Pheroid™ technology for the topical delivery of depigmenting agents transforming growth factor-β1 and tumor necrosis factor-α

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This dissertation is presented in the so-called article format, which includes an introductory chapter with sub-chapters, a full length article for publication in a pharmaceutical journal and appendices containing relevant 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

Pigmentation disorders occur in multiple conditions (Hakozaki *et al.,* 2006:105). Although many modalities of treatments are available, none are completely satisfactory (Briganti *et al*., 2003:101). Two cytokines normally present in the skin, transforming growth factor-beta1 (TGF-β1) and tumour necrosis factor-alpha (TNF-α), have been shown to inhibit melanin synthesis (Martinez-Esparza, 2001:972).

The stratum corneum has been commonly accepted as the main barrier to percutaneous absorption. Many techniques have been applied to overcome this barrier properties and to enhance penetration with varying success (Pellet *et al.,* 1997:92).

The objective of this study was to investigate the topical delivery of the above mentioned peptide drugs with aid of the Pheroid™ drug delivery system. Pheroid™ technology is a delivery system that promotes the absorption and increases the efficacy of dermatological, biological and oral medicines in various pharmacological groups (Grobler *et al*., 2008:4). Pheroid™ entraps drugs with high efficiency and delivers them with remarkable speed to target sites (Grobler, 2004:4). In order to avoid degradation of these peptides, bestatin hydrochloride (an aminopeptidase inhibitor), was used (Lkhagvaa *et al.,* 2008:386).

Topical drug delivery was achieved by means of vertical Franz cell diffusion studies performed over a 6 and 12 h period. ELISA (enzyme linked immunosorbent assay) detection was used to detect cytokine concentrations. Entrapped cytokine solutions were monitored by confocal laser scanning microscopy (CLSM). Upon removal of donor and receptor compartments, skin discs were subjected to tape stripping in order to establish the amount of active present within the stratum corneum and epidermis as well as the remaining dermis (Pellet *et al.,* 1997:92).

When comparing the two studies with each other, it is evident that the diffused concentration values obtained with PBS (phosphate buffer solution, pH 7.4) was lower than that obtained with the Pheroid™ drug delivery system. Both cytokine concentrations were successfully delivered topically as a minimum of concentrations for both actives were detected. This positive result was confirmed as well by the amount of active detected in stratum corneum-epidermis and epidermis-dermis solutions.

Keywords: Pigmentation, Topical delivery, Pheroid™, Transforming growth factor-beta1, Tumour necrosis factor-alpha, Bestatin, Tape stripping

OPSOMMING

Pigmentasieversteurings kom in verskeie toestande voor (Hakozaki *et al.,* 2006:105). Alhoewel daar baie modaliteite vir behandeling beskikbaar is, is hule nie heeltemaal bevredigend nie (Briganti *et al*., 2003:101). Twee sitokiene wat normaalweg in die vel voorkom, nl. transformerende groei faktor-beta1 (TGF-β1) en tumor nekrosis faktor-alpha (TNF-α) het al aangedui dat dit melanien (die pigment verantwoordelik vir die vervaardiging van vel kleur) produksie inhibeer (Martinez-Esparza, 2001:972).

Die hoofskans tot perkutaneuse absorpsie word algemeen beskou as die stratum corneum. Baie tegnieke is al probeer met variëerende sukses om hierdie eienskap te oorkom en penetrasie te bevorder (Pellet *et al.,* 1997:92).

Die doelwit van hierdie studie was om die topikale aflewering van bogenoemde peptied-geneesmiddels, met behulp van die Pheroid™ geneesmiddelafleweringsisteem te ondersoek. Die Pheroid™-tegnologie bevorder die aborpsie en die effektiwiteit van dermatologiese, biologiese en orale medisyne in verskeie farmakologiese groepe (Grobler *et al*., 2008:4). Geneesmiddels word effektief vasgevang in die Pheroid™ en word teen ‘n merkbare spoed na teikengebiede afgelewer (Grobler, 2004:4). Om afbraak van die peptied-geneesmiddels te voorkom, was bestatienhidrochloried, ‘n aminopeptidase-inhibeerder, gebruik (Lkhagvaa *et al.,* 2008:386).

Topikale aflewering was bereik deur middel van vertikale Franz-sel diffusiestudies oor ‘n tydperk van 6 en 12 h. Om sitokienkonsentrasies te bepaal was ELISA deteksietoetse gedoen. Vasgevangde sitokienoplossings is bepaal deur konfokale laserskanderingsmikroskopie (KLSM). ‘n Bandstropings “tape stripping” -tegniek is uitgevoer na verwydering van die velmonsters vanaf die donor- en reseptorkompartemente, om die hoeveelheid aktief in die stratum corneum en epidermis sowel as die oorblywende dermis te bepaal (Pellet *et al.,* 1997:92).

Wanneer die twee studies met mekaar vergelyk word, is dit duidelik dat PBS (pH7.4) laer diffusie konsentrasiewaardes as die Pheroid^{TM}-geneesmiddelafleweringsisteem verkry het. Beide sitokiene was topikaal suksesvol afgelewer, aangesien minimum konsentrasies gemeet kon word. Hierdie positiewe resultaat word ook bevestig deur die hoeveelheid aktief gemeet in die stratum corneum-epidermis- en epidermis-dermisoplossings.

Sleutelwoorde: Pigmentasie, Topkale aflewering, Pheroid™, Transformerende groei faktor-beta1, Tumor nekrosis faktor-alpha, Bestatien, Bandstroping

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

APPENDIX A: STABILITY TESTING OF COSMECEUTICALS

APPENDIX B: STABILITY TESTING OF COSMECEUTICALS

LIST OF TABLES

ABBREVIATIONS

ANOVA	-	Analysis of variance
BCA	-	Bichorionic acid
CLSM	-	Confocal laser scanning microscopy
DCT	-	DOPAchrome tautomerase
DHICA	-	Dhydroxyindole-2-carboxylic cid
DOPA	-	3,4-Dihydroxyphenylalanine
ELISA	-	Enzyme immunosorbent assay
ICH	-	International conference on harmonisation
α-MSH	-	Alpha-melanin stimulating hormone
PBS	-	Phosphate buffer solution
PIH	-	Post-inflammatory hyper-pigmentation
SD	-	Standard deviation
TRP-1	-	Tyrosinase-related protein-1
TRP-2	-	Tyrosinase-related protein-2
TNF-α	-	Tumour necrosis factor-alpha
TGF-β1	-	Transforming growth factor-beta1
UV	-	Ultra violet

CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

Skin pigmentation is one of the major differences between races of the human species (Fluhr *et al.,* 2008:e230). A light and even skin tone is highly valued in world in many regions of the world (Hakozaki *et al.,* 2006:105). While skin lighteners are applied for the prevention and treatment of irregular hyper-pigmentation in Western countries; in Asia, the use thereof is widely extended by traditional beliefs especially for Asian and African women as a lighter skin colour signifies increased wealth and social status (Solano *et al.,* 2006:550).

Skin colour varies with race, age, geographic location and season, parts of the body and between individuals (Mitsui, 1997:21). Skin cells known as melanocytes, give rise to skin colour by generating a substance called melanin. This biosynthesis occurs within membrane-bound organelles known as melanosomes (Costin & Hearing, 2007:978). The amount and regulation of melanosome transfer contributes to the ultimate pigmentation of human skin (Hakozaki *et al.,* 2005:499). The human skin colour consists of two components: constitutive and facultative or inducible melanin pigmentation. The first component is genetically determined and presents as the skin colour of habitually sun yielded areas while the latter component results from deliberately increased exposure to ultraviolet (UV) radiation. Facultative skin colour is reversible as sun exposure is discontinued (Fitzpatrick *et al.,* 1979:132).

The skin reflects internal changes and reacts to changes in the environment. It usually adapts easily and returns to a normal state and when a normal state is not reached, a skin disorder results (Hunter *et al.,* 1995:1). Pigmentation disorders can be inherited, acquired, medication-related and transmitted through infection; affecting just patches of skin while others affect the entire body (Seiberg *et al.,* 2000:162).

The majority of pigmentation disorders are not disorders of melanin quality, but rather of the melanosomes, which may be reduced in number, deficient or hyperactive and generally with regional localization. Hyper-pigmentation (also known as hypermelanoses), refers to an increase in the pigmentation (Ruiz-Maldonado & De la Luz-Covarrubias, 1997:36). Hyper-pigmentation of both types can result from genetic, hormonal or UV radiation tanning resulting from increased melanin, which may occur in the epidermis, dermis, or both. Three of the most common hyper-pigmentation disorders include melasma, lentigines and post-inflammatory hyper-pigmentation (PIH) (Cayce *et al.,* 2004:401).

Despite of the nature of the pigmentary disorder, the general desire is for uniformity of skin colour. Treatments of hyper-pigmentation disorders are often difficult and prolonged; requiring a great deal of patience and knowledge on different therapeutic modalities to achieve success (Pandaya *et al.,* 2000:91). Regardless of the motivation why adults alter their appearance, the psychological and socio-economic impacts of pigmentation problems are great because of visible nature thereof. In most cases pigmented lesions are cosmetically displeasing to individuals and may even cause a significant amount of embarrassment or emotional distress (Petit & Pierard, 2003:169).

Skin lightening agents has been the most commonly practiced method for hyper-pigmentation control. Most of the currently available bleaching or depigmenting agents cause a temporary removal of hyper-pigmentation, which usually recurs after discontinuation of therapy (Katsambas *et al.,* 2001:483). The effect of existing agents is not yet sufficient to fulfil the demanding consumer needs. While one reason for the limited efficacy can be attributed to the weak activity of existing skin-lightening agents themselves, another primary reason has its roots in insufficient transepidermal delivery of skin-lightening agents to the target regions, in and around melanocytes (Hakozaki, 2006:105)

According to Briganti *et al.* (2003:101) the ideal depigmenting compound should have a potent, rapid and selective whitening or lightening effect on hyper-activated melanocytes, carry no short- or long-term side-effects and lead to a permanent removal of undesired pigment, acting at one or more steps of the pigmentation process, as this is a very complex process. It is therefore imperative to understand the underlying mechanisms responsible for melanin synthesis and pigmentation disorders (Briganti *et al.,* 2003:101).

Melanogenesis can be inhibited by several cytokines present in the epidermis. In B16 cells, melanogenesis is strongly down-regulated by two cytokines normally present in the skin, namely tumour necrosis factor-alpha (TNF-α) and transforming growth factor-beta1 (TGF-β1) (Martinez-Esparza *et al.,* 2001:972). TNF-α causes a dose dependent decrease in the activity of tyrosinase, the rate limiting enzyme in melanin synthesis, and inhibits melanocyte proliferation. TGF-β1 down-regulates tyrosinase by decreasing both gene expression and the intracellular half-life of the enzyme (Slominski *et al.,* 2004:1196).

Topical application of formulations to the skin offers delivery of drugs to the local tissues directly under the application site or within tissues under and around the site of application (Ghosh *et al.,* 1997:7). Dermal drug delivery is the topical application of drugs to the skin in the treatment of skin diseases, while transdermal drug delivery uses the skin as an alternative route for the

delivery of systemically acting drugs (Honeywell-Nguyen & Boustra, 2005:67). Up to date, cosmetic scientists have been confronted with the problematic absorption of cosmetic products through the skin layer and substantiation of their clinical efficacy (Morganti, 2001:481).

The most important function of human skin is to act as a barrier by limiting water loss, electrolytes, and other body constituents while barring the percutaneous absorption of harmful or unwanted molecules from the external environment (Morganti, 2001:492). The ultimate purpose in dermatological biopharmaceutics is to design drugs with selective penetrability for incorporation into vehicles or devices that deliver the medicament to the active site (Barry, 2002:507). Pheroid™ drug delivery system, previously known as “Emzaloid™”, is a patented colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures uniformly distributed in a dispersion medium that may be adapted to the indication. These dispersed structures (dispersed phase) can be manipulated in terms of morphology, structure, size and function (Grobler *et al*., 2008:4-5). Pheroid™ entraps drugs with high efficiency and delivers them with remarkable speed to target sites in the body (Grobler, 2004:4).

Proteins are enzymatically attacked in the gastro-intestinal tract, in addition to slow and incomplete penetration through the gut wall. In order to improve the bioavailability of pharmaceutical proteins, such formulations are often co-administered with protease inhibitors to slow down metabolic degradation (Crommelin *et al.,* 2002:550, 552). One such inhibitor is the aminopeptidase inhibitor bestatin hydrochloride. Bestatin exerts its activity by competing with the substrates, binding to the catalytic site of the enzyme, exhibiting a competitive kinetics with the substrate once bound (Lkhagvaa *et al.,* 2008:390).

The aim of this study was to determine whether the Pheroid™ delivery system can be employed to deliver cytokines (TNF-α and TGF-β1) topically, by means of Franz cell diffusion studies, co-administered with bestatin hydrochloride. In order to test the efficacy of this delivery system, a comparative study was conducted with both actives dissolved in phosphate buffer solution (PBS), pH 7.4. The amount of permeant in the stratum corneum-epidermis was determined using a tape stripping technique, while the remaining dermis was incised into smaller pieces. Both samples were placed in separate vials containing PBS (pH 7.4), incubated at 4 °C for 12 h prior to analysis. Receptor phases and samples were subjected to ELISA (enzyme linked immunosorbent assay) tests for detection of protein content.

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CHAPTER 2
PHEROID™ TECHNOLOGY FOR THE TOPICAL DELIVERY OF DEPIGMENTING AGENTS TGF-β1 AND TNF-α

2.1 INTRODUCTION

Skin colour is one of many human physical features used to characterize a particular group or population of people. Such characterization can often be problematic – as skin colour can be influenced by environmental factors or may change with age (Sturm *et al*., 1998:712). Great diversity exists in the colour of human skin across the globe ranging from very pale colour to very dark skin types, commonly spoken of as black, white, red or yellow with a predominant black/white dualism in popular categorization. All organisms exist in different colours and patterns, which arise from the distribution of pigments throughout the body (Jablonski & Chaplin, 2000:58). This is due to the presence of a chemically inert and stable pigment known as melanin, which is produced deep inside the skin, but is displayed at the surface of the body (Costin & Hearing, 2007:976). Melanin accounts for most of the variation in the visual appearance of human skin (Jablonski & Chaplin, 2000:58).

Figure 2.1: The main human skin types of pigmentation: African-American, Asian, Caucasian and Hispanic (left to right, Costin & Hearing, 2007:980)

Genetic, environmental and endocrine factors regulate the highly heritable pigmentation process, modulating the amount, type and distribution of melanin in the skin, hair and eyes. Melanin plays an essential role in defining ethnicity and defending the body against harmful UV rays and other environmental challenges (Costin & Hearing, 2007:980).

Across the array of skin colours, many disorders of the pigmentation system exist, resulting in problems ranging from hypo-pigmentation to hyper-pigmentation (Ortonne & Bissette, 2008:10).

Hyper-pigmentation is quite frequent and troublesome in dark-skinned individuals (Petit & Piérard, 2003:169). Minor changes in the physiological status of the human body or exposure to harmful external factors can affect pigmentation patterns either in transitory manners such as pregnancy or in permanent manners such as age spots (Costin & Hearing, 2007:976).

Regardless of the nature of the problem, the general desire is for uniformity of skin colour. Many modalities of treatment for acquired skin hyper-pigmentation disorders are available. This includes chemical agents or physical therapies, but none is completely satisfactory (Briganti *et al*., 2003:101).

In order to understand the therapeutic approaches intended for treatment of pigmentation diseases, a thorough understanding of different factors and processes contributing to skin pigmentation, as well as an understanding of the cellular and molecular interactions between melanocytes and keratinocytes is needed. The different skin layers and cell types distributed within the skin must be considered as well (Costin & Hearing, 2007:976).

2.2 SKIN STRUCTURE AND FUNCTION

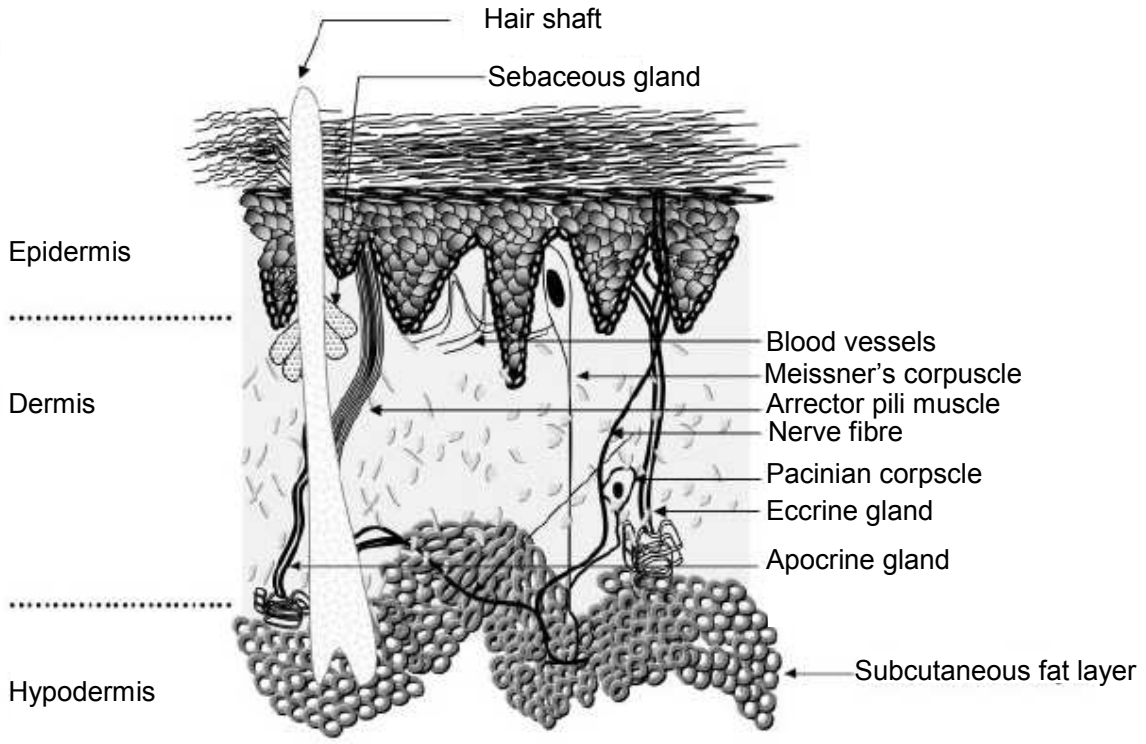

Figure 2.2: Structure of the skin (Costin & Hearing, 2007:977)

The skin of the human body provides both protection and receives stimuli from the environment (Washington *et al*., 2001:182). According to Williams (2003:1), the skin is a highly self-repairing barrier designed to keep the insides in and the outside out and offers ideal and multiple sites to administer therapeutic agents for both local and systemic actions. An understanding of the nature, properties and function of the human skin is essential to understand the routes and mechanisms by which medicaments penetrate the skin barrier (Lund, 1994:135).

The structure of the skin can be categorised into three main layers, namely: the viable epidermis, the underlying dermis and the innermost subcutaneous fat layer (hypodermis) (Lund, 1994:136).

2.2.1 Epidermis

The epidermis is a thin, dry and tough outer protective layer. Its thickness varies, being thick on the plantar and palmer areas and thin behind the ear (Lund, 1994:137). Although nutrients and waste products must diffuse across the dermo-epidermal barrier, the epidermis contains no blood vessels and forms a barrier against water and nutrient loss from the body. The epidermis contains four distinct layers, the stratum germinativum, the stratum spinosum, which is several strata of polyhedral cells lying above the germinal layer, the stratum granulosum, a layer of flattened nucleated cells containing keratohyaline granules and the stratum corneum (Fitzpatrick *et al*., 1979:42; Lund, 1994:135; Williams 2003:5).

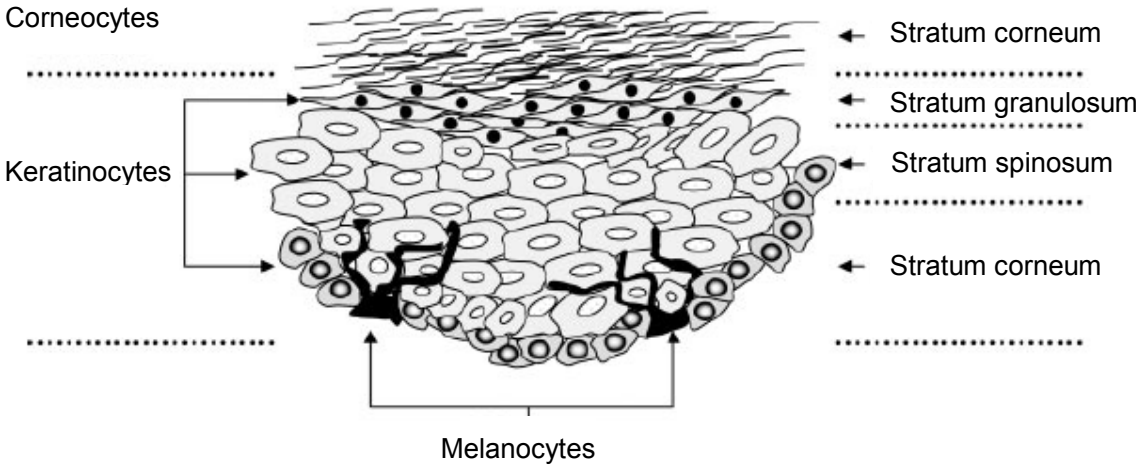

Figure 2.3: Layers of the epidermis (Costin & Hearing, 2007:977)

The stratum germinativum, also referred to as the stratum basale, is the only epidermal layer containing cells that undergo cell division, known as keratinocytes. In addition to the

keratinocytes, the stratum basale contains melanocytes, the cells responsible for the synthesis of melanin (Williams, 2003:7). Other cells found in the stratum basale include Langerhans cells, which play a role in the body's immune defences and Merkel cells involved in sensory reception (Washington *et al*., 2001:183; Williams, 2003:8-9).

Within the stratum basale and the stratum spinosum, keratinocytes are connected through desmosomes. Subsequent to stem cell proliferation new keratinocytes are formed, pushing existing cells towards the surface. During this upward transfer, keratinocytes begin to differentiate, achieving terminal differentiation in the stratum corneum (Delgado-Charro & Guy, 2001:208).

The stratum corneum is recognised as the rate-limiting barrier to the ingress of materials and is considered as the tissue predominantly responsible for the remarkable impenetrability of the skin. This layer is however not an absolute barrier, as trace elements of penetrants can be detected (Lund, 1994:136). It comprises 10 – 15 cell layers, is around 10 µm thick when dry (Williams, 2003:9) and is composed of dead anucleate, keratinised cells in an amorphous matrix of proteins with lipid and water soluble substances. The uppermost layers of the stratum corneum flake off or desquamate, as biochemical and histological components attaching these cells to each other deteriorates, while desmosomal structures tightly packs and holds together the layers which are in close proximity with the viable epidermis (Zatz, 1993:35-36).

2.2.2 The dermis

Below the epidermis is the dermis (Lund, 1994:137), a fibrous layer that supports and strengthens the epidermis. The dermis is 3 – 5 mm thick and constitutes between 15 – 20 % of the total body weight. It comprises of a loose connective tissue composed of fibrous proteins-collagen, elastin, reticulin and an amorphous substance (Washington *et al*., 2001:184; Williams, 2003:2).

The main structural component of the dermis is referred to as a coarse reticular layer while a thin papillary layer adjacent to the epidermis not only provides the nutritive, immune and other support systems for the epidermis, but also plays a role in temperature, pressure and pain regulation (Walters & Roberts, 2002:19). The most important functions of dermis are the protection of the body from mechanical injury and the maintenance of homeostasis (Fitzpatrick *et al*., 1979:58).

2.2.3 Subcutaneous fat layer

The subcutaneous fat layer principally serves to insulate the body and to provide mechanical protection against shock (Williams, 2003:2). It is composed of loose fibrous connective tissue, which contains fat and elastic fibres. If drugs reach this layer, it is considered to have entered the systemic circulation, however entry into the blood can be delayed as the fat deposits may serve as a deep compartment for the drugs (Washington *et al*., 2001:184).

2.2.4 Skin appendages

Epidermal appendages include sweat glands (eccrine and apocrine), sebaceous glands and hair follicles (Barry 2002a:502). The 'intact' barrier provided by the stratum corneum, the appendages may offer a potential route by which molecules could enter the lower layers of the skin (Williams 2003:5).

2.3 PIGMENTATION

2.3.1 Melanocytes, melanosomes and melanin

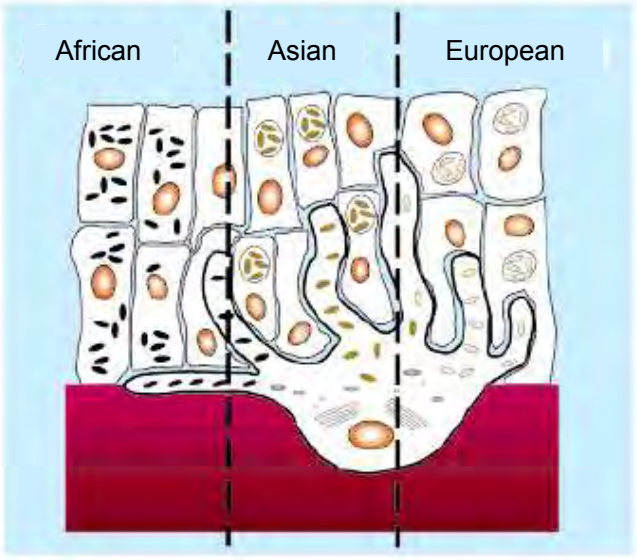

Figure 2.4: The visual gradation of skin and hair colour is determined by chemical differences in the melanin pigments contained within the melanosome as well as differences in the level of melanization (Sturm *et al*., 1998:714)

Variation in the visual appearance of human skin can be accounted for by melanin (Jablonski & Chaplin, 2000:58). Melanocytes remain the key components of the skin's pigmentary system

(Tsatsmali *et al.,* 2002:125). They are derived embryonically from neural crest cells that migrate into the basal layer of the epidermis where they synthesize and store melanin in unique membrane-bound organelles termed melanosomes (Stulberg *et al.,* 2003:1955, Van den Bossche *et al.,* 2006:769). Once produced, melanin is transferred into the neighbouring keratinocytes by the melanosomes (Tsatsmali *et al.,* 2002:126). This process is responsible for the variety of colours in human skin, hair and eyes (Sanchez-Ferrer *et al.,* 1995:2). The major determinant of normal skin colour is not the density but the activity of the normal melanocytes and their interactions with neighbouring keratinocytes (Bolognia & Orlow, 2003:938). Whilst melanosomes remain as singular heavily pigmented particles in African populations, they cluster into membrane bound organelles in Asian and European populations, giving rise to different skin colours as can be seen in Figure 2.4 (Sturm *et al.,* 1998:714; Van den Bossche *et al.,* 2006:770).

