between the average flux 1 and the average flux 2 values with $p < 0.0001$ in both instances. Dermal tape stripping values also depicted a statistical significant difference in the average flux 1 and the average flux 2 values with $p < 0.0001$ and $p = 0.0002$, respectively. Thus, there was a correlation between the tape stripping and the flux values of acyclovir.

D.4.2 Ketoconazole

D.4.2.1 Comparison of ketoconazole's flux values and the 12 h concentration values

In the emulgel a flux 1 and a flux 2 was observed for ketoconazole. The flux 1 value was 6 times higher than the flux 2 value. Median and average flux 1 (0 - 2 h) and flux 2 (2 - 12 h) values were closely related. The Pheroid™ emulgel depicted one flux value (6 - 12 h). The median and the average flux correlated relatively close. The flux value of the Pheroid™ emulgel was higher than the emulgel's flux 1 and flux 2 values. The Pheroid™ cream depicted no transdermal penetration, but the cream, on the other hand, depicted transdermal penetration, but no flux was observed. Comparing the concentrations of ketoconazole diffused after 12 h in the cream (38.86 $\mu g/cm^2$), emulgel (25.68 $\mu g/cm^2$) and Pheroid™ emulgel (415.95 $\mu g/cm^2$), it was clear that the Pheroid™ delivery system increased the transdermal penetration of

ketoconazole. The average amount of ketoconazole that diffused through the skin over the 12 h period was 1.105%, 0.069% and 0.104% for the Pheroid™ emulgel, emulgel and the cream formulae, respectively. Thus, in the cream formulation the Pheroid™ did not increase transdermal delivery. Emulgel formulations showed better transdermal diffusion than the cream formulations. The Pheroid™ delivery system illustrated the best transdermal penetration for ketoconazole.

When comparing the total concentrations diffused over 12 h, a $p < 0.0001$ was obtained. This showed that there was a significant difference between the diffused concentrations of the cream, emulgel and Pheroid™ emulgel formulations. No significant difference in the median and average flux values for ketoconazole was observed. There was no significant difference between flux 1 and flux 2 values of the emulgel or between the flux values of the Pheroid™ emulgel.

D.4.2.2 Comparison of ketoconazole's tape striping data

The epidermis concentrations of ketoconazole in the four different formulations were closely correlated. However, the dermis values varied significantly between the different formulations. The Pheroid™ emulgel formula delivered the highest concentration of ketoconazole to the dermal layer (103.12 µg/ml) of the skin. The cream formula (13.96 µg/ml) delivered more ketoconazole to the dermis than the Pheroid™ cream formula (6.94 µg/ml). Therefore, the emulgel formulae showed enhanced delivery to the dermis layer of the skin. The Pheroid™ emulgel showed that the Pheroid™ increased the delivery of ketoconazole to the dermis layer of the skin in comparison with the cream formula.

The ketoconazole's epidermis tape stripping values were compared within the formulations. There was a significant difference between the Pheroid™ cream and emulgel with $p = 0.0196$; the emulgel and cream showed a significant difference with $p = 0.0321$. Dermis tape stripping values of ketoconazole were compared within the four formulations. There was a significant difference between the Pheroid™ cream and Pheroid™ emulgel with $p = 0.0001$; the emulgel and Pheroid™ emulgel showed a difference with $p < 0.0001$. A significant difference between the cream and emulgel was seen with $p = 0.0152$.

The epidermis and the dermis showed no significant difference with $p = 0.09$. The total of the epidermis tape stripping values of ketoconazole in all four formulations was compared by using the Kruskal-Wallis test. A significant difference between these values was found ($p = 0.0091$). When comparing the total dermis tape stripping values of ketoconazole with all four formulations, a significant difference between these values was found with $p < 0.0001$.

D.4.2.3 Statistical comparison of the flux values and tape stripping values of ketoconazole

There was no significant difference between the flux values and the tape stripping data of ketoconazole.

D.5 CONCLUSION

Experimentally, it was possible to deliver acyclovir transdermally. This was expected when the relatively high aqueous solubility and low molecular weight of acyclovir were considered (McEvoy., 2002; British Pharmacopoeia, 2009). Acyclovir was detected in the receptor compartment of the Franz diffusion cells; this indicated that acyclovir penetrated through full-thickness skin in all four formulations. When comparing the flux values of acyclovir, the Pheroid™ emulgel did not enhance the delivery of acyclovir compared to the emulgel. Acyclovir's transdermal penetration was enhanced by the cream formulations, with the Pheroid™ cream exhibiting the best transdermal delivery of acyclovir.

Transdermal delivery of ketoconazole was poor, as expected, when considering its low aqueous solubility and high molecular weight (McEvoy., 2002; British Pharmacopoeia, 2009). Ketoconazole was detected in the receptor compartment after application of the cream, emulgel and Pheroid™ emulgel formulations, indicating that it penetrated through full-thickness skin. Penetration of ketoconazole was enhanced by the emulgel formulations. Pheroid™ emulgel showed the best delivery of ketoconazole in comparison with the emulgel.

Acyclovir was detected in the dermis and epidermis layers of the skin after the application of all four formulations. The Pheroid™ cream significantly enhanced delivery to the dermis and epidermis of the skin in comparison to the cream formulation. The Pheroid™ emulgel did not enhance the delivery of acyclovir to the skin and showed equal results to that of the emulgel.

Ketoconazole was delivered to the dermis and epidermis layers of the skin, after the application of the four formulations. The Pheroid™ cream did not enhance the delivery to the dermis and epidermis of the skin compared to the cream formulation. The Pheroid™ emulgel enhanced the delivery of ketoconazole to the dermis, but not to the epidermis in comparison with the emulgel.

The reason for the increased delivery of acyclovir by the cream, and ketoconazole by the emulgel formulae, may be explained by the aqueous solubility of the drugs. Ketoconazole is more hydrophobic and acyclovir is more hydrophilic. The cream formula contained more oil and fatty ingredients; and the emulgel contained more water and less oil. Thus, it may be possible that acyclovir did not diffuse out of the emulgel formula, due to its affinity to the surroundings. Ketoconazole may have benefited from the fatty environment and diffusion out of the cream formula was not encouraged. Pheroid™ cream increased the delivery of acyclovir to its target

site, the epidermis, and ketoconazole's delivery to the stratum corneum (included in the epidermis tape stripping) was increased by the Pheroid™ emulgel. In conclusion, the Pheroid™ showed to enhance the flux of, and the delivery to human skin for both the active ingredients.

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APPENDIX E

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