2.3.2 Melanization process

In mammals most visible pigmentation results from the synthesis and distribution of melanins (Hearing & Tsukamoto, 1991:2902). Melanosome production can be increased under hormone stimulation or irritation, leading to hyper-pigmentation (Stulberg *et al.,* 2003:2935). Damage induced to epidermal cells can lead to release of endocrine inducers of pigmentation such as α-melanocyte-stimulating hormone (α-MSH), a hormone known to increase the synthesis of eumelanin in human melanocytes (Ortonne & Ballotti, 2000:S17; Ortonne & Bissett, 2008:10).

Three melanocytic-specific enzymes, tyrosinase, tyrosinase-related protein-1 (TRP1) and tyrosinase-related protein-2 (TRP2), are involved in this enzymatic process (Ortonne & Balotti, 2000:S16). The first and rate-limiting step of melanin formation is mediated by tyrosinase. Tyrosinase catalyzes the hydroxylation of tyrosine into 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA into DOPAquinone. According to Sanchez-Ferrer *et al.* (1995:2) quoted by Villarama and Maibach (2005:148), at this stage of the melanization process, melanocytes may either enter into the classical pathway leading to eumelanin (darker pigment) formation or, pheomelanin (lighter pigment) formation depending on the ratio of sulfhydryl compounds such as cysteine and/or glutathione (GSH) within melanocytes (Villarama & Maibach, 2005:148).

In the absence of cysteine and/or GSH, DOPAquinone is oxidized to form DOPAchrome as the intermediate product of eumelanin, which results in the advance of eumelanogenesis. DOPAchrome is then tautomerized to 5,6-dihydroxyindole-2carboxylic acid (DHICA) in the

presence of TRP-2. TRP-1 promotes the further oxidation and polimerization of DHICA melanins (Petit & Piérard 2003:169).

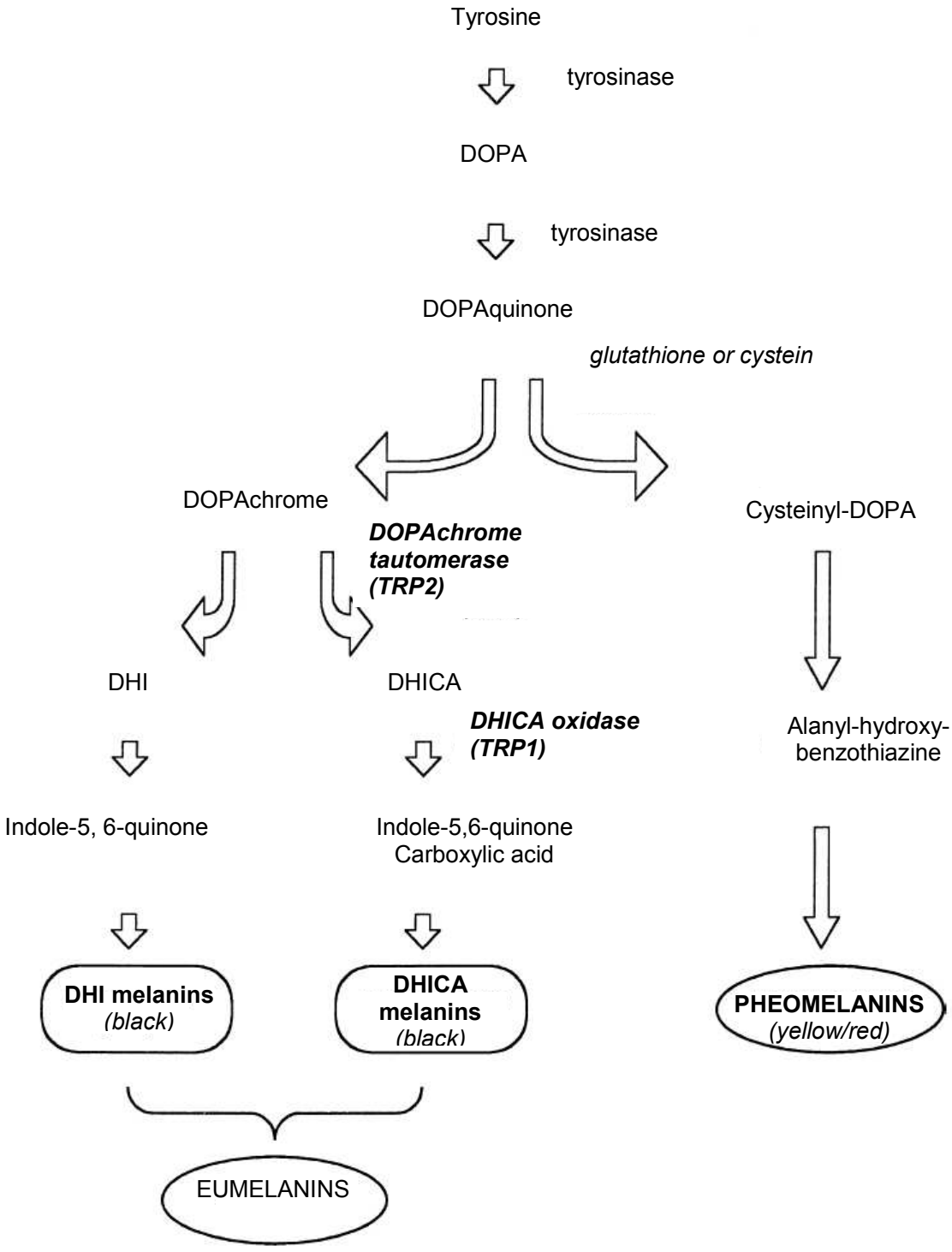

Figure 2.5: A simplified overview of the melanogenic pathway (Petit & Piérard, 2003:171)

In the presence of cysteine or glutathione DOPAquinone is coupled with their SH groups to form cysteinyl-DOPA as a precursor of sulphur-containing pigment known as pheomelanin, which corresponds to the progress of pheomelanogenesis (Petit & Piérard, 2003:169; Tsuji-Naito *et al*., 2007:1967).

During the expression phase, melanosomes are transferred from the melanocytes to the upper skin cell layers followed by incorporation of these melanosomes by keratinocytes. This can occur via three mechanisms: (1) cytophagocytosis, (2) direct injection of melanosomes into keratinocytes, and (3) release of melanosomes into the extracellular space (Fitzpatrick *et al*., 1979:133).

Mature melanosomes (ellipsoidal eumelanosomes or spherical pheomelanosomes) migrate towards the farthest point of the melanocyte dendrites where they are transferred to the surrounding keratinocytes.

The keratinocytes transfer these melanosomes to the surface of the skin, where they are expressed. After this transfer takes place, melanin colour eventually becomes visible on the skin surface (Pigmentation, 2005:2). Figure 2.5 illustrates a simplified overview of the melanocytic-specific enzymes involved in the enzymatic process during melanogenesis (Petit & Piérard, 2003:171).

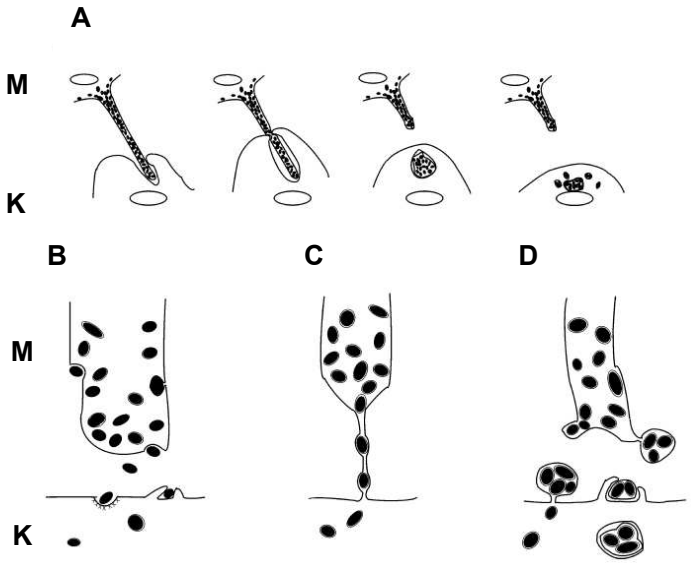

Figure 2.6: Different modes of melanosome transfer: **A**: Cytophagocytosis, **B**: Exocytosis, **C**: Fusion, **D**: Membrane vesicles, **M**: melanocyte and **K**: keratinocyte.

2.3.3 Hyper-pigmentation disorders

Hyper-pigmentation skin disorders occur commonly and manifest in a range of different forms.

Increased pigmentation can be accounted for by two separate mechanisms. Each mechanism may arise in the epidermis, dermis, or mixed (dermis and epidermis). This happens either by increased melanin production by existing melanocytes (melanotic hyper-pigmentation) or from proliferation of active melanocytes (melanocytotic hyper-pigmentation) (Cayce *et al*., 2004:402). The majority of hyper-melanoses transpire as a result of increased melanin production with normal numbers of melanocytes. Numerous internal factors (hormonal influences: melasma; inflammation: PIH) and external stresses (UV radiation: tanning and photoaging; drugs; chemicals) affect human skin pigmentation (Cayce *et al*., 2004:402-403).

2.3.3.1 Hyper-pigmentation induced by internal factors

2.3.3.1.1 Melasma

Melasma, formerly known as chloasma or the "mask of pregnancy", is an acquired form of hyper-pigmentation and is mostly seen on the face (Ting & Barankin, 2005:353). It is exacerbated by exposure to sunlight and is seen most frequently in young women of childbearing age. It is known to appear at any time during a woman's reproductive years and is often associated with pregnancy or oral contraceptive use (Baumann & Martin, 2006:316).

Multiple factors such as UV exposure, hormone therapy, genetic influences, certain cosmetics, endocrine or hepatic dysfunction, and selected anti-epileptic drugs can also contribute to melasma (Cayce *et al*., 2004:403).

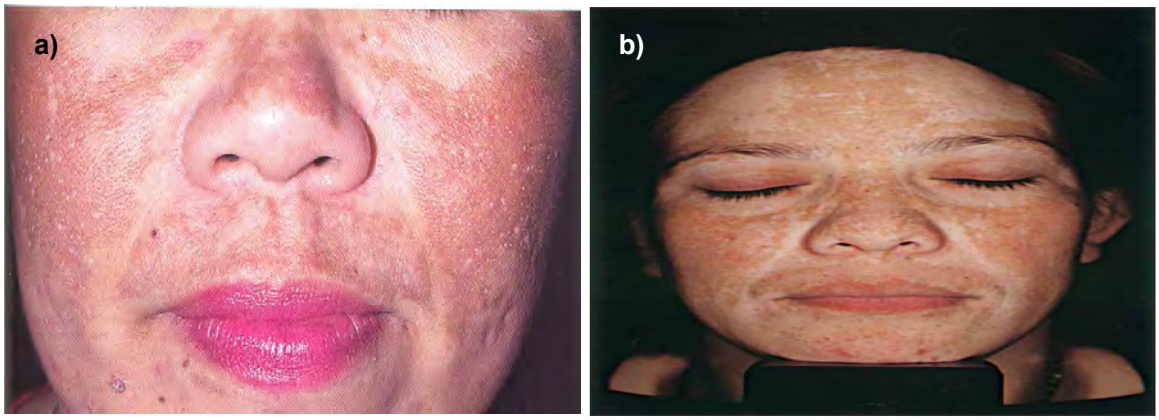

Figure 2.7: Melasma of the face: **a)** Pigmented macules on the upper lip, and cheeks (Habif, 2004:693), **b)** Hyper-pigmented macules on the cheeks, forehead nose and upper lip (Guevara & Pandaya, 2003:969).

Although melasma is more common among women with darker skin types (Pandaya & Guevara, 2000:91), it appears in all racial groups, but occurs more frequently in those persons with Fitzpatrick skin types IV to VI who live in areas of high UV radiation, often deepened by sun exposed hyper-pigmented areas (Rendon *et al*., 2006:S272). The most commonly affected areas are the cheeks, upper lip, nose and forehead (Pandaya & Guevara, 2000:91).

Three patterns of melasma are recognized clinically: centrofocal (most common), malar, and manidbular. Based on Wood's light examination (used in diagnostic areas involving pigmentation disorders, cutaneous infections, and the porphyries) of the skin, melasma can be divided into three types: the epidermal (increased melanin predominantly in the basal and supra-basal epidermis), dermal (melanin-laden macrophages in a perivascular distribution in the superficial and deep dermis) and the mixed type (combination of the epidermis and dermal type, appearing as a deep brown colour (Pandaya & Guevara, 2000:91).

2.3.3.1.2 Post-inflammatory hyper-pigmentation (PIH)

PIH is an acquired excess of pigment in areas of the skin after inflammation. This acquired excess of pigment can be diffuse or circumscribed, depending on the cause and extent of the inflammation. Diagnosis of the condition may be more difficult if the cutaneous inflammation was transient or went by unnoticed by the patient, and relatively easy when the patient gives a history of a preceding cutaneous lesion, eruption or treatment (Ruiz-Maldonado & Orozco-Covarrubias, 1997:37, 39). At cellular level, PIH is characterized by a normal number of melanocytes that have increased melanin production (Costin & Hearing, 2007:989). Persons with darkly pigmented skin have a greater risk of hyper-pigmentation than those with a lighter skin colour, presumably because of an already higher baseline epidermal melanin content, although there is no gender predominance (Cayce *et al.,* 2005:404; Brenner & Hearing, 2008:e193).

PIH is manifested by discrete, hyper-pigmented macules with indistinct, feathered margins which may involve the epidermis and or dermis (Costin & Hearing, 2004:989). Differential diagnosis include nutrition deficiencies, systemic diseases (patterns differ from trauma), skin infections and infestations and itchy conditions (Peel *et al*., 2003:195).

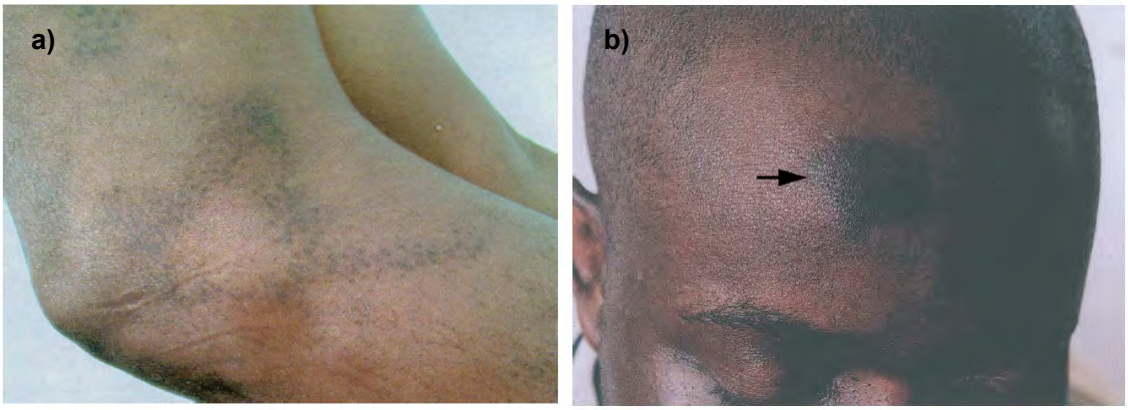

Figure 2.8: PIH: **a)** Male in his early 40's from southern Africa, caustic liquid poured over leg, **b**) Male in his early 30's from West Africa – struck on mid forehead by butt of gun (Peel *et al*., 2003:195)

2.3.3.2 Hyper-pigmentation induced by external factors

2.3.3.2.1 UV influence on human pigmentation

UV radiation is divided in UV-A (320 – 400 nm), UV-B (280 – 320 nm) and UV-C (200 – 280 nm); the latter is normally screened by the ozone layer and does not reach the Earth's surface, like most wavelengths at 280 nm. The skin's reaction to UV radiation results in two defensive barriers: thickening of the stratum corneum and the elaboration of a melanin filter in cells of the epidermis. The palms and soles are the regions with the thickest stratum corneum, and they are exceptionally resistant to UV damage. UV radiation sets in action an integrated mechanism for the formation and delivery of melanin within melanosomes from melanocytes to keratinocytes.

Both UV-A, UV-B stimulate the production of melanin, which constitutes the basis for tanning (Costin & Hearing, 2007:982).

2.3.3.2.2 Lentigines

Solar lentigines are a common dermatologic condition that manifest as localized, hyper-pigmented, macular lesions usually found on sun-exposed areas of the skin (Draelos, 2006:239). These macular lesions range in size from a few millimetres to more than a centimetre in diameter. Synonyms for this condition include actinic lentigines, liver spots, age spots, and sun spots. The potential negative social impact of this condition should not be disregarded in view of the fact that lesions appear on highly visible parts of the body, such as

the face, neck, hands, and forearms (Ortonne *et al.,* 2006:S262). This benign condition is caused by an increased number of active melanocytes and increased melanin production in response to chronic, accumulated UV radiation exposure (Draelos, 2006:239).

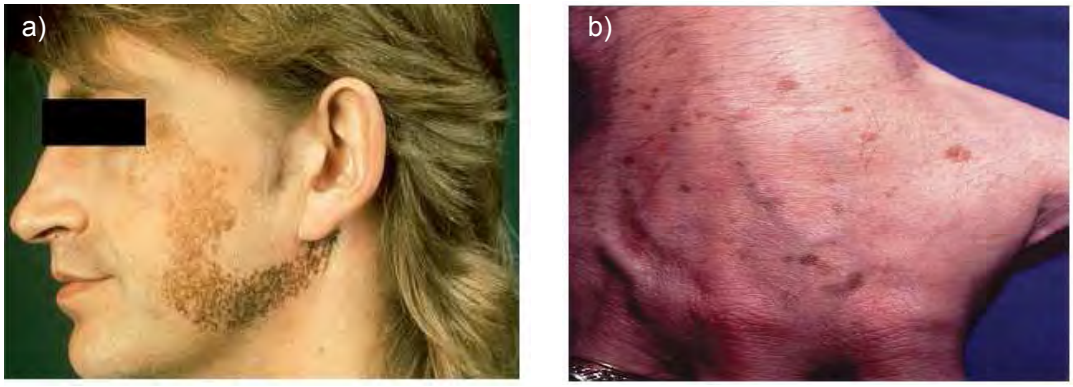

Figure 2.9: Solar lentigo: a) Tightly packed lentigines (Happle *et al.,* 2010:1), b) Solar lentigines on the hand (Ortonne *et al.,* 2006:S263)

2.3.4 Depigmenting agents

Pigmentation disorders have been treated with several depigmenting agents since 1961 when hydroquinone was introduced as a skin lightening agent. Although these agents have been used for the treatment of melasma and PIH, they have been alternatively used for the treatment of ephelides, solar lentigines, nevi, and lentigo maligna. Most of the currently available bleaching or depigmenting agents cause a temporary removal of hyper-pigmentation, which usually recurs after discontinuation of therapy (Katsambas *et al*., 2001:483).

Depigmentation can be achieved by regulating: (i) the transcription and activity of tyrosinase, TRP-1, TRP-2, and/or peroxidase; (ii) the uptake and distribution of melanosomes in recipient keratinocytes and (iii) melanin and melanosome degradation and turnover of pigmented keratinocytes (Briganti *et al.,* 2003:102).

The ideal depigmenting agent has to fulfil certain pharmacologic criteria: (1) it must have a potent bleaching effect with a rapid time of onset, (2) it should carry no short- or long term side-effects and (3) it should lead to a permanent removal of undesired pigment (Katsambas *et al*., 2001:483).

Inhibitors of tyrosinase activity have been reviewed previously by various authors. Many targets exist for controlling melanin synthesis via the regulation of tyrosinase since suppression of

melanin production by melanocytes would be an effective approach to treat a variety of hyper-pigmentation disorders (Ando *et al*., 2007:751-752).

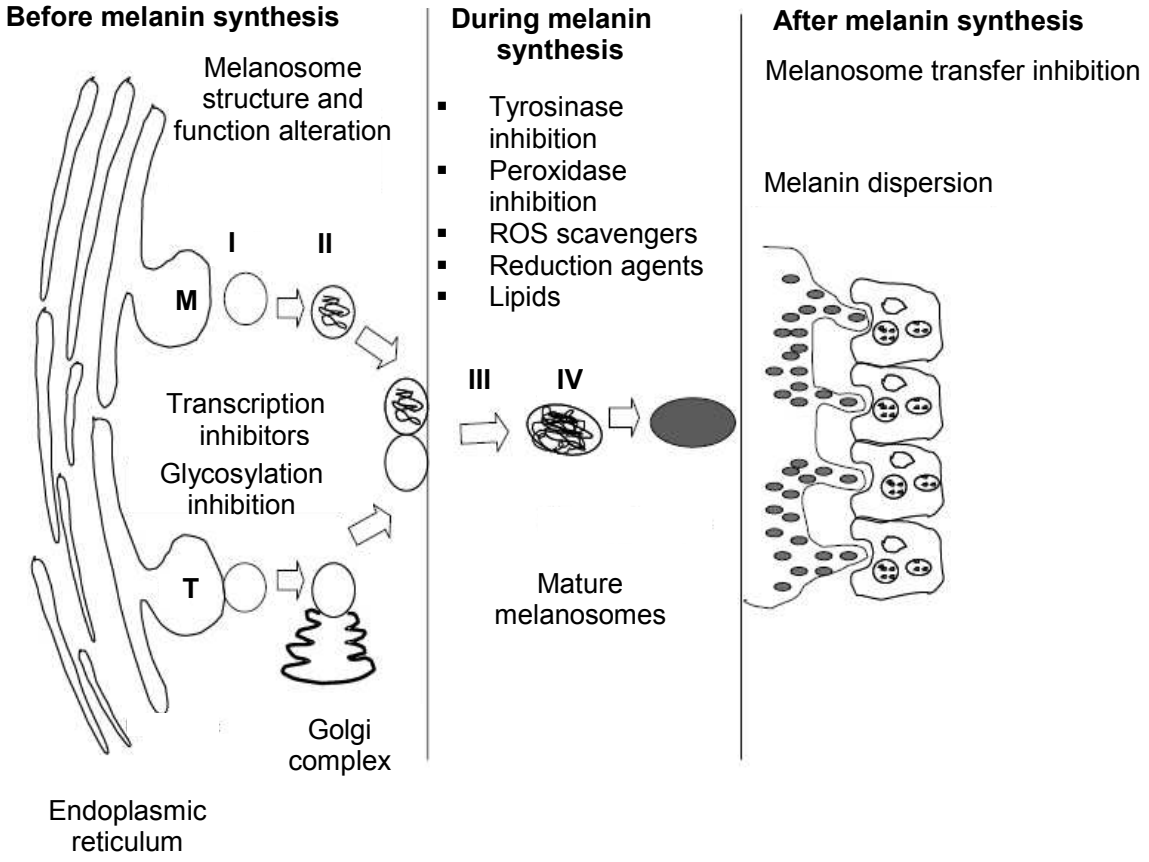

Figure 2.10: Schematic illustration of the possible approaches to interfere with the melanogenesis pathway (T = tyrosinase; M = melanosomes; ROS = reactive oxygen species)

The following section will focus on two of the above mentioned pigmentation control targets and agents namely: hormonal inhibition of tyrosinase by cytokines TGF-β1 and TNF-α.

2.3.4.1 Melanogenesis inhibition by TNF-α

TNF-α induces a heterogeneous array of biological effects and may elicit cell proliferation, differentiation or apoptosis according to the cell type (Englaro *et al*., 1998:1553). At nanomolar concentrations TNF-α has been reported to inhibit both tyrosine hydroxylase and DOPAoxidase activities of tyrosinase in B16 melanoma cells, without affecting levels of tyrosinase-related protein 2/DOPAchrome tautomerase (TRP2/DCT). TNF-α elicits a dose-dependent decrease in the activity of tyrosinase and inhibits melanocyte proliferation. Melanocytes remain viable

despite continuous treatments with TNF-α. Its effects thus appear to be cytostatic, with recovery of cell proliferation upon cessation of TNF-α treatment (Slominski *et al*., 2004:1196). According to Martinez-Esparza *et al*. (1998:141), the effect of TNF-α on the proliferation of B16/f10 melanocytes was found to similar to that observed for TGF-β1.

2.3.4.2 Melanogenesis inhibition by TGF-β1

TGF-β1 exerts its depigmenting effect by decreasing tyrosinase and TRP-1 levels, by means of decreasing both gene expression and the intracellular half-life of the tyrosinase, but does not appear to block tyrosinase stimulation by α-MSH (alpha-melanin stimulating hormone) (Slominski *et al*., 2004:1196). In spite of similar fold-activations by both TGF-β1 and TNF-α, the final tyrosinase activity is lower in cells treated with the cytokines and α-MSH than in the presence of the hormone alone, suggesting independent modes of action for the cytokines on one hand, and α-MSH on the other (Martinez-Esparza *et al*., 2001:973). Its inhibitor effects may reside at the rate-limiting step of the melanogenic pathway since treatment with TGF-β1 does not appear to alter melanosome number, but results in a lowered percentage of fully mature stage IV melanosomes, resulting in the accumulation of incompletely melanized melanosomes, and therefore the inhibition of total melanin formation, and a hypo-pigmenting effect. TGF-β1 thus blocks the α-MSH-induced increase in melanosome number (Martinez-Esparza *et al*., 2001:971).

2.4 PEPTIDES AS DRUGS

The following section will focus on the application of TNF-α and TGF-β1 as peptide/protein drugs.

2.4.1 Peptides/proteins

Peptides and proteins are amphoteric (they either have a positive or negative charge) hydrophilic polyelectrolytes that attain their ionic nature from the weakly acidic or basic side chains of their constituent amino acids. Their molecular weights range from 300 g/mol to greater than 1 000 000 g/mol. Most physiological processes are regulated by peptides at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors (Edwards *et al*., 1999:1). These drugs tend to be specific in their actions, and thus have few side-effects, are effective at low concentrations and can be endogenous (from human origin) and therefore non-allergenic (Amsden & Goosen, 1995:1972).

Due to inactivation by gastrointestinal enzymes, peptides cannot be administered orally. Subcutaneous or intravenous administration is required. Problems such as local targeted delivery and the blood-brain barrier prevent peptides from readily gaining access to the required site of action. Research is thus focussing on alternative routes of delivery including inhaled, buccal, intranasal and transdermal routes with novel delivery systems such as the use of protective liposomes (Edwards *et al*., 1999:1).

2.4.1.1 Challenges facing protein/peptide delivery

According to Davis *et al*. (1986:269), attempts to deliver peptides and proteins to central sties via percutaneous absorption, faces many difficulties (chemical, biological and technical). In most cases, more than one pathway of physical and/or chemical instability may be responsible for the degradation of peptides and proteins (Aboofazeli, 2003:1). Some of the challenges faced by proteins and peptides are:

- Poor intrinsic permeability across biological membranes due to the hydrophobic nature and large molecular size and many functional groups.
- Their structures are stabilized by relatively weak physical bonds and are readily and irreversibly changed. This may directly affect their interaction with the receptor and change their pharmacokinetic characteristics, e.g. their clearance or make them immunogenic and non-biocompatible.
- They are vulnerable to proteolytic attack.
- Proteins/peptides tend to undergo aggregation, adsorption and denaturation (Amsden & Goosen, 1995:1972; Barry, 2002a:545).

2.4.1.2 Administration of Pharmaceutical peptides

Peptide drugs can greatly benefit from controlled release administration technologies as these products provide prolonged delivery of a drug while maintaining its blood concentration within therapeutic limits. According to Lee (1991) and Tauber (1989) (quoted by Amsden & Goosen, 1995:1973), in all the epithelial routes of administration (intranasal, buccal, oral, rectal, vaginal pulmonary and transdermal) enzymatic activities are present that differ strongly among various organs and tissues, whereas the skin exhibits less enzymatic activity although it contains aminopeptidases, leading to increased bioavailability of the delivered peptide or protein (Antosova *et al.,* 2009:631).

2.4.2 TGF-β1

TGF-β denotes a family of structurally related polypeptide growth factors which control proliferation and differentiation of many cell types (Rodeck *et al*., 1994:575). Three differentially regulated mammalian isoforms termed TGF-β1, -β2, and -β3, are important endogenous mediators of growth, maintenance, and repair processes in the developing embryo, neonate, and adult. Each one of the three human isoform genes encodes a product that is cleaved intracellularly to form two peptides, each of which dimerises (Govinden & Bhoola, 2003:258). TGF-β initiates a number of changes in all responsive cells, some of which may lead to proliferation or proliferating arrest (Nilsen-Hamilton 1990:127). Cox and Maurer (1997:25) states that all three isoforms have been shown to be potent endogenous mediators of tissue pair via their stimulatory effects on chemotaxis, angiogenesis, and extracellular matrix (ECM) deposition within the wound environment.

Synthesized as large precursor proteins, TGF-βs consist of an amino-terminal pro-domain (comprising a signal sequence and latency-associated protein or LAP) and a mature carboxy-terminal subunit of 112 amino acids (Cox & Maurer, 1997:25). This LAP, secreted by all cells abundant both in circulating forms and bound to the ECM, is a fundamental component of TGF-β1 that is required for its efficient secretion, preventing it from binding to ubiquitous cell surface receptors, and maintains its availability in a large extracellular reservoir that is readily accessed by activation (Govinden & Bhoola, 2003:258).

2.4.2.1 Functions in the human body

TGF- β1 expression occurs shortly after injury (auto-induction). This important ‘‘triggering’’ signal can be up-regulated by addition of exogenous isoforms. Direct effects on healing have also been reported following local application to other non-cutaneous tissues such as bone, intestine, and the eye. Preclinical data collected over the last 10 years demonstrates that topical or local administration of natural or recombinant TGF-β1 or -β2 improves or accelerates cutaneous healing (Cox & Maurer, 1997:25). Although many of the research conducted reports on TGF-β1 inhibition in melanoma cells, normal melanocytes have been reported to be responsive to TGF-β1 treatment (Martinez-Esparza, 2001:976).

In the adult, TGF-β1 delivers cytostatic and cell death signals, which help maintain tissue homeostasis, and their loss contributes to tumour development. Cancer cells avoiding TGF-β-mediated cytostasis may then use this factor with impunity to exacerbate their own proliferative, invasive, and metastatic behaviour TGF-βs are known for their regulation of chemotaxis and activation of monocytes and fibroblasts, thereby playing an essential role during

tissue repair. TGF-βs have also reported to have selective and potent blocking effects on human bone marrow haematopoietic progenitor cells (Cox & Maurer, 1997:27). In studies conducted by Cox & Maurer (1997:27), it was concludes that the topical application of TGF-βs may provide a safe and effective means for attenuating, or preventing, side-effects of cancer therapies in humans. The diversity of pharmacological effects produced by TGF-βs necessitates evaluation of biologics by review boards with an extensive preclinical safety evaluation program as a prerequisite to studies in humans. A topically applied recombinant human TGF-β will confront its intended target before reaching the systemic circulation, however, since growth factors usually act locally (i.e., with minimal systemic absorption), plasma levels may not be a true reflection of bioavailability. Furthermore, topical doses are usually so low that the plasma and or urine concentrations of the agent are often beyond the limits of quantification and/or detection using current assay techniques. According to Cox & Maurer (1997:27), it is thus important to consider the dose, frequency, and duration of administration, but also the intended target tissue (Cox & Maurer, 1997:27).

2.4.3 TNF-α

Identified as a 17 kDa protein; TNF-α affects multiple responses including signals for cellular differentiation, proliferation and death. Its functions can be both beneficial and deleterious (Oikonomou *et al.,* 2006:e208; Paul *et al.,* 2006:725). When exposed to endotoxin and related stimuli, macrophages release large quantities of TNF-α. Upon reaching the systemic circulation, TNF-α binds to receptors in normal tissues and triggers a wide array of biological effects. In neutrofills it stimulates activation, respiratory burst, degranulation, and adherence to vascular endothelium. Although TNF-α functions locally as a paracrine and autocrine regulator of leukocytes and endothelial cells, signs of septicaemia develops when it gains systemic access which could lead to high lethal levels of the cytokine (Adams, 2001:428-429).

TNF-α mediates its diverse biologic effects through two distinct receptors known as TNF-α receptor type I (TNF-R1) and TNF-α receptor type II (TNF-R2) with apparent molecular masses of 55 – 60 kDa and 75 – 80 kDa, respectively (Paul *et al*., 2006:725). It's signalling involves various pathways and molecules. Binding of TNF-α to TNF-R1 initiates a cascade of events involving the activation of a series mitogen-activated protein kinase kinases (MEKKs) that further phosphorylate and activate a dual-specificity protein kinase (MEK), followed by a mitogen-activated protein kinase (MAPK). Activated MAPK then phosphorylates downstream kinases and nuclear factor-κB (NF-κB). The inappropriate activation of NF-κB by TNF-α in diseases triggers inflammatory diseases (Paul *et al*., 2006:726).

2.4.3.1 Functions in the body

TNF-α production can be triggered by numerous stimuli resulting in the induction of acute inflammation by enhancing endothelial permeability, inflammatory cell recruitment, and release of superoxide anion and addition cytokines by polymorphonuclear leukocytes (Granel *et al*., 2004:187). Inflammation is the normal immune response to infection or injury, however chronic or high levels of TNF-α can lead to either a persistent or an overly robust inflammatory response.

Though many literature articles reports on the inflammatory responses elicited by TNF-α, it plays an important role in normal host resistance to infection and growth of malignant tumours. In a study conducted by Naessens *et al*. (2005:408), with *T. congolense* infected TNF-α-deficient mice, it was concluded that TNF-α does not mediate anaemia and major pathology. It affects multiple responses that extend well beyond its well-characterized pro-inflammatory properties to include diverse signals for cellular differentiation, proliferation and death; its functions can be both beneficial, as well as detrimental (Oikonomou *et al*., 2006:1). The net effect of TNF-α depends upon its concentration and its interaction with other inflammatory mediators (Shendurnikar & Shastri, 1993:487).

2.4.4 Peptide/protein drug delivery by means of enzyme inhibition

Skin metabolism by aminopeptidases occurs mainly in the viable dermis, giving rise to active or inactive metabolites, more or less active than the parent drug (Amsden & Goosen, 1995:1975). Physical instability and chemical instability may occur for a given peptide or protein, due the presence of multiple susceptible sites as proteolytic enzymes present as most routes of administration are able to quickly metabolize most peptides (Rishabh *et al.,* 2009:232; Aboofazeli, 2003:1). In order to overcome this problem, protein solutions are treated with protease inhibitors to slow down metabolic degradation (Barry, 2002a:552).

2.4.4.1 Aminopeptidase inhibition by bestatin hydrochloride

Bestatin hydrochloride is a specific inhibitor of aminopeptidase B and leucine aminopeptidase. First isolated from a culture filtrate of *Streptomyces olivoreticuli* by Umezwa *et al.* (1976:97-99), bestatin hydrochloride has been shown to inhibit cytosolic exopeptidases in mammalian cells, resulting in the accumulation of di- and tripeptide intermediates in cellular protein degradation (Scornick & Botbol, 1997:798). Although a wide array of enzyme inhibitors exists, bestatin hydrochloride was selected for this study, based on the successful inhibition of aminopeptidases the transdermal delivery of arginine vasopressin by Coetzee (2007).

2.4.4.2 Mechanism of bestatin's inhibition

Bacterial aminopeptidases can be sub-divided into three main catalytic groups based on their mechanism of catalysis and the structure of their active sites (Jankiewicz & Bielawski, 2003:217). Bestatin exerts its activity by competing with the substrates, binding to the catalytic site of the enzyme, exhibiting a competitive kinetics with the substrate once bound. A delayed reaction is obtained at lower concentrations, with a lag time of up to one hour, whereas higher concentrations render shorter lag times (Scornick & Botbol, 2001:71). In mammalian cells, bestatin permits the degradation of cellular proteins to di- and tripeptides, but inhibits the further degradation of these peptides to amino acids (Scornick & Botbol, 1997:798).

2.4.4.3 Utilisation of bestatin as a therapeutic agent

Aminopeptidases N is a major bestatin-sensitive enzyme involved in the degradation of oligopeptides on the surface of intestine and kidney brush borders, and the inactivation of encephalin in the brain. Bestatin is one of the first aminopeptidase inhibitors that could be administered to cultured cells, intact animals and humans, with low toxicity with a multitude of effects on the immune system, both *in vivo* and *in vitro* (Scornick & Botbol, 2001:72). It suppresses the production of the pro-inflammatory cytokines and stimulates the anti-inflammatory cytokine by activated monocytes and macrophages. In a study conducted by Lkhagvaa *et al*. (2008:390), bestatin was shown to be useful as an immunomodulator for the control and treatment of various inflammatory diseases. It has developed into a useful tool for:

- the elucidation of the physiological role of some mammalian exopeptidases in the regulation of the immune system,
- in the growth of tumours and their invasion of surrounding tissues, in the degradation of cellular proteins,
- in the digestion and absorption of peptides on the brush border of the intestine and the kidney,
- in the reproductive system, and
- in the metabolism of opioid peptides and leukotrienes.

2.5 PERCUTANEOUS ABSORPTION AND TOPICAL DRUG DELIVERY

Medicated applications are placed on the skin to ameliorate infections and diseases or to provide relief of disease symptoms. Three types of drug delivery can be distinguished: (1) delivery to the local tissues immediately beneath the application site, (2) delivery to the deep

regions in the vicinity, but still remote from the application site, and (3) the systemic circulation to mediate pharmacological changes somewhere totally removed from the application site pivotal (Flynn & Weiner, 1993:33).

Percutaneous absorption can be described as the penetration of a substance through the skin and subsequent movement into the systemic circulation (Lund, 1994:135). Since percutaneous absorption is an essential aspect to the effectiveness of both topical and transdermal systems, there is a tendency to view these systems as being functionally closely related (Flynn & Weiner, 1993:33-34).

Topical delivery can be defined as the shallow penetration of the active ingredients to directly treat cutaneous disorders or the manifestations of general disease with the intent of confining the pharmacological effect of the drug to the surface of the skin or within the skin (Ghosh *et al.,* 1997:7). The objective of a transdermal delivery system is aimed at achieving systemically active levels of a drug (Washington *et al.*, 2001:182; Flynn & Weiner, 1993:33-34). While topical dermatological therapy is aimed at increasing the retention of therapeutic drugs in the skin, rather than the penetration of the drug through the skin, the goal of transdermal drug therapy is to maximise flux of the drug through the skin into the systemic circulation and simultaneously minimizing the retention and metabolism of the drug in the skin (Hsieh, 1994:11-12).

Topical drug delivery offers the advantages of:

- Avoidance of first pass metabolism.
- Better patient compliance.
- Sustained drug release (useful for drugs with short biological half-lives requiring frequent oral or parenteral administration).
- Continuous intervention (system repositioning, removal or replacement).
- A relatively large and readily accessible surface area for absorption.
- Decreased side-effects (peak plasma levels of drugs are reduced).
- Easy termination of drug delivery in case of toxicity.
- Controlled input kinetics which is mainly indispensable for drugs with narrow therapeutic indexes (Naik *et al.,* 2000:319; Dmochowski *et al.,* 2006:515; Kumar & Philip, 2007:634).

Disadvantages to topical drug delivery are:

- Limited number of drugs may be used.
- Local dermatologic reactions at the application site.
- Intolerance of or reaction to the transdermal formulation itself.
- Variable absorption secondary to individual skin characteristics and local dermal metabolism.
- Patient overdose (Dmochowski *et al.,* 2006:515; Kumar & Philip, 2007:634).

To maintain an effective concentration at the site of action, topical products should be applied in sufficient quantity at a suitable rate to achieve a therapeutic effect. Where skin penetration for local activity is the aim, the topically active substance should be retained for as long as possible in the viable dermis and epidermis (Lund, 1994:135).

2.6 ROUTES OF TOPICAL DRUG DELIVERY

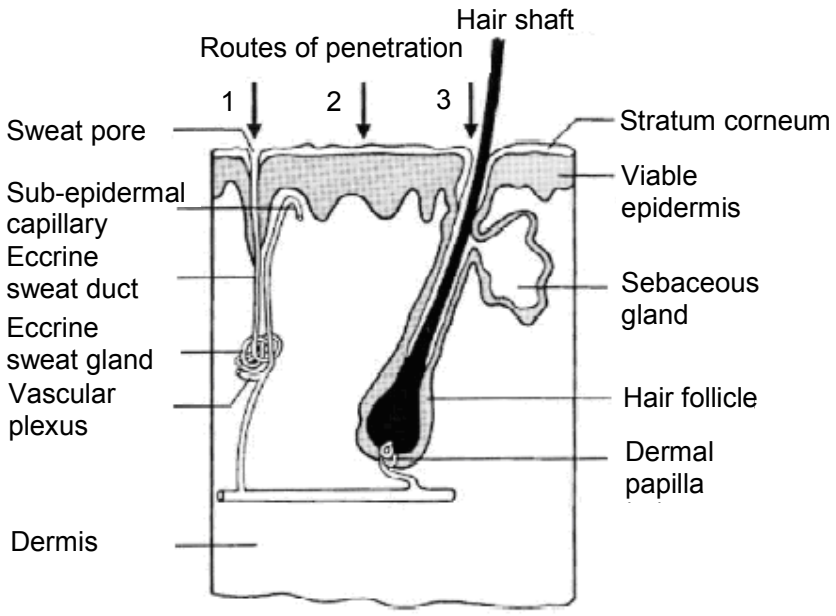

Figure 2.11: Simplified diagram of skin structure and macro-routes of drug penetration: (1) via the sweat ducts; (2) across the continuous stratum corneum or (3) through the hair follicles with their associated sebaceous sweat glands (Barry, 2001:102)

Depending on the target area of drug delivery, drug diffusion can be considered as the passage through a series of diffusional barriers. Such a drug would have to pass first through the

stratum corneum, the epidermis and the dermis, each of which has different barrier properties (Washington *et al*., 2001:186).

As soon as a molecule reaches the skin, it comes into contact with cellular debris, micro-organisms, sebum and other materials. Subsequently , the diffusant can pursue one of three possible entry routes: (1) through the hair follicles with their associated sebaceous glands, (2) via the sweat ducts or (3) across the incessant stratum corneum between these appendages. Drug absorption through routes (1) and (2) is known as the transappendageal route, whilst drug absorption across the stratum corneum, termed transepidermal drug absorption occurs via two possible pathways: the intercellular and intracellular route (Lund, 1994:138; Barry, 2002b:S33).

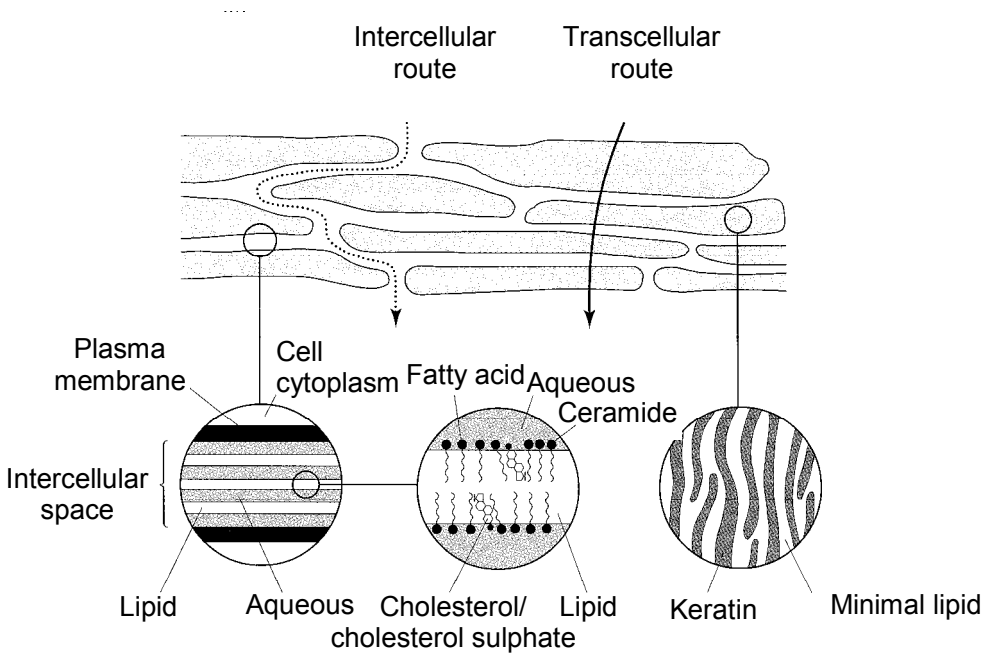

Figure 2.12: Stratum corneum and two micro-routes of drug penetration (Barry, 2001:102)

2.6.1 Transappendageal permeation

The transappendageal route does not contribute substantially to the steady state flux of a drug as the fractional appendageal area available for transport is only about 0.1 %. This route may however be important for ions and large polar molecules that cross the intact stratum corneum with difficulty (Barry, 2001:102; Barry, 2002a:506).

2.6.2 Transepidermal permeation

Transepidermal permeation first involves partitioning into the stratum corneum, followed by diffusion across this tissue. Resistance encountered along the transepidermal pathway arises in the stratum corneum. According to Scheuplein (quoted by Benson, 2005:24), it was traditionally thought that hydrophilic chemicals diffuse within the aqueous regions near the outer surface of intracellular keratin filaments (intracellular or transcellular route) whilst lipophilic chemicals diffuse through the lipid matrix between the filaments (intercellular route) (Benson, 2005:24).

2.6.2.1 Transcellular permeation

Molecules traversing the intact stratum corneum via the transcellular route face numerous challenges. Solutes diffuse through highly hydrated keratin-filled cells bound to a lipid envelope connecting the intercellular multiply bilayered lipid domains. A molecule traversing via the transcellular route must partition into the keratinocyte and diffuse through the hydrated keratin and in order to move to the next keratinocyte, the molecule must partition into and diffuse through the estimated 4 – 20 lipid lamellae between each keratinocyte (Williams, 2003:33).

2.6.2.2 Intracellular permeation

Topically applied products mainly permeate the stratum corneum along the tortuous intercellular route, through the lipid domains which plays a crucial role in proper skin barrier function. According to Barry (2001:102), many enhancing techniques are aimed at the disruption or bypass of the elegant molecular architecture of the intercellular permeation route (Barry, 2001:102). The diffusional path length of this route is much longer than the thickness of the stratum corneum and has been estimated as long as 500 mm (Hadgraft, 2004:292). Depending on their physicochemical properties, permeants traverse the stratum corneum at different path lengths (Williams, 2003:35).

2.7 TOPICAL KINETICS

Diffusion of compounds across a membrane is described by Fick's first law:

$$Js = \frac{KmDCs}{E} \qquad \textbf{Equation 2.1}$$

Where Js is the steady state flux of solute, Km is the distribution coefficient of the drug between the solvent or vehicle and the stratum corneum, Cs is the concentration difference of

solute across the stratum corneum, E is the thickness of the stratum corneum and D is the average membrane diffusion coefficient for the solute of the stratum corneum (Lund, 1994:139). The mathematical theory for isotropic materials is underlined by the hypothesis that the rate of transfer of a diffusing substance per unit area of a section is proportional to the concentration gradient (Barry, 2002a:506). The quantity of drug absorbed over unit area in unit time depends on:

- the drug's solubility and distribution characteristics,
- the concentration difference of the drug across the membrane,
- the nature of the solvent or vehicle in which the drug is presented, and
- the thickness of the stratum corneum (Barry, 2002a:506).

The condition of the skin, physicochemical characteristics of the active substances and effects due to the vehicle are factors to be considered in skin penetration (Lund, 1994:139).

2.8 FACTORS INFLUENCING TOPICAL DRUG DELIVERY

The skin comes into contact with a number of compounds daily. In most cases no local or systemic side-effects are elicited, and thus the skin is considered impermeable to many substances. However, local application of drugs is the basis of dermatological therapy and the concomitant therapeutic or toxic effects elicited systemically, are a direct proof of the permeability of the skin (Schalla & Schaefer, 1982:41).

The choice of therapeutic agent for topical delivery is dependent on a number of factors, including physicochemical properties of the drug, its interactions with the membrane and its pharmacokinetic properties. The physicochemical ones determine the rate at which it can penetrate the skin, while the pharmacokinetic factors control its clearance in order to estimate concentrations either in the lower regions of the skin or the plasma (Hadgraft & Wolff, 1993:161; Kalia & Guy, 2001:160). The most important processes to consider are the partitioning and diffusion steps that occur in the transport into, through, and out of the stratum corneum (Hadgraft & Wolff, 1993:161). A preparation applied to diseased skin clinically results from a sequence of processes:

- release of the medicament from the vehicle;
- penetration through the skin barriers; and
- activation of the pharmacological response.

These steps are affected by three components, the drug, the vehicle and the skin (Barry, 2002a:508-509).

2.8.1 Physiological factors

2.8.1.1 Skin hydration

Hydration of the stratum corneum is one of the most important factors in increasing the penetration rate of most substances permeating the skin. When water saturates the skin, the tissues swell, soften and wrinkle resulting in increased permeability (Barry, 2002a:511). Occlusive dressings and patches are highly effective strategies to increase skin delivery since they elevate stratum corneum hydration (Williams, 2003:17).

2.8.1.2 Skin age

Skin condition and structure varies with age. In the elderly, the stratum corneum thickens and is less hydrated, increasing the skin's barrier function (Washington *et al*., 2001:188). Blood concentrations from topically applied drugs are much higher in infant than adult skin although the ultra-structure of both groups is indistinguishable. This is due to the fact that epidermal enzymes capable of metabolising applied medicaments may not be fully developed in infants (Lund, 1994:140). Infants and children are also at increased risk for dermal exposure to toxic compounds because of their greater surface-to-volume ratio. Pre-term infants may demonstrate an increased percutaneous absorption of some compounds, because of the lack of a fully developed stratum corneum, unlike full-term infants who have a completely functional stratum corneum (Bronaugh *et al*., 1991:2-20).

2.8.1.3 Site of application

According to Lund (1994:140), Fick's law of diffusion states that the diffusion of a solute will be inversely proportional to the thickness of the stratum corneum. The stratum corneum is thicker on the palms of the hands and soles of the feet than on the lips or eyelids. Skin of the head and neck is also relatively permeable compared to skin on the arms and legs (Williams, 2003:16). Other than thickness, the size and lipid composition of the cells in the stratum corneum, their number of layers and associated stacking pattern, as well as the depth and distribution of the appendages play a role in deciding the extent of percutaneous absorption at a particular body site (Lund, 1994:140).

2.8.1.4 Sex and race

Both pharmacokinetic (pk) and pharmacodynamic (pd) responses are influenced by genetic and physiological differences between men and women. Pharmacological outcomes can also be affected by genomic imprinting, body size, organ size, body fat, absorption, distribution, metabolism, and elimination (Mattisson & Mattison, 2008:21). The tendency of keratinocytes to be slightly larger in females (37 – 46 μm) than males (34 – 44 μm), also suggests that the permeability characteristics of male and female skin differ (Williams, 2003:17). According to Weingand (quoted by Washington *et al*., 2001:189), race appears to influence penetration to small extent as Negroid stratum corneum has more layers and is generally less permeable, although no definite stratum corneum differences between Negroids and Europeans exists (Washington *et al*., 2001:189).

2.8.1.5 Cutaneous metabolism

A multitude of enzymes including a cytochrome P450 system have been identified in the skin. Since the epidermis is a bio-chemically active tissue with metabolic capability (Delgado-Charro & Guy, 2001:212), is likely that some topically applied drugs may be metabolized prior to penetrating the skin (Williams, 2003:21). Skin metabolism by aminopeptidases occurs mainly in the viable dermis, giving rise to active or inactive metabolites, more or less active than the parent drug. In order to overcome this problem, protein solutions are treated with protease inhibitors to help preserve the activity and nature of proteins. Bestatin hydrochloride, a specific inhibitor of aminopeptidase B and leucine aminopeptidase was selected for this study, based on the successful inhibition of aminopeptidases the transdermal delivery of arginine vasopressin by Coetzee (2007).

2.8.1.6 Pathological disorders

Although intact skin presents a barrier to absorption of substances, this barrier function can be reduced considerably when the skin is damaged or in a diseased state, resulting in higher drug absorption. Skin damage can be caused by dryness, irritation, allergic reactions, infections or by abrasion (Lund, 1994:139; Washington *et al*., 2001:189). Bacterial, viral and fungal infections may erupt from the skin as well. As many of these infections vary in their severity, so does the damage they cause to the skin barrier integrity. For many skin disorders where the barrier function is compromised, conditions improve as treatment progresses with a subsequent decrease in drug delivery (Williams, 2003:22).

2.8.1.7 Miscellaneous aspects

Lund (1994:140) reports that the application of vasoconstrictors such as steroids to the skin surface may slow penetration because of the reduced blood supply. An increased blood flow could reduce the amount of time a penetrant remains in the dermis and raises the concentration gradient across the skin as well (Barry, 2002a:510). Blood flow is influenced by temperature as well. Elevation of the skin temperature can induce structural alterations within the stratum corneum, and these modifications can increase diffusion through the tissue. Another factor most likely to cause an increase in drug permeation is the level of hydration of the skin (Williams, 2003:18). Absorption of active substances is enhanced as the skin becomes more hydrated (Lund, 1994:140).

2.8.2 Physicochemical factors

2.8.2.1 Partition coefficient

The partition coefficient is a measurement of the distribution of molecules between two phases (Williams, 2003:40), and aids penetration of the skin barrier (which has both hydrophilic and hydrophobic properties) (Lund, 1994:140) in knowing what the distribution would be. Partition coefficients are the gate-keepers controlling access of permeant to the stratum corneum. The passage of permeant through the stratum corneum cannot begin until it has been transferred from the vehicle to one of the stratum corneum components (Rieger, 1993:43). In order to cross the stratum corneum, a permeant must first partition into the membrane which could be a rate-limiting step in the permeation process, as the partition coefficient of a permeant is usually the governing factor in dictating which pathway to follow through the skin. In transdermal studies, a partition coefficient between octanol and water is often used as guide to how well a molecule will distribute between the stratum corneum lipids and water (Williams, 2003:27).

A phase change is encountered by a drug when it reaches the viable tissue. It has to transfer from the predominantly lipophilic intercellular channels of the stratum corneum into the living cells of the epidermis which will be largely aqueous in nature and essentially buffered to pH 7.4 (Hadgraft & Wolf, 1993:165). Molecules with good solubility in both oils and water will permeate well. This would encompass molecules with a log Koct of 1 – 3 (Hadgraft, 2004:292). Hydrophilic molecules ($\log P < 1$) are expected to permeate largely via the intracellular route whereas the intercellular route will dominate for lipophilic ($\log P > 3$) molecules (Williams, 2003:35).

2.8.2.2 Diffusion coefficient (D)

The diffusion coefficient measures the penetration rate of a molecule through the skin under specified conditions (Barry, 2002b:S33). Diffusion describes the access of a substance for either absorption, the bulk invasion of various layers of the skin, or adsorption which is a highly specific interaction effecting retention of the invading chemical on certain sites within the skin (Zatz, 1993:34). The diffusional speed of a molecule depends mainly on the state of matter of the medium. Gases and air have large diffusion coefficients due to greater void space between molecules, while liquids have reduced diffusion coefficients due to a much smaller free volume. Diffusivities drop progressively in the skin and reach their lowest values within the compacted stratum corneum matrix (Barry, 2002b:S33).

2.8.2.3 Permeability coefficient (kp)

The permeation coefficient describes the rate of permeant transport per unit concentration, given in units of distance/time (cm/h). The steady-state flux of drugs traversing the stratum corneum is the product of the permeability coefficient and the applied concentration (Williams, 2003:27, 37). The stratum corneum vehicle partition coefficient is therefore crucially important in establishing a high initial concentration of diffusant in the first layers of the skin, as it is influenced by hydrophobicity, size of penetrants, and the presence or absence of the electric charges and many other factors due to the characteristics of the application area (Morganti *et al*., 2001:494). Even though lipophilic permeants provide relatively high permeability coefficients, their lipophilicity usually dictates relatively low aqueous solubility, with a consequent impact on drug flux through the stratum corneum (Williams, 2003:37).

2.8.2.4 Ionisation, pH and pka

The ambient pH and the pKa of a permeant will give the relative amounts of ionised and unionised species (Hadgraft & Valenta, 2000:243). The stratum corneum is remarkably resistant to alterations in pH, tolerating a range of 3 – 9. Weak acids and bases dissociate to different degrees depending on the pH of their pKa or pKb values (Barry, 2002a:511-512). While ionised species do not penetrate the skin very well, unionised molecules pass readily across lipid membranes (Barry, 2002a:511-512). Drug flux is the product of the permeability coefficient and effective drug concentration in the vehicle. Although the permeability coefficient of unionised species through the lipid membrane may be high, its aqueous solubility will be low. The opposite applies to ionised species as this group is associated with low permeability coefficients but high solubility (Williams, 2003:38-39). TGF-β1 and TNF-α were prepared at physiological pH (7.4).

2.8.2.5 Solubility and melting point

According to Williams (2003:37), it is well known that most organic compounds with high melting points and enthalpies of melting have relatively low aqueous solubility values at normal temperatures and pressures, thus stating a clear relationship between melting point and solubility. In any assessment of the feasibility of transdermal or topical delivery, it should be considered that the partitioning behaviour of the drug will be linked with its solubility characteristics (Hadgraft & Wolf, 1993:164). Thus the degree to which a drug released from a formulation partitions into the stratum corneum depends on the amount of drug applied and its solubility limit in the stratum corneum, as the portioning behaviour of a drug is linked to its solubility characteristics (Hadgraft & Wolff, 1993:161).

2.8.2.6 Molecular weight

Small molecules penetrate faster than larger ones as absorption is inversely related to molecular weight (Barry, 2002b:S33). For therapeutic agents within a relatively narrow range of molecular weight (100 – 500 Dalton), the influence of such narrow ranges is inconsequential. When selecting larger molecules like peptides and proteins, the influence on drug flux is more apparent (Williams, 2003:37).

2.9 SKIN PENETRATION ENHANCEMENT TECHNOLOGIES

Dermal conditions are still treated with many useful drugs still administered orally or via other systemic applications. Unfortunately, the physicochemical characteristics of these drugs do not allow them permeation through the skin. Three major barriers can be associated with the topical delivery of drugs (Hsieh, 1994:13): poor permeation across the stratum corneum; permeated drugs are not easily retained in the skin for localized therapy; and skin irritation caused by many drugs delivered topically.

Strategies to improve dermal conditions include the development of permeation enhancement technologies to facilitate not only the permeation of drugs across the stratum corneum, but also to increase the retention time of drugs in the stratum corneum and/or epidermis. This targeted delivery system is an important aspect with regards to skin care product development in the area of dermatology and cosmeceuticals (Hsieh, 1994:13). Compounds with low solubility and affinity for the hydrophilic and lipophilic components of the stratum corneum would, theoretically partition into the skin at a slow rate. Addition of a chemical adjunct to the delivery system promotes drug partitioning into the stratum corneum (Walker & Smith, 1996:296). Penetration enhancers, also known as sorption promoters or accelerants (Lund,1994:144), increase the

penetration rate of drugs through the skin and are believed to operate by increasing the permeability of the stratum corneum either in the lipid or keratinized protein regions (Washington *et al*., 2001:191). Although penetration enhancers should be compatible with a wide variety of drugs, it should still be non-toxic, non-irritant, non-allergenic, devoid of pharmacological activity and contain excipients, with rapid, predictable and reversible action. The enhancer should not facilitate the loss of any body fluids, electrolytes, and other endogenous material from the skin or underlying layers of the skin should immediately regain its barrier properties on its removal (Lund, 1994:144; Sinha & Kaur, 2000:1131).

Three pathways are suggested for drug penetration through the skin: polar, non-polar and polar/non-polar. The polar pathway can be altered by causing protein conformation or solvent swelling, while the key of altering non-polar pathway is to alter the rigidity of the lipid structure and fluidize the crystalline pathway (Shah, 1994:20).

2.9.1 Chemical modulation of topical permeation

This type of enhancers exert their effects by partitioning into the stratum corneum, interacting with tissue components to reduce the barrier properties of the membrane without causing damage to the underlying skin cells (Williams, 2003:86). Chemical enhancer effects on the stratum corneum, termed lipid-protein-partitioning theory (LPP), can be explained as the process by which enhancers act by altering skin lipids and/or proteins by affecting partitioning behaviour (Williams & Barry, 2004:86).

According to the LPP theory, enhancers would act by one or more of six mechanisms:

(1) Disruption of the highly ordered structure of stratum corneum lipid with an increase in intercellular diffusivity.

(2) Interaction with intracellular protein partition to enhance penetration through the corneocytes.

(3) Improvement in partitioning of a drug, co-enhancer, or co-solvent into the stratum corneum.

(4) Disruption of the corneocyte envelope.

(5) Manipulate protein junctions such as desmosomes.

(6) Change in the partitioning, between the stratum corneum components and the diffusion pathway lipids (Magnusson *et al*., 2001:211; Kanikkannan *et al*., 2006:18).

Examples of chemical penetration enhancers include water, hydrocarbons, sulphoxides, (especially dimethylsulfoxide [DMSO]) and their analogues, pyrrolidones, esters and alcohols, various surfactants, amides, polyols, essential oils, terpenes, oxazolidines, epidermal enzymes, polymers, lipid synthesis inhibitors, bio-degradable enhancers and synergistic mixtures (Lund, 1994:144; Barry, 2001:9).

2.9.2 Physical enhancers

Physical techniques of enhancement may provide an effective alternative method for improved permeation when the enhancement of drug molecules is not accomplished effectively with chemicals (Walker & Smith, 1996299). Physical penetration enhancers share the common goal of disrupting the stratum corneum structure in order to create 'holes' big enough for molecules to permeate (Thong *et al*., 2007:273). Such methods include microneedles, laser ablation, electroporation or physically enhanced flux such as iontophoresis, ultrasound, magnetophoresis and the use of vesicular carriers (Williams, 2003:123).

Table 2.1: Commonly investigated technologies of physical penetration enhancement

METHOD	DEFINITION	MECHANISMS
Ionotophoresis	The electrical driving of charged molecules into tissue by passing a small direct current through a drug-containing electrode in contact with skin.	1) Electrical repulsion from the driving electrode drives charged molecules. 2) The flow of electric current enhances skin permeability. 3) Electro-osmosis affects uncharged and large polar molecules.
Electroporation	Application of an electric pulse to reversibly permeabilise lipid bilayers.	Application of short (µs - ms) electrical pulses of 100 – 1000 V/cm creates transient aqueous pores in the lipid bilayers.
Sonoporation	Ultrasound-mediated delivery of therapeutic agents/drugs into biological cells.	1) Low energy frequency disturbs the lipid packing in stratum corneum by cavitation. 2) Shock waves increase free volume space in bimolecular leaflets, thus enhance permeation.
Microneedle enhanced delivery systems	A method using arrays of microscopic needles to open pores in the stratum corneum, thus facilitating drug permeation.	Bypasses the stratum corneum and delivers drugs directly to the skin capillaries; also has the advantage of being too short to stimulate the pain fibres.

The generally low bioavailability of proteins and peptides is caused by their large molecular weight and variable solubility. It is therefore necessary to facilitate the protein or peptide permeation through the skin in order to achieve therapeutic drug levels. Ultrasound, iontophoresis or electroporation can be use to increase the permeability of the lipid lamellae (Antosova *et al.,* 2009:631). Topical delivery of either TGF-β1 or TNF-α can thus be enhanced by the above mentioned methods.

2.9.3 DRUG DELIVERY SYSTEMS

Vesicles composition is influenced by their physicochemical characteristics such as: size, charge, thermodynamic phase, lamellarity and bilayer elasticity. The uses of vesicles in dermal and transdermal drug delivery are as follows: Vesicles might:

- act as drug carriers to deliver entrapped drug molecules into or across the skin;
- act as penetration enhancers owing the penetration of the individual lipid components into the stratum corneum and subsequently the alteration of the intercellular lipid lamellae within this skin layer; serve as a depot for sustained release of dermally active compounds;
- serve as a rate-limiting membrane barrier for the modulation of systemic absorption, hence, providing a controlled transdermal delivery system (Honeywell-Nguyen & Bouwstra, 2005:67).

The discussion below will focus on four of the most common vesicle types of current drug and cosmetic delivery to and through the skin namely conventional vesicles (liposomes, ethosomes, niosomes) and highly deformable or elastic liposomes known as transfersomes (Williams, 2003:124; Honeywell-Nguyen & Bouwstra, 2005:67).

2.9.3.1 Liposomes

Liposomes are colloidal particles, typically consisting of phospholipids and cholesterol arranged in one or more bilayers (Williams, 2003:124; Honeywell-Nguyen & Bouwstra, 2005:67). They provide lower systemic drug levels as drugs are localised within the skin membrane. Beside from the delivery of drugs across the bulk stratum corneum, liposomes have been used to target therapeutic and cosmetic agents to the pilosebaceous units for treatment of alopecia as well as systemic delivery (Williams, 2003:126).

2.9.3.2 Ethosomes

Ethosomes are liposomes high in ethanol content (up to 45 %) (Barry, 2001:105). Their mechanisms of actions are not clear, though ethanol, typically at 30 % in the formulation, acts as a chemical penetration enhancer and phospholipids have the ability to disrupt the intercellular lipid domains to facilitate permeation. Controls such as hydro-ethanolic solutions of the permeant appear less effective for promoting drug delivery than the ethosomes, suggesting that the vascular structure and permeant entrapment is important for efficacy (Williams, 2003:134).

2.9.3.3 Noisomes

Noisomes (or non-ionic surfactant vesicles) have been found to form vesicles capable of entrapping hydrophilic and hydrophobic solutes (Yoshioka *et al.,* 1994:1). They are regarded as inexpensive alternatives, of non-biological origin to liposomes. Noisomes possess greater stability and alleviate disadvantages associated with liposomes such as chemical stability, variable of phospholipids and high cost (Vora *et al*., 1998:150). Drug delivery from noisomes appears to be less effective compared to liposomes, though the increased flux values reported from liposomes when compared to noisome may result from using an optimized liposome system in comparison with a suboptimal noisomal formulation (Williams, 2003:130).

2.9.3.4 Transfersomes

Transfersomes are highly deformable (or elastic, ultra-flexible) liposomes comprising of a phospholipid as their main ingredient with 10 to 24 weight percent of a surfactant added. Transfersomes labelled with a radioactive marker applied onto the skin, resulted in the observation of radioactivity in the liver, indicating the presence of radioactive particles in the systemic blood as reported by Honeywell-Nguyen & Bouwstra (2005:71). This detection might propose that transfersomes permeate across the skin (Honeywell-Nguyen & Bouwstra, 2005:71).

2.9.4 Pheroid™ technology as therapeutic drug delivery system

Pheroid™ technology (previously known as Emzaloids™), is a delivery system that promotes the absorption and increases the efficacy of dermatological, biological and oral medicines in various pharmacological groups and has been used in several preparations for the treatment of many skin disorders such as psoriasis, eczema, and dermatitis (Saunders *et al*., 1999:99; Grobler *et al.,* 2008:284). The entrapment of actives within the Pheroid™ generally creates a

safer, more effective formulation which includes advantages such as decreased time to onset of action, reduction of minimal effective concentration, increased therapeutic efficacy, reduction in cytotoxicity, penetration of most known barriers in the body and in cells, ability to target treatment areas, lack of immunological response, ability to transfer genes to cell nuclei and reduction of drug resistance (Grobler, 2004:3).

2.9.4.1 Structural characteristics of Pheroid™

The basic Pheroid™ has a vesicular structure with a size ranging from 200 – 440 nm (Grobler *et al*., 2008:283) and contains ethyl esters of the essential fatty acids (ethylated and pegylated polyunsaturated fatty acids, including the omega-3 and omega-6 acids buy excluding arachidonic acid (Grobler *et al*., 2008:285), linoleic acid and linolenic acid, as well as oleic acid, emulsified in water saturated with nitrous oxide (Saunders *et al*., 1999:99). Its membranes contain pores with a proposed fatty acid packing. The addition of nitrous oxide to the respective oil and water phases adds another dimension to the basic Pheroid™ (Grobler *et al.,* 2008:289).

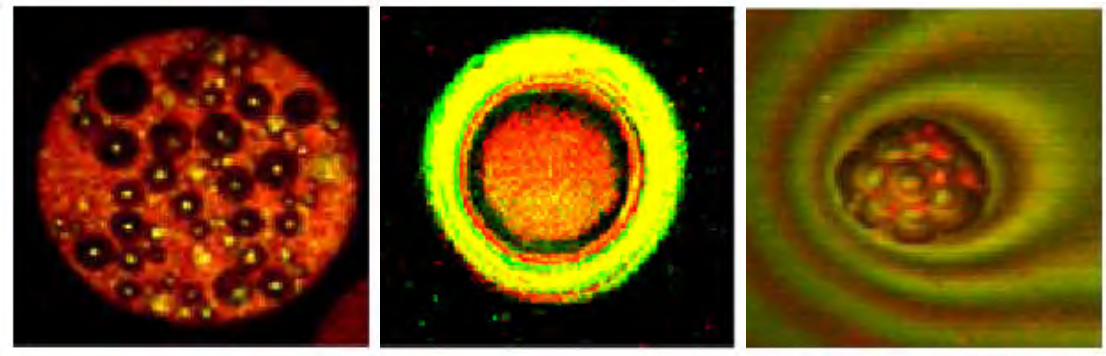

Figure 2.13: Confocal laser scanning microscopy micrographs show active compounds entrapped in several Pheroid™-Emzaloid™ types. Each type has a specific composition. The size and shape of vesicles can be reproducibly controlled.

Pheroid™ delivery systems are stable lipid-based submicron- and micron-sized structures in a colloidal system, dispersed in a medium that may be adapted to the indication and consists mainly of plant and essential fatty acids. This delivery system is stable within a system and can be manipulated in terms of morphology, structure, size and function in a very specific manner to ensure its high entrapment capabilities, very fast rate of transport, delivery and stability (Grobler, 2004:4). Although the ultra-fine particles that remain suspended in the dispersion medium range between 1 – 100 nm, Pheroid™ is typically formulated to have a diameter of between 200 nm and 2 µm. The amount and size of active compound to be entrapped, the rate of delivery and the administration route are parameters to be taken into account when deciding on

the type and diameter of the Pheroid™. Just like liposomes, Pheroid™ contains a lipid bilayer, but no phospholipids or cholesterol and is formed by a self assembly process similar to that of low-energy emulsions and micro-emulsions (Grobler *et al*., 2008:285).

The design of the Pheroid™ allows for manipulation of both its structural and functional features by:

- Modifying the fatty acid composition or concentration.
- The addition of non-fatty acids or phospholipids such as cholesterol.
- The addition of cryo-protectants.
- Altering in the hydration medium (ionic strength, pH).
- Altering the character and the concentration of the active compounds.
- Including sunscreen formulations (Grobler *et al.*, 2008:292).

2.9.4.2 Functional characteristics of Pheroid™

2.9.4.2.1 Pliability

Pheroid™ are exceptionally elastic and do not shatter under moderate pressure or extravasations due to the use of nitrous oxide as well as the addition of the pliable pegylayted tails added to the fatty acids, which serves to sterically stabilize the Pheroid™ and maintain their interior spaces (Grobler *et al.*, 2008:294).

2.9.4.2.2 Entrapment efficiency

Confocal laser scanning microscopy (CLSM) is generally used to determine the entrapment efficiency of Pheroid™-based products, which is aimed at 90 % for all products in development. The size, charge and solubility of the active ingredient influences the number of molecules entrapped within one Pheroid™. The number of colloidal particles per volume can be increased or decreased to suit the required concentration of active compound (Grobler *et al.*, 2008:294).

2.9.4.2.3 Penetration efficiency

The penetration efficacy of entrapped compounds is determined by comparison of the testing compound with a reference product. This types of studies has repeatedly confirmed that Pheroid™ traverse the stratum corneum with its entrapped compound. The determination of an enhancement factor at different depths in the skin over time can be calculated. With each

depth, an area under the curve enhancement factor is obtained, resulting in the calculation of the total enhancement of the respective total areas under the curve (Grobler *et al.*, 2008:297).

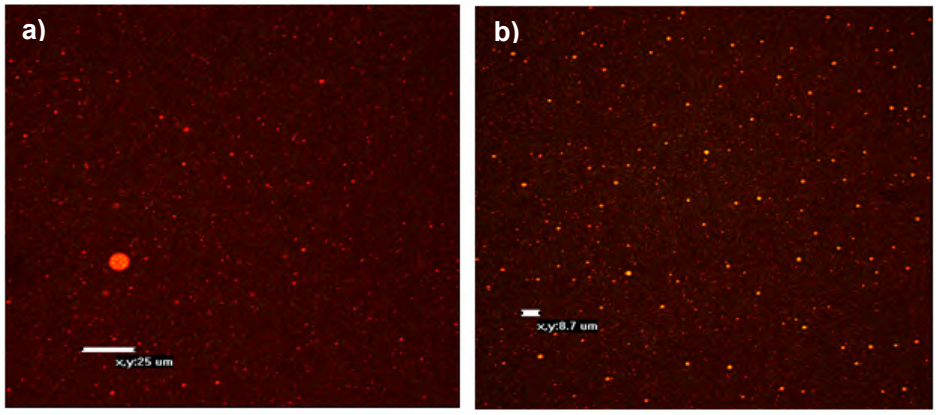

Figure 2.14: Pheroid™ vesicles containing **a)** TGF-β1 (1000 pg/ml) and **b)** TNF-α (500 pg/ml)

2.9.4.2.4 Cellular uptake of Pheroid™ and entrapped compounds

The cellular uptake of Pheroid™ by cells is to some extent still speculative, although preliminary evidence suggests that the uptake is actively facilitated by the fatty acid membrane binding proteins generally present within lipid rafts in the cell membrane (Grobler *et al.*, 2008:299).

Cholesterol results in rigid vesicular structure with an inability to extravagate from capillaries without fracturing, whereas the electro-chemical stabilization of the Pheroid™ creates a very elastic vesicular structure. Cellular uptake of Pheroid™ may be influenced by the Pheroid™ formulation as well as the mechanism of cellular uptake (Grobler *et al.*, 2008:297).

Factors influencing the permeation of the Pheroid™ formulation include the:

- size and morphology of the Pheroid™,
- molecular geometry of fatty acids,
- concentration ratio of the fatty acids,
- hydration medium (ionic strength, etc.),
- pH of the preparation,
- presence of ionisable molecules or molecules that influence the electrostatic environment, and
- character and concentration of the active, and gel or fluid state of Pheroid™ (Grobler *et al.*, 2008:297).

2.10 SUMMARY

Many modalities of treatment for acquired skin hyper-pigmentation are available. Compounds with depigmenting activity are abundant and the classification of these molecules is based on their mechanism of action. Such topical remedies allow for a high local concentration of the drug at the site of the action and lower or negligible systemic drug levels, producing fewer or no adverse drug effects.

Depigmentation can be achieved by regulating: i) the transcription and activity of tyrosinase, TRP-1, TRP-2, and/or peroxidase; ii) the uptake and distribution of melanosomes in recipient keratinocytes and iii) melanin and melanosome degradation and turnover of pigmented keratinocytes. The control of hyper-pigmentation disorders poses a great challenge for dermatologists as they are confronted with numerous therapeutic options often resulting in unsatisfactory effects.

Successful depigmenting treatments need the combination of two or more agents acting on different mechanisms to achieve a synergistic effect. This project was selective in the coverage of two hypo-pigmenting agents: TGF-β1 and TNF-α (regulation of the transcription and activity of tyrosinase, TRP-1 and TRP-2).

In the course of this study, the topical delivery of depigmenting agents TGF-β1 and TNF-α encapsulated in Pheroid™ vesicles across the skin will be investigated.

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

Chapter 3 is given in an article format for publication in The International Journal of Pharmaceutics. The guide for authors is provided in Appendix C.

Pheroid™ technology for topical delivery of depigmenting agents: TGF-β1 and TNF-α

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ABSTRACT

The objective of this study was aimed at the topical delivery of depigmenting agents, transforming growth factor-beta1 (TGF-β1) and tumour necrosis factor-alpha (TNF-α). Dermal drug delivery of peptide drugs is often problematic because the skin, as a natural barrier, has a very low permeation rate. To possibly overcome these barrier properties, a drug delivery system, the Pheroid™ technology was used together with bestatin (aminopeptidase inhibitor) to circumvent any skin-related degradation. Actives were entrapped within Pheroid™ vesicles for 6 h (TGF-β1) and 12 h (TNF-α) at concentrations of 1000 pg/ml (TGF-β1) and 500 pg/ml (TNF-α). Entrapped actives were traced by means of confocal laser scanning microscopy. Vertical Franz diffusion studies were conducted with Caucasian female abdominal skin. The amount of permeant in the stratum corneum-epidermis and epidermis-dermis was determined by tape stripping. The diffused concentration values obtained with both actives dissolved in phosphate buffered solution (PBS) and Pheroid™ vesicles was compared. For both actives a higher diffused concentration was obtained when compared to PBS profiles. Pheroid™ stratum corneum-epidermis and epidermis-dermis solutions revealed a higher amount of the actives as well. TGF-β1 and TNF-α were successfully delivered across the stratum corneum by the Pheroid™ drug delivery system in conjunction with bestatin hydrochloride.

Keywords: Transforming growth factor-beta1, Tumour necrosis factor-alpha, Topical delivery, Hyper-pigmentation, Pheroid™ technology

1 Introduction

Skin colour is one of the most evident ways in which humans vary. Most of the variation in the visual appearance of human skin can be accounted for by melanin, the pigment responsible for skin colour (Jablonski and Chaplin, 2000). Three structurally related enzymes, tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2) are required to synthesize different types of melanin (Costin and Hearing, 2007).

Pigmentary disorders can be acquired, inherited or transmitted through skin diseases or induced by medication (Costin and Hearing, 2007). Cutaneous hyper-pigmentation transpires in multiple conditions (Hakozaki et al., 2006). Various hyper-pigmentation skin disorders such as melasma (irregular brown macules on sun exposed skin; common sites are the cheeks forehead, upper lip, nose and chin), post-inflammatory hyper-pigmentation (PIH) (localised hyper-pigmented resulting from trauma or injury to the skin) and senile lentigines (lesions) can be intensified by sun exposure (Pandaya et al., 2000; Hakozaki et al., 2002; Stulberg et al., 2003; Draelos, 2006).

Regardless of the nature of the problem, the general desire is for uniformity of skin colour. Many modalities of treatment for acquired skin hyper-pigmentation disorders are available. This includes chemical agents or physical therapies, but none are completely satisfactory (Briganti et al., 2003).

Pigmentary control agents can be screened alongside a wide array of targets. Among the various existing target zones, one mechanism was selected for the purpose of this study: inhibition of melanin synthesis enzymes TRP-1 and TRP-2 by cytokines transforming growth factor-beta1 (TGF-β1) and tumour necrosis factor-alpha (TNF-α) (Martinez-Esparza et al., 2001).

TNF-α has been reported to inhibit both tyrosine hydroxylase and 3,4-dihydroxyphenylalanine (DOPA) oxidase activities of tyrosinase in B16 melanoma cells at nanomolar concentrations, affecting activity levels of TRP-1. It elicits a dose-dependent decrease in the activity of tyrosinase and inhibits melanocyte proliferation. Melanocytes remain viable despite continuous

treatments with TNF-α. Its effects thus appear to be cytostatic, with recovery of cell proliferation upon cessation of TNF-α treatment (Slominski et al., 2004). TGF-β1 exerts its depigmenting effect by decreasing tyrosinase and TRP-1 levels, by means of decreasing both gene expression and the intracellular half-life of the tyrosinase, but does not appear to block tyrosinase stimulation by α-melanocyte-stimulating hormone (α-MSH). Its inhibitor effects results in a lowered percentage of fully mature stage IV melanosomes, resulting in the accumulation of incompletely melanized melanosomes (Martinez-Esparza et al., 2001; Slominski et al., 2004).

Topical dermatological therapy is aimed at increasing the retention of therapeutic drugs in the skin (Lund, 1994); however, few drugs are able to passively diffuse across the uppermost layer of the skin, the stratum corneum, as a result of its effective barrier properties (Asbill and Michniak, 2000). The use of *in vitro* techniques such as Franz type diffusion cells permits the evaluation of the dermal kinetics of drug uptake and diffusion. The choice of therapeutic agent for topical delivery is dependent on a number of factors, including physicochemical properties of the drug, its interactions with the membrane and its pharmacokinetic properties (Kalia and Guy, 2001). To maintain an effective concentration at the site of action, topical products should be applied in sufficient quantity at a suitable rate to achieve a therapeutic effect (Lund, 1994). This penetration can be measured by a tape stripping technique, investigating the amount of active present in the stratum corneum-epidermis after application of supersaturated solutions (Pellet et al., 1997).

In an attempt to overcome the barrier properties of the skin, a novel drug delivery system known as Pheroid™ was used. Pheroid™ technology (previously known as Emzaloid™), is a delivery system that promotes the absorption and increases the efficacy of dermatological, biological and oral medicines in various pharmacological groups and has been used in several preparations for the treatment of many skin disorders such as psoriasis, eczema, and dermatitis (Saunders et al., 1999; Grobler, 2004). The entrapment of actives within the Pheroid™ generally creates a safer, more effective formulation which includes advantages such as

decreased time to onset of action, reduction of minimal effective concentration, increased therapeutic efficacy, reduction in cytotoxicity, penetration of most known barriers in the body and in cells, ability to target treatment areas, lack of immunological response, ability to transfer genes to cell nuclei and reduction of drug resistance (Grobler, 2004).

Due to inactivation by gastrointestinal enzymes, peptides cannot be administered orally as problems such as local targeted delivery and the blood-brain barrier prevent peptides from readily gaining access to the required site of action. Subcutaneous or intravenous administration is thus required (Edwards et al., 1999).

The therapeutic efficacy of topical applied compounds may be determined by skin metabolism as it is capable of metabolizing a number of compounds (Amsden and Goosen, 1995; Barry, 2002). In order to improve the bioavailability of pharmaceutical proteins, such compounds can be co-administered with inhibitors to slow down metabolic degradation (Crommelin et al., 2002). One such enzyme inhibitor employed in the topical delivery of TGF-β1 and TNF-α, was bestatin hydrochloride, a specific inhibitor of aminopeptidase B and leucine aminopeptidase (Sigma, 2008). Bestatin exerts its activity by competing with the substrates, binding to the catalytic site of the enzyme, exhibiting a competitive kinetics with the substrate once bound. Aside from its inhibitor action, bestatin suppresses the production of the pro-inflammatory cytokines and stimulates the anti-inflammatory cytokine by activated monocytes and macrophages. In a study conducted by Lkhagvaa et al. (2008), bestatin was shown to be useful as an immuno-modulator for the control and treatment of various inflammatory diseases (Lkhagvaa et al., 2008).

This first objective of this study was to investigate the *in vitro* topical delivery of TGF-β1 and TNF-α in conjunction with enzyme inhibitor bestatin hydrochloride entrapped in the Pheroid™ delivery system. The efficiency of penetration was determined by comparative study with the actives dissolved in PBS (phosphate buffered solution). The second objective was to investigate the amount of active in the stratum corneum-epidermis and remaining epidermis-dermis by means of a tape stripping technique.

2 Materials and Methods

2.1 Materials

Enzyme linked immunosorbent assay (ELISA) detection kits for both cytokines were obtained from Biocombiotech (South Africa). Both kits consisted of two 96 well uncoated plates, detections antibodies, cytokine standards, assay diluents, detection enzymes and substrate solutions. Wash buffers consisted of a 1 x PBS and tween 20 % (Sigma-Aldrich) solution. A 1 M phosphoric acid solution was employed as well. Potassium dihydrogen orthophosphate and sodium chloride (Merck South Africa) were used during preparation of the PBS (pH 7.4). A tape stripping extraction solution consisting of PBS and methanol AR was prepared for both cytokines. Pheroid™ was prepared by the Unit for Drug Research and Development, North-West University. Vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) and a type JB5 Grant water bath (Grant instruments, Cambridge, England) were used during the *in vitro* permeation studies. All solutions were prepared using HPLC (high performance liquid chromatography) (deionised) water, purified by a Milli-Q® Academic purification system (Millipore, Milford, USA).

2.2 Methods

2.2.1 Stability testing

In an attempt to establish the degradation rates of cytokines, TGF-β1 and TNF-α, they were subjected to stability testing. The respective intervals were determined in a trial stability test whereby 95 % of the active had to be intact for the duration of a diffusion study. Constituted solutions of the cytokines with or without Pheroid™ were subjected to temperatures of 25 and 37 °C for a period of 12 h. Each solution (200 µl) was withdrawn hourly and cytokine concentrations were detected by means of ELISA detection after 12 h.

2.2.2 Entrapment of TGF-β1 and TNF-α in Pheroid™ drug delivery system

Depigmenting agents TGF-β1 (1000 pg/ml) and TNF-α (500 pg/ml) were entrapped into a Pheroid™ vesicle formulation. The individual concentrations were based on the respective standard curve ranges as stipulated by the product information. These formulations were stirred

overnight at 32 °C in order to mimic the *in vivo* situation (Williams, 2003) for 6 h (TGF-β1) and 12 h (TNF-α). Each formulation (50 µl) was placed on a glass slide and covered with a glass cover slip. Entrapment was monitored with the aid of confocal laser scanning microscopy (CLSM). For this purpose a Nikon-D-eclipse Ci si CLSM, equipped with a violet diode laser (400 – 405 nm) and argon ion laser (457 – 514 nm) was used. Pheroid™ vesicle formulations were stained with the fluorophore Nile Red, staining the fatty acid components of the Pheroid™. Nile red only emits fluorescence when associated with lipid-like molecules (Grobler *et al.,* 2008). Samples were subjected to laser scanning at 488 and 505 nm.

2.2.3 The preservation of TGF-β1 and TNF-α with bestatin hydrochloride

Due to the limited amount of cytokine active, one set of diffusion studies (6 Franz diffusion cells) were conducted. TGF-β1 (1000 pg/ml) and TNF-α (500 pg/ml) were prepared in conjunction with bestatin at a concentration of 300 µg/ml in both PBS and Pheroid™ vesicles. Solutions were vortexed for a few seconds with subsequent application to donor cells.

2.2.4 Skin permeation method

In the course of this study, Caucasian skin obtained from abdominal plastic surgeries was employed for conduction of Franz cell diffusion studies. Ethical approval for the procurement and exploitation of the skin tissue was provided by the Research Ethics Committee of the North-West University (reference number 04D08). Informed consent was obtained from the patients in advance and their identities were masked to assure anonymity. After procurement, the full thickness skin was examined for defects and stored frozen at -20 °C for no longer than 6 months. To obtain full thickness skin discs, the subcutaneous fat layer was removed and skin was cut into circles of about 15 mm in diameter. Skin sections were drifted on Whatman® filter paper with the stratum corneum side of the epidermal layer facing upwards and were subsequently left to air dry. The prepared skin discs were covered in aluminium foil and were kept frozen at -20 °C until used.

2.2.5 Franz cell diffusion method

In vitro permeation studies were conducted by means of Franz diffusion studies with a receptor capacity of 2 ml and a diffusion area of 1.075 cm^2. Prior to conduction of *in vitro* permeation studies, full thickness skin discs were thawed at room temperature. The skin discs were clamped between chambers with the epidermis facing the donor compartment. Both the receptor and donor compartments were clamped together with a metal clamp and sealed with Dow Corning® vacuum grease. The receptor compartments were slowly filled with PBS (pH 7.4), taking care to avoid the accumulation of air bubbles, while the donor compartments were filled with the respective depigmenting agents, TGF-β1 (1000 pg/ml) and TNF-α (500 pg/ml). Diffusion cells were placed in a water bath at 37 °C, with only the receptor compartments submerged in the water, stirred at 75 rpm by means of a Variomag® magnetic stirrer. Donor compartments were covered with Parafilm® to avoid evaporation. For both cytokines, TGF-β1 and TNF-α, the entire contents of the receptor compartments were withdrawn at an interval of 12 h (TNF-α) and 6 h (TGF-β1). Each sample (100 µl) was directly assayed by means of ELISA in order to determine the amount of peptide concentration within the respective receptor compartments. Each permeation study was conducted with 6 Franz diffusion cells.

2.2.6 Tape stripping

Upon removal of the donor and receptor phases, skin discs were carefully dismantled, dabbed dry with tissue and stapled on a piece of Parafilm® to a solid surface. Adhesive tape (3M Scotch® Magic™ Tape) was cut into pieces, into the size of the skin dismounted from each cell disk, covering only the diffusion area. The first tape strip was discarded as it is considered to be part of the cleaning procedure. The following 15 tape strips were placed in a vial containing 5 ml PBS (pH7.4). Excess skin was trimmed away from the flange imprints and the remaining viable epidermis and dermis was placed in vials as well. Collected samples were kept in a fridge at 4 °C overnight to allow extraction of the respective depigmenting agents into the PBS (Pellet et al., 1997). Samples were then assay by ELISA analysis.

2.2.7 Enzyme linked immunosorbent assay (ELISA) detection

Subsequent to collection of receptor compartment fluid, protein concentrations of both cytokines was measured with human TGF-β1 and TNF-α quantitative sandwich ELISA kits following the supplier's protocol. Each incubation step was followed by aspiration with wash buffer (1 x PBS). Plates were coated with the respective capture antibodies and left to incubate for 6 h (TGF-β1) and 12 h (TNF-α). Standards (2-fold serial dilutions) and samples were added to the plates and left for incubation with additional incubation with a detection antibody and subsequent secondary antibody. The absorbance was read on a BioTek ELX800™ microplate reader with GEN5™ software. The amount of antibody was inferred from the known concentrations of the standards. Results were expressed relative to protein content.

2.2.8 Protein quantitation

The standard bicinchoninic acid (BCA) method for protein determination was done using 1 $\mu g.\mu l^{-1}$ bovine serum albumine (BSA) as a standard. Protein quantitation of both cytokines was conducted in order to measure the total amount of protein concentration in the samples. Reagents used in this process were bicinchoninic acid solution (BCA) and copper(II)sulphate 4 % solution in a ratio of 50:1. This solution (2 ml) was added to 0.1 ml of protein solution. Solutions were mixed for 15 min until the BCA working agent turned light green (PBS) and purple (Pheroid™). Plates were read at 450 and 570 nm and absorbance data was analysed.

2.2.9 Statistical data analysis

Statistical techniques are used to analyse a set of data collected. The fundamental characteristics of data can be determined by making use of descriptive statistics which refers to the graphical and tubular methods used to summarise and order data, while statistical inference (methods used to make conclusions about a population from sample data) draws conclusions about the population from which a random sample was drawn, using the descriptive measures that have been calculated (Ellis and Steyn, 2003, Swanepoel et al., 2004).

2.2.9.1 Descriptive measures of a location and spread

A simple description of a sample distribution can be explained by measuring its central tendency and spread (De Coster, 1998). For the purpose of this study, two measures of

location, the sample average and median values were used. The average of the total set of observations is the sum of all observations divided by the total number of observations (Bewick et al., 2004), while a sample median is the number situated in the middle of the observations if they are arranged from the smallest to the highest value. In the event of an even number of observations, the median is calculated as the mean of the two middle observations (Swanepoel et al., 2004).

The spread of a population portrays how widely data is distributed over the area of the data by a single value. The standard deviation of a sample attempts to describe the average distance of the observations from the mean of the observations (Swanepoel et al., 2004).

2.2.9.2 Probability testing

A common task in data analysis is to compare two or more sets of data to establish whether they are basically the same or one set is significantly different from the others. A statistical test of comparison is usually needed to test for such significant differences. The result of such tests is a probability (*p*-value) that the 'null hypothesis' (which always states that there is no difference between the sets of data) is true. If p is less than 0.05 ($p < 5$ %) then it can be concluded that there is a significant difference between the sets of data and the null hypothesis is rejected. If $p > 0.05$, there is no significant difference between the sets of data and the null hypothesis is accepted (Millar, 2001).

2.2.9.3 Comparative statistics

A general task in data analysis is to compare two or more sets of data to conclude whether they are basically the same (i.e. they could come from the same population) or one set is significantly different from the others (Millar, 2001).

Parametric tests are applied when normality (and homogeneity of variance) assumptions are satisfied otherwise the equivalent non-parametric test will be used (Chan, 2003). Based on the result of statistical analysis on data, one parametric (ANOVA) and non-parametric test (Mann Whitney U) was used to compare significant differences between data sets.

2.2.9.4 Analysis of variance

Analysis of variance (ANOVA) is a technique for analyzing the way in which the mean of a variable is affected by different types and combinations of factors (Bewick et al., 2004). When conducting a statistical analysis, values of one variable are often used to predict or explain the values of another variable. The variable used to make this prediction is called the independent variable (IV) while the variable one tries to predict is called the dependent variable (DV) (De Coster, 2006). One-way, ANOVA tests measure significant effects of one factor only (Bewick et al., 2004), whereas two-way ANOVA tests measure the effects of two factors simultaneously. Three *p*-values are generated by a two-way test, one for each parameter independently, and one measuring the interaction between the two parameters (Cornish, 2006).

2.2.9.5 Mann Whitney U-test

The Mann-Whitney U-test is the non-parametric equivalent of the T-test (Millar, 2004). This test can be used to establish whether a difference exists between groups and has the great advantage of possibly being used for small samples of subjects. Where two independent groups have to be compared, each group contains a number (*n*) of observations. The Mann Whitney U-test is based on the comparison of each observation from the first group with each observation from the second group. The data from each group are then individually compared together. The Mann Whitney U-test null hypothesis (H_0) stipulates that the two groups come from the same population while the alternative hypothesis (H_1) against which the null hypothesis is tested, stipulates that the first group data distribution differs from the second group data distribution (Nachar, 2008).

3 Results and Discussion

3.1 Entrapment of TGF-β1 and TNF-α in Pheroid™ vesicles

CLSM images of TGF-β1 and TNF-α can be seen in Figure 1. Entrapped molecules (yellow) can be seen within the Pheroid™ vesicles in Figure 1b) and Figure 1c). Both cytokine standard solutions were labelled with Nile red, as both cytokines are not auto-fluorescent. Red (fatty acid) and yellow (active entrapped) spots were observed in the CLSM micrographs.

Figure 1: CLSM micrographs: a) Placebo Pheroid™ vesicles; b) TGF-β1 entrapped in Pheroid™ vesicles and c) TNF-α entrapped in Pheroid™ vesicles

3.2 Stability

The aim of the test was to assess the stability of both cytokines in order to establish what the duration of their diffusion studies would be.

3.2.1 TNF-α

TNF-α was very stable throughout the stability study. 99 % of the 37 °C sample solutions (PBS and Pheroid™) were still detectable after 12 h with only 0.5 – 0.8 % degradation that have occurred, while at 25 °C, 72 % (PBS) and 85 % Pheroid™ was detected. Therefore the diffusion studies (37 °C) were conducted over a 12 h period.

3.2.2 TGF-β1

After 12 h, 90 – 94 % all of all four TGF-β1 samples (PBS at 25 and 37 °C; Pheroid™ 25 and 37 °C) could still be detected. The highest concentration after 12 h was detected for the Pheroid™ 25 °C sample (94 %), while 93 % of the Pheroid™ (37 °C) sample was detected. Both PBS final concentrations detected were between 90 % (25 °C) and 92 % (37 °C). In order to detect ± 95 % of both PBS and Pheroid™, it was concluded to conduct the Franz cell diffusion studies at an interval of 6 h, because after 6 h PBS (25 and 37 °C) as well as Pheroid™ (25 °C) would be ± 97 % stable; although Pheroid™ at 37 °C was still 96 % stable.

3.3 *In vitro* permeation studies

Figure 2: Comparison of TNF-α and TGF-β1 average diffused concentration (pg/cm^2) in both PBS and Pheroid™

Figure 3: Box and whisker-plots of the median diffused concentration values (pg/cm^2) in PBS and Pheroid™™ of a) TNF-α and b) TGF-β1

3.3.1 TNF-α

The amount of active traversing the stratum corneum was determined by investigative comparison. Results obtained showed that TNF-α was detected in the receptor solutions, collected for both testing mediums (PBS and Pheroid™). The average percentage detected was within the detection ranges as stipulated by the product information sheet (4 – 500 pg/ml). Obtained diffused concentrations for the Pheroid™ (88.478 ± 0.976 pg/cm²) was slightly higher than that of PBS (82.664 ± 1.437 pg/cm²). The higher Pheroid™ concentration is definitely attributable to its effectiveness as a drug delivery system, as both testing mediums contained the same amount of active in conjunction with bestatin hydrochloride. Entrapment within the Pheroid™ vesicles generally leads to an enhanced delivery of compounds, facilitated by the fatty acid membrane binding proteins present within the lipid rafts in the cell membrane (Grobler et al., 2008).

A normal distribution was obtained ($p > 0.05$). Average (PBS: 82.664 pg/cm²; Pheroid™: 88.478 pg/cm²) and median (PBS: 82.790 pg/cm²; Pheroid™: 88.437 pg/cm²) concentrations was more or less the same for both testing mediums. From the results it can be concluded that either one of these parameters can be used to describe the centre of the data although the average is the most commonly used measure of the centre. The distribution of the data is normal and appears to be symmetrical (Sonnad, 2006, 2002:623). Results obtained is of practical importance ($p = 4.04$).

3.3.2 TGF-β1

From the results obtained it is evident that the Pheroid™ drug delivery system enhanced the permeation of TGF-β1 across the stratum corneum. A two-fold increase in the concentration diffused was detected for the Pheroid™ (302.441 pg/cm^2) when compared to that obtained for

PBS (148.274 pg/cm^2), with a % standard deviation (SD) of 8.76 (Pheroid™) and 17.75 (PBS). The higher drug concentration detected for the Pheroid™ samples is indicative of the successful entrapment of TGF-β1 that led to an enhancement in the absorption and transport of the active (Grobler et al., 2008). As the cellular uptake of Pheroid™ is actively facilitated by the fatty acid membrane-binding proteins normally present within lipid rafts in the cell membrane, it can be proposed that the Pheroid™ vesicles traversed the stratum corneum through the transcellular route (Grobler et al., 2008). The lower amount of active delivered by PBS could be attributable to the hydrophobic nature of the stratum corneum (Boss and Meinardie, 2000).

Normality could not be assumed ($p < 0.05$) for PBS whereas Pheroid™ data was distributed normally. The observed average for Pheroid™ (302.441 pg/cm^2) was greater than the median (294.617 pg/cm^2), while the opposite was observed for PBS, as the median (162.719 pg/cm^2) was greater than the average (148.274 pg/cm^2), resulting in a skewed distribution to the left. Due to the presence of outliers in the case of the Pheroid™ values, the median proved to be a better representation of central tendency as it is not affected by outliers (Gerber et al., 2008; Swanepoel et al., 2004). Results obtained was of practical importance ($p = 5.8$).

3.4 Tape stripping

Figure 4: Comparison of TNF-α and TGF-β1 average tape stripping concentrations (pg/ml) in both PBS and Pheroid™

Figure 5: Box and whisker-plots of the tape stripping values (pg/ml) in PBS and Pheroid™™ of a) TNF-α and b) TGF-β1

3.4.1 TNF-α

TNF-α concentrations in the stratum corneum-epidermis (PBS: 2.695 pg/ml; Pheroid™: 4.54 pg/ml) and epidermis-dermis (PBS: 6.684 pg/ml; Pheroid™ 8.636 pg/ml) solutions were higher for Pheroid™ than that of PBS. More active was delivered to the epidermis-dermis and less retained within the stratum corneum-epidermis (target zone). Although not to a great extent and less to what was expected, it is evident that better penetration and subsequent delivery of TNF-α was obtained with the Pheroid™ drug delivery system.

A significant difference in sample means was obtained for both mediums and skin ($p < 0.05$). A p-value of 0.898 was obtained for medium/skin interaction which implies no interaction between the two groups and the null hypothesis could be accepted. No significant difference between testing mediums or the skin was found ($p = 0.05$). Although both PBS and Pheroid™ individually affect the amount of active in the epidermis and dermis, no interaction was found and the effect is not of statistical significance. The skin and testing medium medians were statistical significant at the 5 % level. This concluded the both groups are different.

3.4.2 TGF-β1

Dermal (PBS: 24.995 pg/ml); Pheroid™: 47.299 pg/ml) concentrations were almost the same (PBS) or higher (Pheroid™) than the epidermal (PBS: 25.648 pg/ml; Pheroid™: 55.614 pg/ml) concentrations. This result was in accordance with the objective of this project, as the delivery of TGF-β1 was aimed at the epidermis where melanocytes are located. The higher concentration detected from the tape solutions (stratum corneum-epidermis) is also indicative of better penetration and delivery of the Pheroid™ entrapped compound across the stratum corneum.

The null hypothesis was rejected in terms of a significant difference between sample means for the testing mediums but not for the skin ($p = 0.00$). However, an interaction between testing mediums and the skin was established as a p-value of 0.447 was generated. Although a significant difference was not found between groups, it was established that both testing mediums had an effect on the skin. The alternative hypothesis (sample medians differ) was accepted in terms of the skin (epidermis and dermis). It can thus be concluded that both testing mediums had no significant effect on the drug concentrations detected in the skin.

4 Conclusion

Stability tests conducted in this study was successful in the prediction of the degradation rates for both cytokines as the duration of their diffusion studies were established. Both cytokines were still detectable after 12 h although to different extents. The Pheroid™ vesicles increased the stability of TNF-α in both 25 °C and 37 °C, but not for TGF-β1 as only 95 % of the cytokine was detectable after 6 h whereas 99 % of TNF-α could still be detected after 12 h. Based on the results obtained, the respective diffusion rates were determined as 12 h (TNF-α) and 6 h (TGF-β1).

The transdermal/topical delivery of cytokines (TGF-β1 and TNF-α) were successfully enhanced by the Pheroid™ technology, although not to a great extent for TNF-α. Although both cytokine solutions included aminopeptidase inhibitor bestatin hydrochloride, both TNF-α solutions (PBS and Pheroid™) were not delivered across the skin to a great extent when compared to TGF-β1 detected concentrations. This could be attributable to the presence of micro-organisms such as *Staphylococcus epidermidis* present on the skin surface which could be responsible for metabolising topically applied drugs (Williams 2003). As TNF-α was still detectable in both receptor and tape stripping (stratum corneum-epidermis and epidermis-dermis) solutions, it can be concluded that the Pheroid™ vesicles were successful in delivering a higher concentration of TNF-α across the skin.

The transdermal/topical delivery of TGF-β1 was enhanced by the Pheroid™ drug delivery technology. This conclusion was evident from the amount of active detected in the receptor as well as tape stripping (stratum corneum-epidermis and epidermis-dermis) solutions. When comparing the tape stripping results, TGF-β1 was detected in both solutions (PBS and Pheroid™); a higher concentration was detected in the epidermis solutions. From this result it can be concluded that TGF-β1 was successfully delivered to its target zone, the melanocytes located within the epidermal skin layer. When comparing diffusion and tape stripping data, it can be concluded that in the case of both cytokines, the larger the amount of active diffused, the more active ingredients was delivered to the epidermis and dermis.

Although a comparative study was not conducted in order to establish the success of bestatin hydrochloride as an enzyme inhibitor, it can be concluded that the addition of bestatin hydrochloride to cytokine solutions enhanced the permeation of both TGF-β1 and TNF-α, as both actives could still be detected form tape stripping solutions.

The higher amount of diffused concentrations obtained from Pheroid™ solutions, as well as the elevated concentrations obtained from tape stripping (stratum corneum-epidermis and epidermis-dermis) data, is definitely suggestive that the Pheroid™ technology proved to be advantageous for the topical delivery of the above mentioned cytokines.

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Figure legends:

Figure 1: CLSM micrographs: a) Placebo Pheroid™ vesicles; b) TGF-β1 entrapped in Pheroid™ vesicles and c) TNF-α entrapped in Pheroid™ vesicles

Figure 2: Comparison of TNF-α and TGF-β1 average diffused concentration (pg/cm^2) in both PBS and Pheroid™

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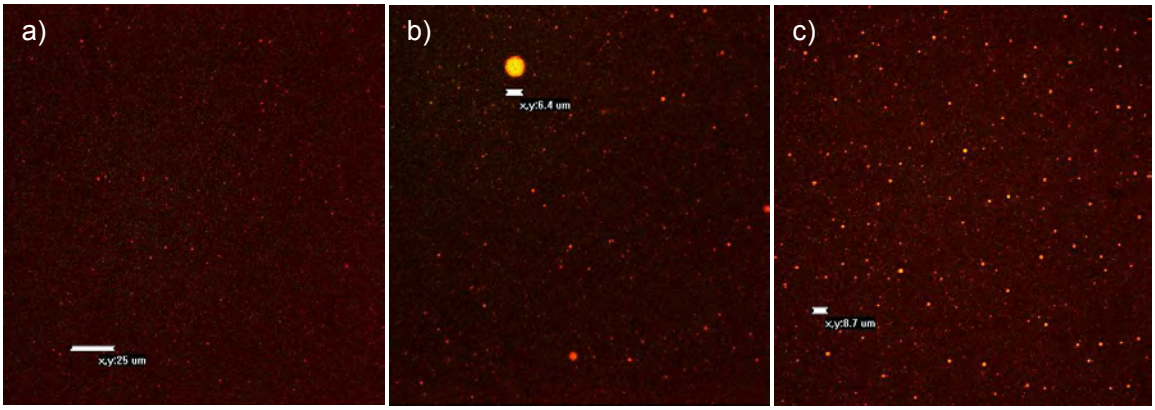
Figures:



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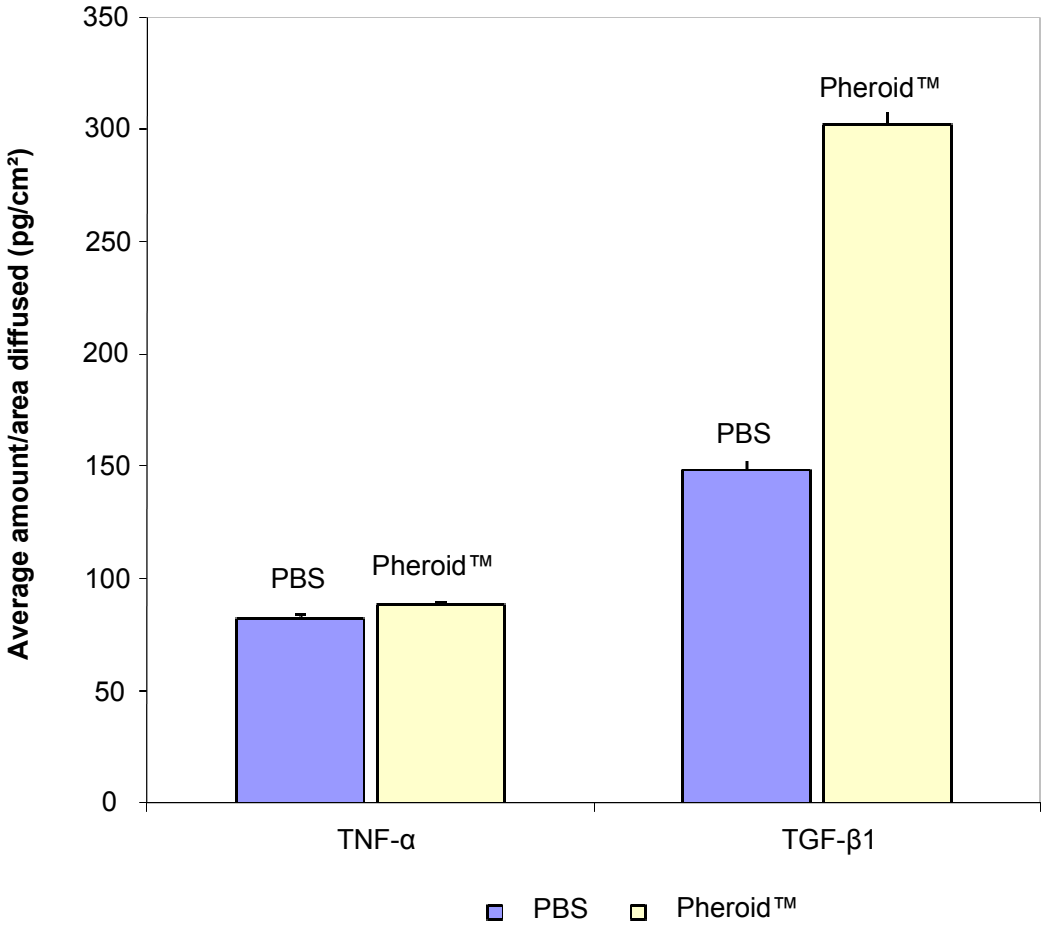

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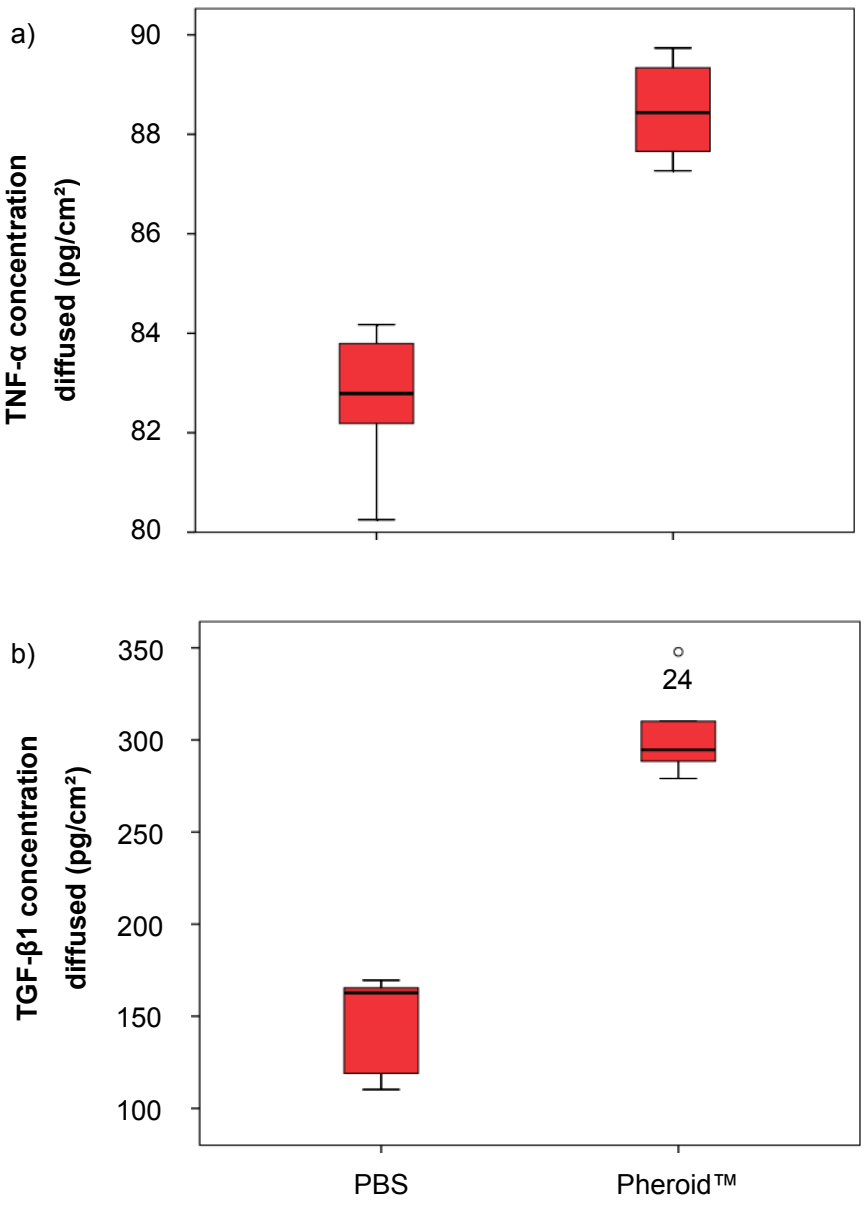

Figure 3: Box and whisker-plots of the median diffused concentration values (pg/cm^2) in PBS and Pheroid™ of a) TNF-α and b) TGF-β1

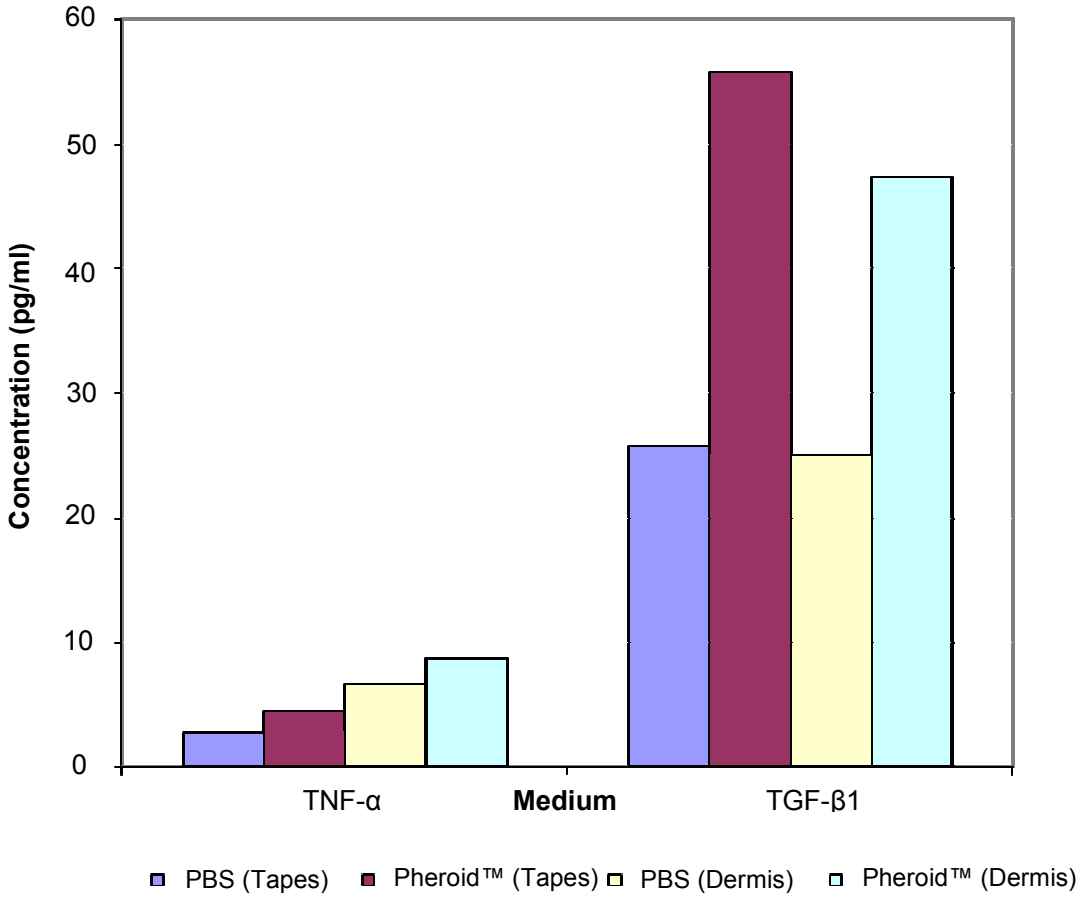

Figure 4: Comparison of TNF-α and TGF-β1 average tape stripping concentrations (pg/ml) in both PBS and Pheroid™

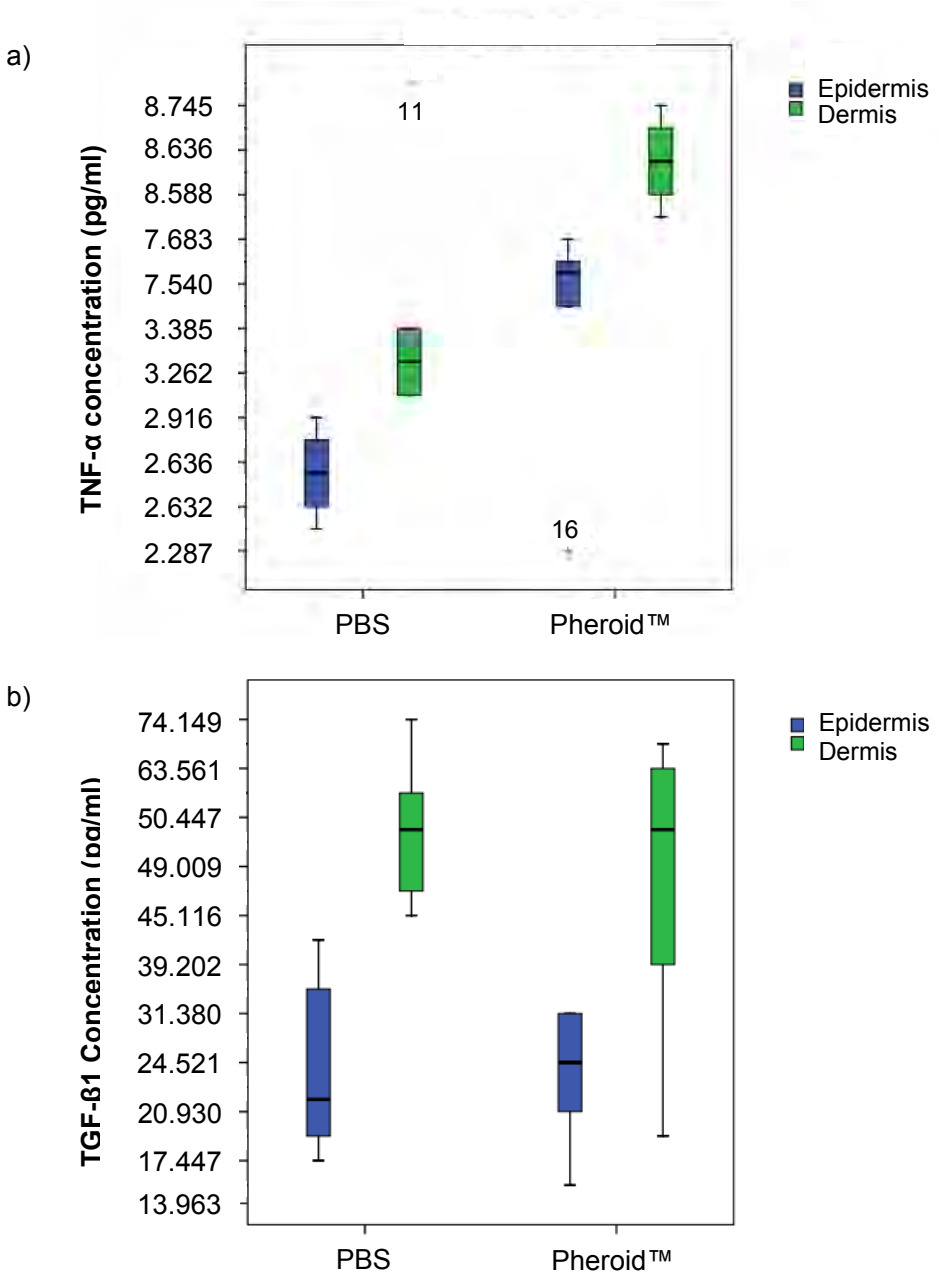

Figure 5: Box and whisker-plots of the tape stripping values (pg/ml) in PBS and Pheroid™ of a) TNF-α and b) TGF-β1

CHAPTER 4: FINAL CONCLUSIONS AND FUTURE PROSPECTS

The aim of this study was to establish whether depigmenting cytokines, TNF-α and TGF-β1, could be topically delivered across the skin with the aid of the Pheroid™ drug delivery technology.

The principle 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 factors. Such factors include temperature, humidity and light (ICH Tripartite Guidelines, 2003:5). The main objective for the stability tests conducted during this study was to establish the degradation rate of cytokines (TGF-β1 and TNF-α) in PBS and Pheroid™ over 12 h. In order to ensure maximum detectable concentrations of both cytokines during diffusion studies, a cut off concentration of 95 % would be acceptable. Results obtained revealed that TNF-α diffusion study could be conducted at 12 h and TGF-β1 at 6 h.

Topical dermatological therapy is aimed at increasing the retention of therapeutic drugs in the skin (Lund, 1994); however, few drugs are able to passively diffuse across the uppermost layer of the skin, the stratum corneum, as a result of its effective barrier properties (Asbill & Michniak, 2000:36). The use of *in vitro* techniques such as Franz type diffusion cells permits the evaluation of the dermal kinetics of drug uptake and diffusion (Kalia & Guy, 2001:160).

In an attempt to overcome the barrier properties of the skin, a novel drug delivery system known as the Pheroid™ drug delivery system was employed. Pheroid™ technology (previously known as Emzaloid™), is a delivery system that promotes the absorption and increases the efficacy of dermatological, biological and oral medicines in various pharmacological groups and has been used in several preparations for the treatment of many skin disorders such as psoriasis, eczema and dermatitis (Saunders *et al.,* 1999:99).

In order to improve the bioavailability of pharmaceutical proteins, such compounds can be co-administered with inhibitors to slow down metabolic degradation (Crommelin *et al.,* 2002). One such enzyme inhibitor employed in the topical delivery of TGF-β1 and TNF-α, was bestatin hydrochloride a specific inhibitor of aminopeptidase B and leucine aminopeptidase (Umeza *et al.,* 1975:99). Bestatin exerts its activity by competing with the substrates, binding to the catalytic site of the enzyme, exhibiting a competitive kinetics with the substrate once bound (Lkhagvaa *et al.,* 2008:390). A tape stripping technique was used to investigate the amounts of

drug that were present in the stratum corneum-epidermis of the excised human skin after the application of the supersaturated solutions (Pellet *et al.,* 1997:92).

During the course of the study the following observations were made:

- Successful entrapment of the above mentioned depigmenting agents in Pheroid™ vesicles by means of CLSM.
- Stability profiles of TNF-α and TGF-β1 in Pheroid™ vesicles was established.
- Topical drug delivery was confirmed by data obtained from tape stripping.
- When compared to PBS, a higher amount of concentration diffused was obtained with the Pheroid™ drug delivery system.

Aspects rendering further investigation:

- If possible, the utilisation of the same donor skin in order to monitor possible drug degradation on the skin surface for both cytokines (TNF-α and TGF-β1) and mediums (PBS and Pheroid™).
- Reduction of cytokine concentrations in order to obtain topical rather than transdermal drug delivery.
- Determination of depigmentation by means of quantitative image analysis of melanosomes in order to determine melanin contents.
- Comparative study with and without bestatin in order to establish the exact enhanced protection of protein concentrations.

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APPENDIX A
STABILITY TESTING

A.1 INTRODUCTION

The principle 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 factors. Such factors include temperature, humidity and light (ICH Tripartite Guidelines, 2003:5). Investigation of the intrinsic stability of drugs provides the possibility to advise on formulation approaches, indicate types of excipients, protective additives and packaging (Barry, 2002:129). The main objective for this study was to establish the degradation rate of cytokines (TGF-β1 and TNF-α) in PBS and Pheroid™ over 12 h. The result of this study would then provide the necessary criterion to determine the length of diffusion studies to be conducted at a later stage.

A.2 GUIDELINES FOR STABILITY TESTING

A.2.1 Stress testing

Stress testing of the drug substance can help identify the likely degradation products. The nature of these tests will depend on the individual drug substance and the type of drug product involved. Stress testing is likely to be carried out on a single batch of the drug substance and should include the effect of temperature, humidity (where appropriate), oxidation, and photolysis on the drug substance (ICH Tripartite Guidelines, 2003:5). Under accelerated conditions, studies may provide useful support data for establishing the expiration date, provide product stability information for future product development, assist in validation of analytical methods for the stability program, or generate information which may help reveal the degradation profile of the drug substance or drug product (ICH Tripartite Guidelines, 2003:6).

The stress criterion selected for this study was temperature. Studies were conducted at 25 and 37 °C in order to establish the degradation rate of actives at room- and water bath temperature during *in vitro* penetration studies.

A.2.2 Selection of batches

The overall quality of the batches of drug substance, placed on formal stability studies, should be representative of the quality of the material used in preclinical and clinical studies to and of

the quality of the material to be made at manufacturing scale (ICH Tripartite Guidelines, 2005:3). Batches were selected according to maximum detection ranges as specified by the manufacturer at quantities of 10 ml.

A.2.3 Specifications

Suggested maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis (ICH Tripartite Guidelines, 1995:7). Release limits to be derived from consideration of all the available stability information should relate to the limits of acceptance (MCC, 2009:8).

The limit of acceptance for detection of both actives would be determined by the time interval whereby 80 % of the actives are still detectable.

A.2.4 Storage conditions

A drug product should be evaluated under storage conditions that test its thermal stability and, if applicable, its sensitivity to moisture or potential for solvent loss. Stability testing of the drug product after constitution or dilution, if applicable, should be conducted to provide information for the labelling on the preparation, storage condition, and in-use period of the constituted or diluted product (ICH Tripartite Guidelines, 2003:11).

Actives were stored at 25 and 37 °C for 12 h. The duration and temperatures were chosen based on the conduction and length of transdermal diffusion studies.

A.2.5 Packaging/containers

Stability studies should be conducted on the drug substance packaged in a container closure system that is the same as or simulates the packaging proposed for storage and distribution (ICH Tripartite Guidelines, 2003:5). The containers to be used in the long-term, real-time stability evaluation, should be the same as, or closely simulate, the actual packaging, to be used for storage and distribution (MCC, 2009:6).

Both actives were reconstituted in glass politops and sealed with fitted plastic tops.

A.2.6 Evaluation

A systematic approach should be adopted in the presentation and evaluation of the stability information (MCC, 2009:9). The nature of any degradation relationship will determine whether

the data should be transformed for linear regression analysis. This relationship can be represented by a linear, quadratic, or cubic function on an arithmetic or logarithmic scale (ICH Tripartite Guidelines, 2003:9).

A.2.7 Guidance for stability testing

The "significant change" for a drug product is defined as:

- a 5 % change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
- any degradation product's exceeding its acceptance criterion; and
- failure to meet the acceptance criteria for appearance, physical attributes, and functionality test.

The stability of the drug products after reconstituting or diluting according to labelling should be addressed in order to provide appropriate and supportive information (ICH Tripartite Guidelines, 2003:12). The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts (ICH Tripartite Guidelines, 1995:7).

A.3 Test methods

A.3.1 Sample preparation

Cytokine standards were reconstituted to their respective concentrations in conjunction with aminopeptidase inhibitor, bestatin hydrochloride. Concentrations are shown in Table A.1 below.

Table A.1: Sample concentrations

TGF-β1	1000 pg/ml
TNF-α	500 pg/ml
Bestatin hydrochloride	300 µg/ml

Analytes were reconstituted to a final volume of 5 ml.

A.3.2 Enzyme linked immunosorbent (ELISA) detection

Protein assay techniques of high sensitivity and discrimination are provided by immunochemical procedures. Radio-immunoassays and ELISAs are extensively used to detect small amounts of specific proteins and other biological substances (Voet & Voet, 1995:74).

A.3.2.1 Experimental procedure

The experimental procedure consisted of four main steps: coating of plates and capture with the respective antibody; addition of standards (2 fold serial dilutions) and samples; incubation with detection antibody and subsequent secondary antibody and detection and analysis of the antibody-protein-antibody-enzyme complex (Voet & Voet, 1995:74). During incubation intervals plates were stirred between 200 – 240 rpm with a Stuart microtitre plate shaker.

Figure A.1: Stuart microtitre plate shaker (SSM5)

Appropriate wells were filled with 100 µl of samples. Plates were read at 450 and 570 nm using a BioTek Elx800 plate reader.

Figure A.2: BioTek Elx800 plate reader

A.4 Results and discussion

A.4.1 Results

Results obtained from the respective study are presented in the sections below.

A.4.1.1 Degradation of TNF-α

Table A.2: Stability testing profile of TNF-α in PBS and Pheroid™ (T0 – 12)

TNF-α (pg/ml)				
Time	**25 °C PBS**	**25 °C Pheroid™**	**37 °C PBS**	**37 °C Pheroid™**
0	553.978	552.14	495.237	467.657
1	555.204	523.948	495.237	467.131
2	526.272	523.367	494.684	467.131
3	525.691	523.367	494.132	466.604
4	523.948	522.786	493.58	466.604
5	499.102	523.367	493.028	466.604
6	475.022	452.108	492.475	466.078
7	498.55	495.789	468.183	444.572
8	433.093	432.131	469.235	445.577
9	433.093	473.447	446.079	425.402
10	416.111	432.612	410.58	444.572
11	398.707	414.267	447.084	422.031
12	399.149	472.918	467.657	443.576
TNF-α % Degradation				
Time	**25 °C PBS**	**25 °C Pheroid™**	**37 °C PBS**	**37 °C Pheroid™**
0	100.000	100.000	100.000	100.000
1	100.221	94.894	100.000	99.890
2	94.999	94.789	99.888	99.888
3	94.894	94.789	99.777	99.775
4	94.579	94.684	99.665	99.775
5	90.094	94.789	99.554	99.775
6	85.747	81.883	99.442	99.662
7	89.995	89.794	94.537	95.064
8	78.179	78.265	94.750	95.279
9	78.179	85.748	90.074	90.965
10	75.113	78.352	82.906	95.064
11	71.972	75.029	90.277	90.244
12	72.051	85.652	94.431	94.851

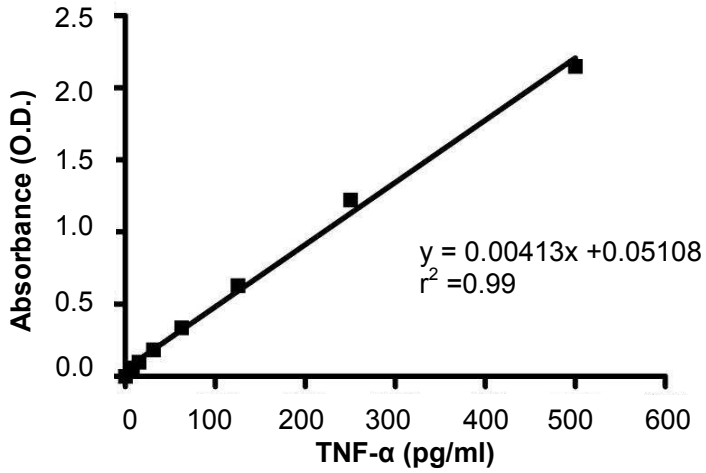

Figure A.3: TNF-α standard curve

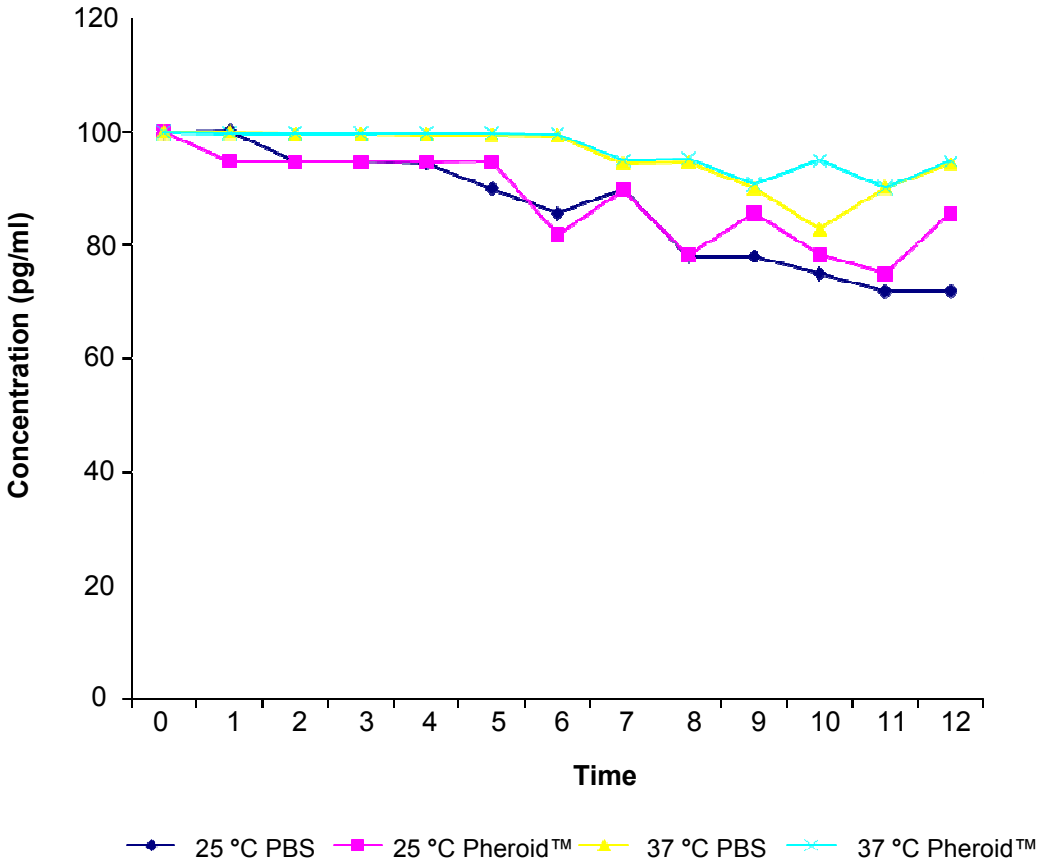

Figure A.4 Visual representation of TNF-α degradation (T0 – 12)

A.4.1.2 Degradation of TGF-β1

Table A.3: Stability testing profile of TGF-β1 in PBS and Pheroid™ (T0 – 12)

TNF-α Detection (pg/ml)				
Time	**25 °C PBS**	**25 °C Pheroid™**	**37 °C PBS**	**37 °C Pheroid™**
0	548.51	548.51	558.94	554.89
1	538.18	543.33	549.71	549.71
2	539.48	551.04	549.84	549.71
3	530.49	542.03	544.56	548.4
4	530.49	539.48	544.56	536.89
5	527.92	538.18	543.25	536.89
6	533.05	534.34	542.02	533.05
7	526.69	538.18	538.14	527.95
8	522.81	531.77	536.89	533.05
9	520.38	534.34	535.6	529.22
10	502.38	515.12	534.34	529.22
11	511.42	526.65	526.69	508.82
12	497.35	517.71	517.71	516.47
TNF-α % Detection				
Time	**25 °C PBS**	**25 °C Pheroid™**	**37 °C PBS**	**37 °C Pheroid™**
0	100.000	100.000	100.000	100.000
1	98.117	99.056	98.349	99.066
2	98.354	100.461	98.372	99.066
3	96.715	98.819	97.427	98.830
4	96.715	98.354	97.427	96.756
5	96.246	98.117	97.193	96.756
6	97.181	97.417	96.973	96.064
7	96.022	98.117	96.279	95.145
8	95.315	96.948	96.055	96.064
9	94.872	97.417	95.824	95.374
10	91.590	93.913	95.599	95.374
11	93.238	96.015	94.230	91.697
12	90.673	94.385	92.624	93.076

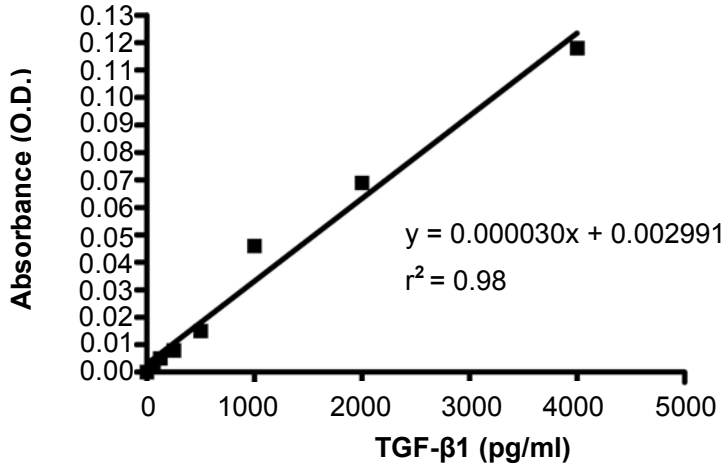

Figure A.5 TGF-β1 standard curve

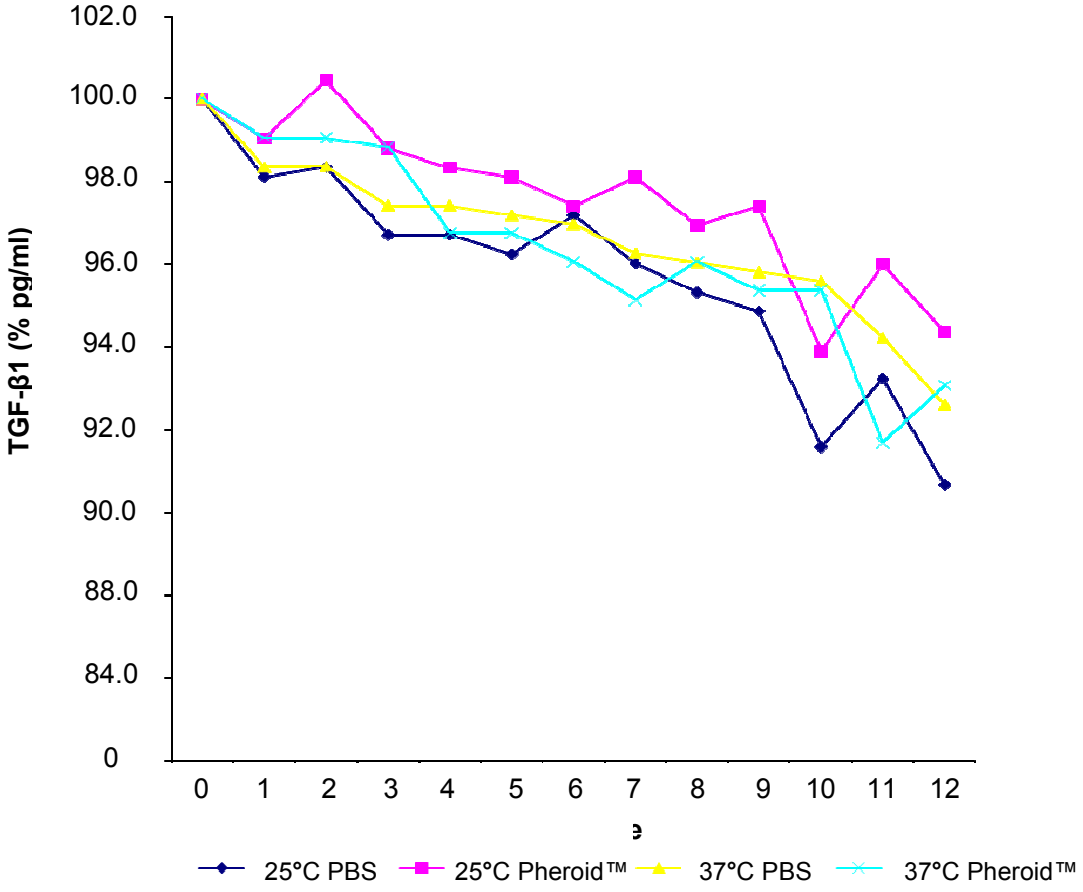

Figure A.6 Visual representation of TGF-β1 degradation (T0 – 12)

A.4.2 Discussion

The respective stability test intervals were determined in a trial stability test whereby 95 % of the actve had to be intact after 12 h as the duration of diffusion study can last up to 12 h. More than 95 % was still detectable for TNF-α at T12, whereas 95 % and more of TGF-β1 was detected at T6.

A.4.2.1 TNF-α

TNF-α was more stable at 37 °C in the Pheroid™ formulation with only 5 % degradation as opposed to 15 % degradation in the 25 °C sample after 12 h. No significant difference in degradation for both 37 °C PBS (6 %) and Pheroid™ (5 %) samples was detected. TNF-α was least stable in the 25 °C PBS medium (total degradation of 27 %) after 12 h. The elevated sample concentrations at certain intervals could be due to a loss of moisture which may lead to apparent increases in concentration of actives (Pugh, 2002:111). When compared to PBS, the Pheroid™ medium increased the stability of TNF-α at both temperatures. As ± 95 % TNF-α was still detectable at 37 °C after 12 h, its *in vitro* permeation study would subsequently be conducted at a 12 h interval.

A.4.2.2. TGF-β1

After 12 h 90 – 94 % all of all four TGF-β1 samples could still be detected. The highest concentration at T12 was detected for the Pheroid™ 25 °C sample (94 %), while 93 % of the Pheroid™ 37 °C sample was detected. Both PBS final concentrations detected were between 90 % (25 °C) and 92 % (37 °C). In order to detect ± 95 % of the Pheroid™ solution, it was concluded to conduct the respective Franz cell diffusion studies at an interval of 6 h (TGF-β1).

A.5 CONCLUSION

The duration of the degradation study was 12 h for each formlation. The aim of the test was to assess the stability of both cytokines in order to establish what the duration of their diffusion studies would be. In order to ensure maximum detectable concentrations of both cytokines during diffusion studies, a cut off concentration of 95 % would be acceptable.

By means of linear regression a correlation was found between the amount of drug absorbed over time. For both TNF-α (R^2 = 0.99) and TGF-β1 (R^2 = 0.98) the correlation coefficients were close to 1.0, implying a perfect fit of the least squares curve to the observed data.

From the results obtained, it can be concluded that the stability studies conducted was successful in its aim to establish the stability of both cytokines in PBS and Pheroid™ at 25 and 37 °C and the subsequent duration of Franz cell diffusion studies as the respective degradations rates was established.

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APPENDIX B
DIFFUSION STUDIES

B.1 INTRODUCTION

Permeation of chemicals through the skin can be measured by *in vivo* and *in vitro* techniques. *In vitro* techniques are generally used due to simple experimental conditions. Data from these studies allow with confidence the prediction of *in vivo* dermal absorption and percutaneous penetration of compounds (Varvaresou, 2006:52). Franz diffusion cell is extensively used for studying *in vitro* permeation (Mahajan *et al.,* 2009:314). Upon completion of diffusion studies, the concentration of test substances in each compartment of the skin (stratum corneum, epidermis and dermis) and receptor fluid is analysed. The stratum corneum adsorption represents the amount of topically applied test substance found in the stratum corneum after cessation of the experiment. Dermal absorption represents the amount of topically applied test substance that is found in the epidermis, whilst dermis and percutaneous absorption represents the amount of topically applied substance found in the receptor fluid (Varvaresou, 2006:52). In this study, Franz cell diffusion and tape stripping technique was used to determine the amount of test substances that penetrated through (collected receptor fluids) and into the skin, respectively.

B.2 METHODS

B.2.1 Skin preparation

In the course of this study, female, Caucasian skin obtained from abdominal plastic surgeries was employed for conduction of Franz cell diffusion studies in order to reduce the variability in skin permeability properties (Cross *et al.,* 2007:149).

Ethical approval for the procurement and exploitation of the skin tissue was provided by the Research Ethics Committee of the North-West University (reference number 04D08). Informed consent was obtained from the patients in advance and their identities were masked to assure anonymity.

After procurement, the full thickness skin examined for defects and stored frozen at -20 °C for no longer than 6 months. To obtain full thickness skin discs, the subcutaneous fat layer was removed and skin was cut into circles of about 15 mm in diameter with a punch and hammer.

Skin sections were placed on Whatman® filter paper with the stratum corneum side of the epidermal layer facing upwards and were subsequently left to air dry. The prepared skin discs were covered in aluminium foil and were kept frozen at -20 °C until used.

Figure B.1: The punch and hammer

B.2.2 Preparation of receptor and donor solutions

The receptor solution consisted of a PBS (pH7.4) solution. The PBS was prepared by adding 250 ml of 0.2 M KH_2PO_4 (potassium dihydrogen orthophosphate) (6.8 g KH_2PO_4 dissolved in 393.4 ml distilled water) to 393.4 ml of 0.1 M NaOH (sodium hydroxide) (1.5736 g NaOH dissolved in 250 ml distilled water).

Donor solutions were reconstituted to their respective cytokine concentrations (TGF-β1 (1000 pg/ml); TNF-α (500 pg/ml)) in conjunction with bestatin hydrochloride (300 µg/ml).

B.2.3 Franz cell diffusion method

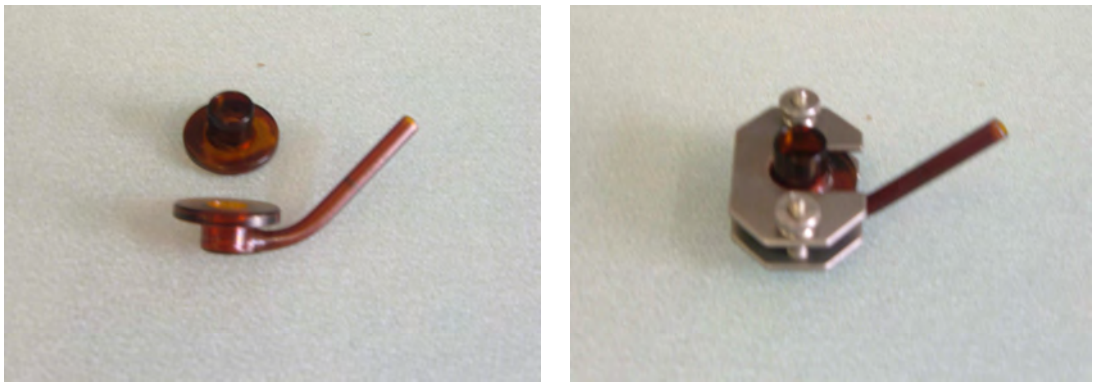

Figure B.2: a) Franz cell diffusion donor and receptor compartments; b) Donor and receptor compartments clamped together

In vitro permeation studies were conducted by means of Franz diffusion studies with a receptor capacity of 2 ml and a diffusion area of 1.075 cm^2. Prior to conduction of *in vitro* permeation studies, full thickness skin discs were thawed at room temperature. The skin discs were clamped between Franz type diffusion cells with the epidermis facing the donor compartment. Both the receptor and donor compartments were clamped together with a metal clamp and sealed with Dow Corning® vacuum grease.

Figure B.3: Dow Corning high vacuum grease

The receptor compartments were slowly filled with PBS (pH 7.4), taking care to avoid the accumulation of air bubbles, while the donor compartments were filled with the respective depigmenting agents, TGF-β1 (1000 pg/ml) and TNF-α (500 pg/ml). Diffusion cells were placed in a water bath at 37 °C, with only the receptor compartments submerged in the water, stirred at 75 rpm by means of a Variomag® magnetic stirrer.

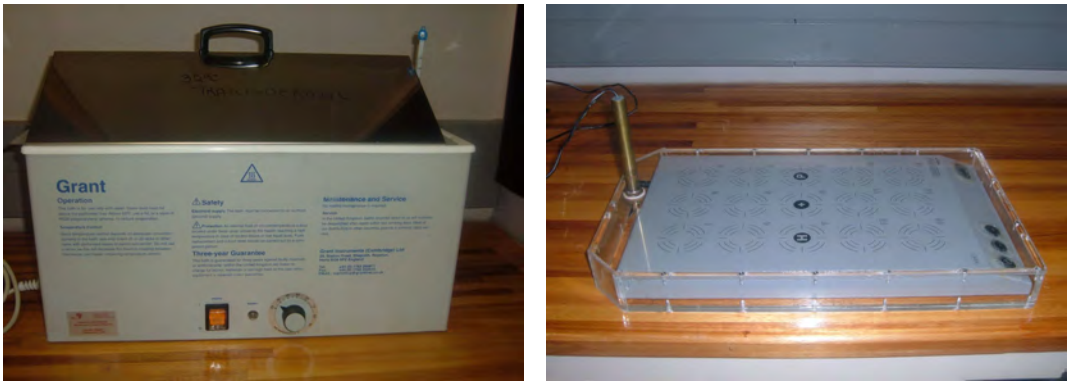

Figure B.4: a) Grant water bath; b): Variomag magnetic stirrer

Donor compartments were covered with Parafilm® to avoid evaporation. For both cytokines, TGF-β1 and TNF-α, the entire contents of the receptor compartments were withdrawn at an interval of 12 h (TNF-α) and 6 h (TGF-β1), respectively. Each sample (100 µl) was directly

assayed by means of ELISA in order to determine the amount of peptide concentration within the respective receptor compartments.

B.2.4 Tape stripping

In vitro methods reveal the potential of a substance to penetrate the skin. After determination of diffusion periods, residual material in the stratum corneum-epidermis and epidermis-dermis was analysed by using a tape stripping method (Varvaresou, 2006:54). Upon removal of the donor and receptor phases at 12 h (TNF-α) and 6 h (TGF-β1), skin discs were carefully dismantle dabbed dry with tissue and stapled on a piece of Parafilm® to a solid surface. Adhesive tape (3M Scotch® Magic™ Tape) was cut into pieces, into the size of the skin (diffusional area) dismounted from each cell disk. The first tape strip was discarded as it is considered to be part of the cleaning procedure (Pellet *et al.,* 1997:94). The following 15 tape strips were placed in a vial containing 5 ml PBS. Excess skin was trimmed away from the flange imprints and the remaining viable epidermis and dermis was placed in vials as well. Collected samples were kept in a fridge at 4 C overnight to allow extraction of the respective depigmenting agents into the PBS (Pellet *et al.,* 1997:94). Samples were then assayed by ELISA analysis the following day.

B.2.5 Analysis of samples by enzyme linked immunosorbent assay (ELISA) detection

Subsequent to collection of receptor compartment fluid, protein concentrations of both cytokines was measured with human TGF-β1 and TNF-α ELISA kits following the supplier's protocol. Each incubation step was followed by aspiration with wash buffer (PBS 7.4). The experimental procedure consisted of four main steps: 1) coating of plates and capture with the respective antibody; 2) addition of standards (2-fold serial dilutions) and samples; 3) incubation with detection antibody and subsequent secondary antibody and detection and 4) analysis of the antibody-protein-antibody-enzyme complex (Voet & Voet, 1995:74).

During incubation intervals plates were stirred between 200 – 240 rpm with a Stuart microtitre plate shaker. Appropriate wells were filled with 100 µl of samples. Plates were read at 450 and 570 nm using a BioTek Elx800 plate reader.

Figure B.5: Stuart microtitre plate shaker; b) BioTek Elx800 plate reader

B.2.6 Topical drug delivery and statistical analysis

The cumulative amount (pg/ml) of active that traverse the skin was detected by ELISA tests as mentioned above. Samples were detected at 450 and 570 nm. Values detected at 570 nm were subtracted from those detected at 450 nm and cytokine (TGF-β1 or TNF-α) concentrations were obtained. Total protein concentrations were detected by means of protein quantitation. Final protein concentrations were determined by dividing the initial cytokine concentration by the total protein value. The % diffusion was determined by division of the diffused concentration by the initial concentration. The diffused concentration was expressed as the amount in the donor solution multiplied by the correlation factor (μg/cm^2). Measure of spread was determined by standard deviation (SD) and % SD.

Statistical analysis was determined by using SPSS analysis. Equality of variances was determined by the Levene's test for equal variances for diffused concentrations while a two-way analysis of variance (ANOVA) was used for tape stripping data. The Mann Whitney U-test was used to determine normality of diffused concentrations and tape stripping values (Millar 2001:29). Practical significance of both diffused concentrations and tape stripping data was determined by effect size measurement. A *p*-value smaller than 0.05 was considered as evidence that results were of statistical significance (Ellis & Steyn, 1994:1). Comparison between data sets was visualised by means of box-plots (Swanepoel *et al*., 2004:88).

B.2.7 Enzyme inhibition by bestatin hydrochloride

Two diffusion studies were conducted with a total of 12 diffusion cells for each test medium (Pheroid™ and PBS). The respective concentrations are shown in the Table B.1 below.

Table B.1: Cytokine and bestatin concentrations

TGF-β1	1000 pg/ml
TNF-α	500 pg/ml
Bestatin hydrochloride	300 µg/ml

Bestatin hydrochloride, an aminopeptidase inhibitor (Umezwa *et al.,* 1976:99) was included in donor solution in order to combat drug metabolism within the skin (Barry 2002:552), 2006:263).

B.3 Results

Cytokine (TNF-α and TGF-β1) ELISA detection, the amount of diffused concentration as well as detected cytokine tape stripping concentrations can be seen in the following section.

B.3.1 Protein detection

B.3.1.1 TNF-α

Table B.2: TNF-α protein detection in PBS and the Pheroid™ (protein detection at 450 and 570 nm) for time interval 0 – 12 h

TNF-α in PBS and Pheroid™						
Spectrophotometry O.D. (nm)				Protein concentration (pg/ml)		
T0	450	570	450 – 570	[TNF-α]	Total protein	[TNF-α]/ Total protein
PBS	0.914	0.041	0.873	190.612	5.933	818.832
Pheroid™	0.917	0.041	0.876	191.308	0.233	902.471
PBS						
Spectrophotometry O.D. (nm)				Protein concentration (pg/ml)		
Cell	450	570	450 – 570	[TNF-α]	Total protein	[TNF-α]/ Total protein
1	0.302	0.041	0.302	48.683	1.086	44.840
2	0.302	0.044	0.302	47.987	1.086	44.199
3	0.300	0.043	0.300	47.755	1.107	43.158
4	0.301	0.043	0.301	47.987	1.065	45.062
5	0.304	0.041	0.263	49.147	1.086	45.267
6	0.303	0.041	0.262	48.915	1.107	44.206
Pheroid™						
Spectrophotometry O.D. (nm)				Protein concentration (pg/ml)		
Cell	450	570	450 – 570	[TNF-α]	Total protein	[TNF-α]/ Total protein
1	0.318	0.042	0.276	52.161	1.086	48.044
2	0.318	0.041	0.277	52.393	1.086	48.257
3	0.319	0.043	0.276	52.161	1.107	47.140
4	0.318	0.043	0.275	51.929	1.107	46.931
5	0.318	0.041	0.277	52.393	1.086	47.350
6	0.319	0.040	0.263	52.857	1.107	47.769

* O.D. = Optical detection

B.3.1.2 TGF-β1

Table B.3: TGF-β1 protein detection in PBS and the Pheroid™ (protein detection at 450 and 570 nm) for time interval 0 – 6 h

TGF-β1 in PBS and Pheroid™						
Spectrophotometry O.D. (nm)				Protein concentration (pg/ml)		
T0	450	570	450 – 570	[TGF-β1]	Total protein	[TGF-β1]/ Total protein
PBS	0.194	0.039	0.155	5046.78	8.928	565.248
Pheroid™	0.193	0.042	0.151	4913.98	8.928	550.374
PBS						
Spectrophotometry O.D. (nm)				Protein concentration (pg/ml)		
Cell	450	570	450 – 570	[TGF-β1]	Total protein	[TGF-β1]/ Total protein
1	0.068	0.046	0.022	0.299	7.181	87.886
2	0.065	0.049	0.016	0.299	7.285	59.287
3	0.067	0.044	0.023	0.299	7.285	91.189
4	0.062	0.045	0.017	0.299	7.264	64.027
5	0.065	0.043	0.022	0.299	7.243	87.129
6	0.064	0.042	0.022	0.299	7.098	88.916
Pheroid™						
Spectrophotometry O.D. (nm)				Protein concentration (pg/ml)		
Cell	450	570	450 – 570	[TGF-β1]	Total protein	[TGF-β1]/ Total protein
1	0.073	0.043	0.03	896.713	5.974	150.092
2	0.074	0.043	0.031	929.914	5.995	155.109
3	0.075	0.043	0.032	963.114	5.954	161.770
4	0.078	0.045	0.033	996.315	5.974	166.764
5	0.074	0.043	0.031	929.914	5.995	155.109
6	0.079	0.042	0.037	1129.117	6.037	187.038

* O.D. = Optical detection

B.3.2 Diffusion studies

B.3.2.1 TNF-α

Table B.4: TNF-α % diffused and concentration diffused (pg/cm^2)

Diffusion: TNF-α						
	Concentration (pg/ml)		Concentration after correlation factor (pg/cm^2)		% Diffused	
	PBS	Pheroid™	PBS	Pheroid™	PBS	Pheroid™
Initial	818.832	902.471	-	-	-	-
1	44.840	48.044	424.715	797.081	17.789	26.793
2	44.199	48.257	438.661	791.973	18.373	26.621
3	43.158	47.140	442.168	786.615	18.520	26.441
4	45.062	46.931	439.939	787.374	18.426	26.467
5	45.267	47.350	437.714	790.187	18.333	26.561
6	44.206	47.769	438.139	789.162	18.351	26.527

Figure B.5: Average diffused concentration of TNF-α and spread (SD and % SD) after 12 h

	PBS	Pheroid™
Ave % diffused	14.145	30.505
Ave diffused concentration (pg/cm^2)	82.664	88.478
SD	1.437	0.976
% SD	1.738	1.103

B.3.2.1 TGF-β1

Table B.6: TGF-β1 % diffused and concentration diffused (pg/cm^2)

Diffusion: TGF-β1						
	Concentration (pg/ml)		Concentration after correlation factor (pg/cm^2)		% Diffused	
	PBS	Pheroid™	PBS	Pheroid™	PBS	Pheroid™
Initial	565.248	550.374	-	-	-	-
1	87.886	150.092	150.778	184.782	15.548	27.271
2	59.287	155.109	164.855	201.620	10.489	28.183
3	91.189	161.770	143.006	191.082	16.133	29.393
4	64.027	166.764	140.117	236.942	11.327	30.300
5	87.129	155.109	159.443	203.993	15.414	28.183
6	88.916	187.038	161.684	202.592	15.731	33.984

Figure B.7: Average diffused concentration of TGF-β1 and spread (SD and % SD) after 6 h

	PBS	Pheroid™
Ave % diffused	14.107	29.552
Ave diffused concentration (pg/cm^2)	148.274	302.441
SD	26.316	24.724
% SD	17.748	8.175

B.3.3. Tape stripping data

B.3.3.1 TNF-α

Table B.8: TNF-α stratum corneum-epidermis protein detection (450 and 570 nm) in PBS and Pheroid™ for time interval 0 – 12 h

TNF-α in PBS (stratum corneum-epidermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	450	570	450 – 570	[TNF-α]	Total protein	[TNF-α]/ Total protein
1	0.141	0.040	0.101	11.577	4.310	2.686
2	0.140	0.040	0.100	11.345	4.310	2.632
3	0.139	0.039	0.100	11.345	4.289	2.645
4	0.140	0.035	0.105	12.505	4.289	2.915
5	0.140	0.039	0.101	11.577	4.289	2.699
6	0.141	0.042	0.099	11.113	4.289	2.591
TNF-α in Pheroid™ (stratum corneum-epidermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	450	570	450 – 570	[TNF-α]	Total protein	[TNF-α]/ Total protein
1	0.288	0.041	0.110	13.664	4.206	3.170
2	0.285	0.041	0.110	13.664	4.310	3.170
3	0.289	0.041	0.112	14.128	4.331	3.262
4	0.152	0.041	0.113	14.360	4.352	3.300
5	0.288	0.043	0.245	51.002	4.310	10.434
6	0.289	0.042	0.114	14.592	4.310	3.385

* O.D. = Optical detection

Table B.9: TNF-α dermis protein detection (450 and 570 nm) in PBS and Pheroid™ for time interval 0 – 12 h

TNF-α in PBS (dermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	**450**	**570**	**450 – 570**	**[TNF-α]**	**Total protein**	**[TNF-α]/ Total protein**
1	0.288	0.039	0.249	45.900	5.974	7.683
2	0.285	0.043	0.242	44.276	5.995	7.385
3	0.289	0.042	0.247	45.436	5.974	7.605
4	0.152	0.042	0.110	13.664	5.974	2.287
5	0.288	0.042	0.246	45.204	0.110	7.540
6	0.289	0.042	0.247	45.436	5.974	7.605
TNF-α in Pheroid™ (dermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	**450**	**570**	**450 – 570**	**[TNF-α]**	**Total protein**	**[TNF-α]/ Total protein**
1	0.314	0.041	0.273	51.466	5.933	8.675
2	0.313	0.041	0.272	51.234	5.933	8.636
3	0.315	0.041	0.274	51.698	5.912	8.745
4	0.312	0.043	0.269	50.538	5.891	8.579
5	0.312	0.042	0.270	50.770	5.912	8.588
6	0.313	0.042	0.271	51.002	5.933	8.597

* O.D. = Optical detection

B.3.3.2 TGF-β1

Table B.10: TGF-β1 stratum corneum-epidermis protein detection (450 and 570 nm) in PBS and Pheroid™ for time interval 0 – 6 h

TGF-β1 in PBS (stratum corneum-epidermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	**450**	**570**	**450 – 570**	**[TGF-β1]**	**Total protein**	**[TGF-β1]/ Total protein**
1	0.052	0.042	0.010	232.703	9.490	24.520
2	0.050	0.042	0.008	166.301	9.551	17.485
3	0.052	0.042	0.008	166.301	9.511	17.485
4	0.055	0.042	0.013	332.304	9.511	34.939
5	0.058	0.043	0.015	398.705	9.490	42.013
6	0.050	0.042	0.008	166.301	9.532	17.447
TNF-α in Pheroid™ (stratum corneum-epidermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	**450**	**570**	**450 – 570**	**[TGF-β1]**	**Total protein**	**[TGF-β1]/ Total protein**
1	0.058	0.042	0.016	431.906	9.573	45.116
2	0.061	0.041	0.020	564.708	9.428	59.899
3	0.058	0.041	0.017	465.106	9.490	49.010
4	0.058	0.041	0.017	465.106	9.220	50.447
5	0.065	0.041	0.024	697.510	9.407	74.149
6	0.061	0.042	0.016	431.906	9.469	67.992

* O.D. = Optical detection

Table B.11: TGF-β1 dermis protein detection (450 and 570 nm) in PBS and Pheroid™ for time interval 0 – 6 h

TGF-β1 in PBS (dermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	**450**	**570**	**450 – 570**	**[TGF-β1]**	**Total protein**	**[TGF-β1]/ Total protein**
1	0.055	0.043	0.012	299.104	9.532	31.380
2	0.053	0.043	0.01	232.703	9.553	24.360
3	0.052	0.043	0.009	199.502	9.532	20.930
4	0.053	0.042	0.011	265.903	9.511	27.958
5	0.051	0.044	0.007	133.101	9.532	13.964
6	0.055	0.043	0.012	299.104	9.532	31.380
TNF-α in Pheroid™ (dermis)						
Spectrophotometry O.D.* (nm)				Protein concentration (pg/ml)		
Cell	**450**	**570**	**450 – 570**	**[TGF-β1]**	**Total protein**	**[TGF-β1]/ Total protein**
1	0.061	0.04	0.021	597.908	9.407	63.561
2	0.055	0.041	0.014	365.505	9.324	39.202
3	0.058	0.041	0.017	465.106	9.469	49.117
4	0.058	0.041	0.017	465.106	9.220	50.447
5	0.059	0.042	0.017	465.106	9.220	17.485
6	0.062	0.041	0.021	597.908	9.344	63.985

* O.D. = Optical detection

Table B.12: Summary of TNF-α and TGF-β1 tape stripping concentration data (pg/ml)

TNF-α	PBS TS*	Pheroid™ TS*	PBS Dermis	Pheroid™ Dermis
Cell	**Concentration (pg/ml)**		**Concentration (pg/ml)**	
1	2.686	3.170	7.683	8.675
2	2.632	3.170	7.385	8.636
3	2.645	3.262	7.605	8.745
4	2.915	3.300	2.287	8.579
5	2.699	10.434	7.540	8.588
6	2.591	3.385	7.605	8.597
Ave	**2.695**	**4.454**	**6.684**	**8.636**

TGF-β1	PBS TS*	Pheroid™ TS*	PBS Dermis	Pheroid™ Dermis
Cell	**Concentration (pg/ml)**		**Concentration (pg/ml)**	
1	24.521	45.116	31.380	63.561
2	17.485	59.899	24.360	39.202
3	17.485	49.010	20.930	49.117
4	34.939	74.149	27.958	50.447
5	42.013	59.899	13.964	17.485
6	17.447	45.611	31.380	63.985
Ave	**25.648**	**55.614**	**24.995**	**47.299**

* TS = tape stripping (stratum corneum-epidermis)

B.3.4 Statistical analysis and summary of data

B.3.4.1 TNF-α

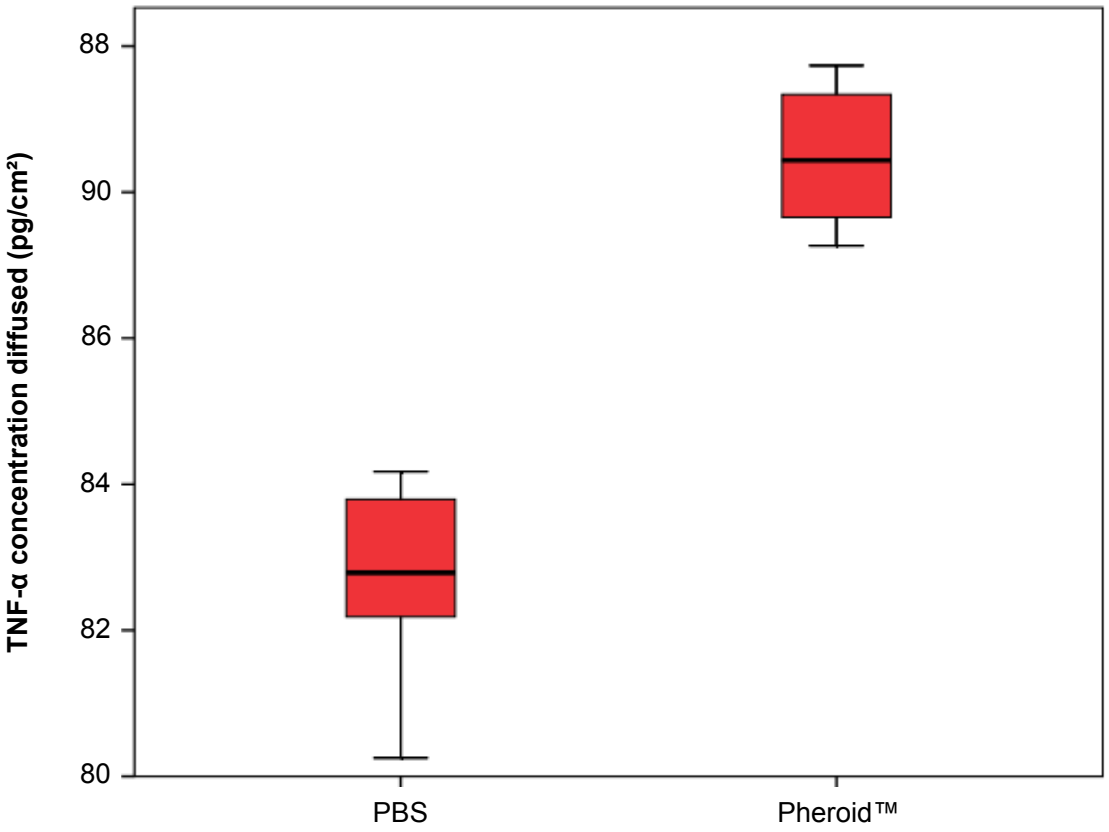

Figure B.6 Box-plots to illustrate the median concentration diffused (μg/cm^2) values of TNF-α in PBS and Pheroid™

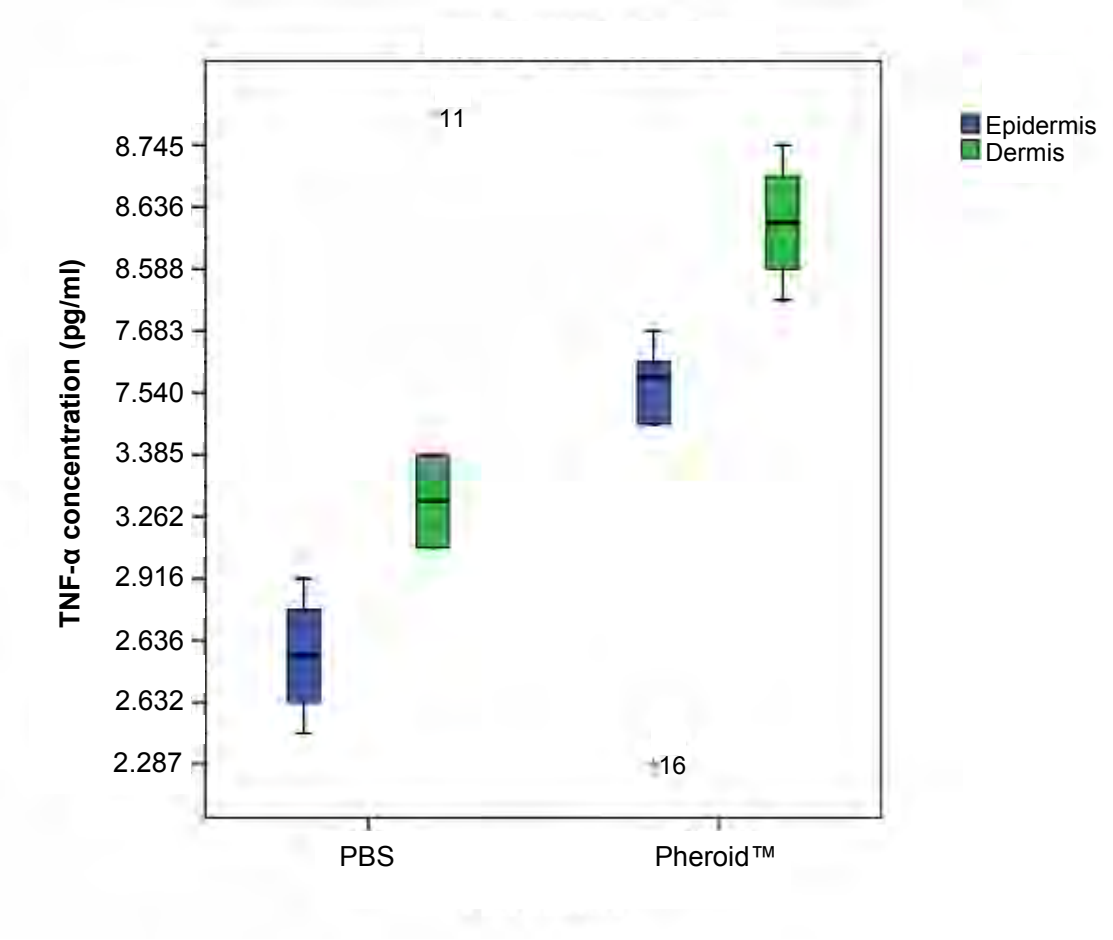

Figure B.7: Box- and whisker-plots of the tape stripping values (pg/ml) of TNF-α in PBS and Pheroid™

B.3.4.2 TGF-β1

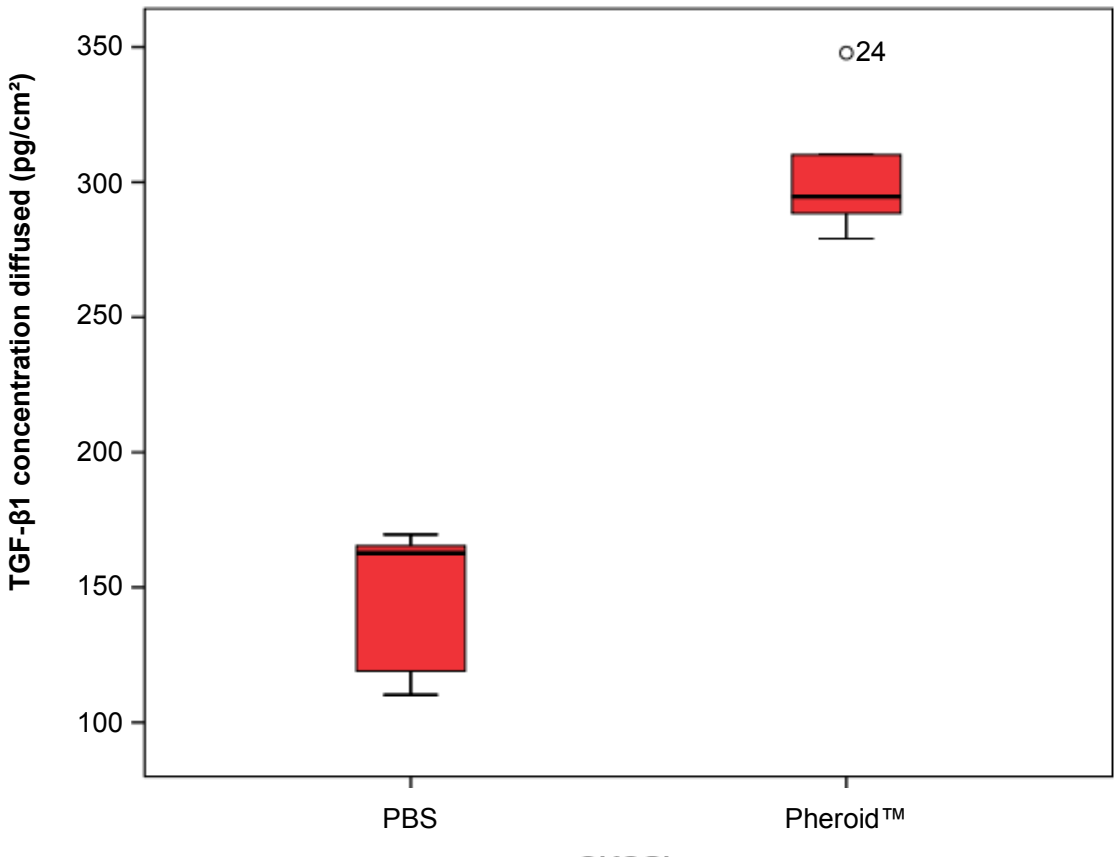

Figure B.8: Box-plots to illustrate the median concentration diffused (μg/cm^2) values of TGF-β1 in PBS and Pheroid™

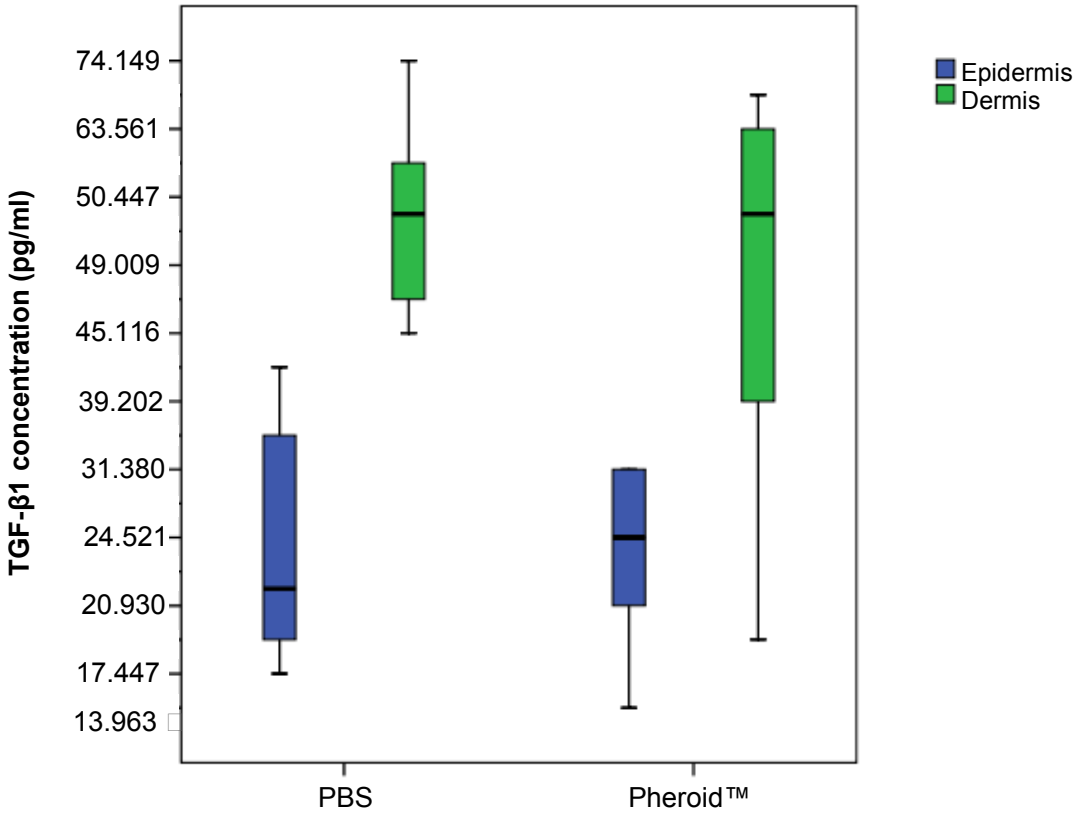

Figure B.9: Box- and whisker-plots of the tape stripping values (pg/ml) of TGF-β1 in PBS and Pheroid™

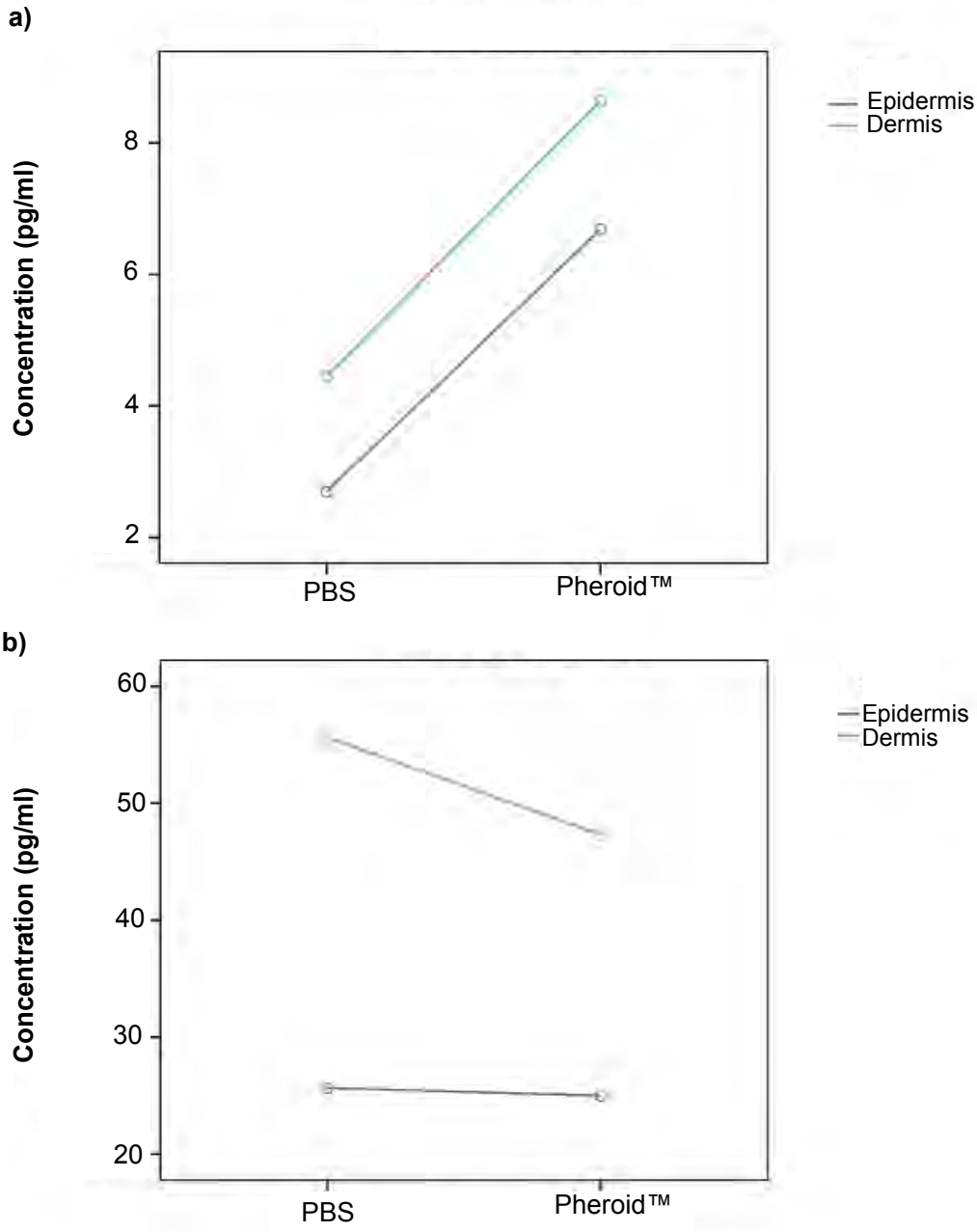

Figure B.10: Statistical interaction profiles of topical data obtained for a) TNF-α and b) TGF-β1

Table B.13: Summary of average and median concentration diffused (pg/cm²) as well as average stratum corneum-epidermis and dermis concentration data (pg/ml) of TNF-α and TGF-β1

Cytokine	Medium	Average[a]	Median[b]	Average TS[c]	Average dermis
TNF-α	PBS	82.664	82.790	2.695	4.454
	Pheroid™	88.478	88.437	6.684	8.636
TGF-β1	PBS	148.274	162.719	25.648	55.614
	Pheroid™	302.441	294.617	24.995	47.299

[a] average concentration diffused

[b] median concentration diffused

[c] TS = tape stripping (stratum corneum-epidermis)

B.4 DISCUSSION

Average and median concentration diffused values of the actives in PBS and Pheroid™ were compared for each active. The amount of cytokine detected in stratum corneum-epidermis and dermis solutions were compared as well. In order to establish to what extent actives traversed into the skin, concentration diffused and topical data was compared. These comparisons are discussed in the section below.

B.4.1 Diffusion studies

B.4.1.1 TNF-α

The amount of active traversing the stratum corneum was determined by means of investigative comparison. TNF-α was detected well within its specified detection range as specified by the product information (4 – 500 pg/ml) for both PBS and Pheroid™ receptor solutions. As expected, a higher concentration was detected for the Pheroid™ (88.478 ± 0.976 pg/cm²) when compared to PBS (82.664 ± 1.437 pg/cm²). Although not to a great extent, the higher amount of active delivered across the stratum corneum is definitely attributable to the effectiveness of the Pheroid™ drug delivery technology as a drug delivery system. Results obtained also serves as evidence that better entrapment in a carrier or drug delivery system leads to better drug delivery (Grobler *et al.,* 2008:296).

Normality could be assumed ($p > 0.05$). Average (PBS: 82.664 pg/cm²; Pheroid™: 88.478 pg/cm²) and median (PBS: 82.790 pg/cm²; Pheroid™: 88.437 pg/cm²) concentrations was more or less the same. Based on the obtained result, it can be concluded that either one of

the parameters can be used to describe the centre of the data. In a normally distributed population the average and median would be the same (Sonnad, 2006:623). Results obtained was of practical importance (p = 4.04).

B.4.1.2 TGF-β1

The dermal drug delivery of TGF-β1 was more successful when compared to TNF-α data. A two-fold increase in drug concentration was detected for the Pheroid™ (302.441 ± 8.78 pg/cm²) when compare do PBS (148.274 ± 17.75 pg/cm²). Better entrapment and subsequent drug delivery was achieved in terms of the Pheroid™ technology.

Normality could not be assumed (p< 0.05). The observed average value for the Pheroid™ (302.441 pg/cm^2) was greater than the median (294.617 pg/cm²), while the opposite was observed for PBS, as the median (162.719 pg/cm²) was greater than the average (148.274 pg/cm²). Due to the presence of outliers in the case of the Pheroid™ values, the median proved to a better representation of central tendency as it is not affected by outliers (Gerber *et al.,* 2008:190, Swanepoel *et al.,* 2004:64).

A statistical difference between samples was found (p < 0.05). Equal variances could be assumed by the Levene's test of equality (p > 0.05). Results obtained are of practical importance (p = 5.8).

B.4.2 Tape stripping

B.4.2.1 TNF-α

A higher concentration was once again detected for the Pheroid™ stratum corneum-epidermis (4.454 pg/ml) and epidermis-dermis (8.636 pg/ml) solutions in comparison of that obtained for PBS (epidermis: 2.695 pg/ml; dermis: 6.684 pg/ml). A higher concentration was delivered to the epidermis-dermis and less retained within the stratum corneum-epidermis (target zone). Detection of TNF-α in the respective skin layers and receptor solutions serves as evidence that the active was delivered across the stratum corneum. The Pheroid™ technology contains slightly modified essential fatty acids, which are inherent components of the skin (Grobler *et al.,* 2008:305). Morganti *et al.,* 2001:489; 491, states that although ceramides, free fatty acids and choletsrol play an important role in corneocyte cohesion, contributing to the permeability barrier of the skin, a greater amount of unsaturated fatty acids makes the membrane more permeable, whereas ceramides and cholesterol has the opposite effect. The increased amount of active obtained with the Pheroid™technology could be attributable to naturally occurring fatty acids in the skin and the content thereof within the Pheroid™ formulation.

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A significant difference in sample means was obtained for both mediums and skin ($p < 0.05$). A p-value of 0.898 was obtained for the medium/skin interaction. There was no interaction between both groups and the null hypothesis could be accepted. Although PBS and Pheroid™ individually affect the amount of active in the epidermis and dermis, no interaction was found and this effect is not of statistical significance. The skin and testing medium medians was statistical significant at the 5 % level. In conclusion to the former, both groups were found to be different and not from the same population.

B.4.2.2 TGF-β1

Dermal (PBS: 24.995 pg/ml; Pheroid™: 47.299 pg/ml) concentrations were almost the same (PBS) or higher (Pheroid™) than the epidermal (PBS: 25.648 pg/ml; Pheroid™: 55.614 pg/ml) concentrations. As the drug delivery was aimed the melanocytes located in the epidermal layer, the obtained result was in accordance to what was expected. The higher concentration detected from the tapes solutions (stratum corneum-epidermis and epidermis-dermis) is also indicative of better penetration and delivery of the entrapped Pheroid™ compound across the stratum corneum.

The null hypothesis was rejected in terms of a significant difference between sample means for the testing mediums ($p = 0.375$), but not for the skin ($p = 0.00$). An interaction between testing mediums and the skin was established ($p = 0.447$). Although a significant difference was not found between groups, it could be established that both testing mediums had an effect on the skin. No significant difference was found between the medians of both PBS and Pheroid™. The alternative hypothesis concluded that the sample means differ and that both testing mediums had no significant effect on the drug concentrations in the epidermis and dermis.

B.5 Conclusion

The topical/transdermal delivery of the TNF-α and TGF-β1 were successfully delivered as both cytokines was detected in the receptor solutions. By means of comparison of data obtained with PBS and the Pheroid™ drug delivery technology, it is evident that the Pheroid™ drug technology enhanced the delivery of these drugs.

The amount of diffused concentration obtained for TNF-α Pheroid™ solution was slightly higher than that of PBS, while a two-fold increase in drug concentration from the stratum corneum-epidermis was obtained for the Pheroid™ drug delivery technology. Results obtained showed

that the amount of TNF-α delivered to the skin was enhanced by entrapment in Pheroid™ which could be attributable to the fatty acid content within the Pheroid™ formulation (Grobler *et al.,* 2008:3057) or inherent fatty acids in the skin (Morganti *et al.,* 2001:491). It is evident that a greater concentration of the cytokine was retained within the skin and that enzymatic degradation due the presence of aminopeptidases, might have occurred (Lee, 1991; Tauber, 1989 quoted by Antosova *et al.,* 2009:631) even though enzyme inhibitor bestatin hydrochloride was included in both PBS and Pheroid™ solutions.

The topical drug delivery of TGF-β1 was definitely enhanced by the Pheroid™ drug delivery technology. Both Pheroid™ formulations diffused concentration and tape stripping values were double that of the PBS formulations. When comparing results obtained for TNF-α and TGF-β1, the Pheroid™ drug delivery technology was advantageous in the topical drug delivery of both cytokines, but even more so in the case of TGF-β1.

As mentioned before, the low detection of TNF-α could be attributable to enzymatic degradation. This degradation could be due the accumulation of peptide intermediates as bestatin permits the degradation of cellular proteins to di- and tripeptides, but inhibits the further degradation of these peptides to amino acids. The accumulation of peptide intermediates in mammalian cells requires higher extracellular bestatin concentrations (Scornik & Botbol 1997:798). The aforementioned degradation could also be attributable to the presence of micro-organisms such as *Staphylococcus epidermidis* present on the skin surface which could be responsible for metabolising topically applied drugs (Williams 2003).

When comparing the results of both cytokines, it is evident that greater success was obtained with the topical delivery of TGF-β1 by the Pheroid™ drug delivery technology and that the larger the amount of diffused amount of active, the larger the amount of active delivered to the epidermis and dermis. It can also be ruled that bestatin hydrochloride was responsible for the low detection of TNF-α as it is obvious that the same did not occur with TGF-β1. The occurrence of enzymatic degradation of the skin surface can however not be ruled out.

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APPENDIX C
GUIDE FOR AUTHORS

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The International Journal of Pharmaceutics publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligo-nucleotides, gene constructs and radiopharmaceuticals.

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