PHEROID™ TECHNOLOGY FOR THE TOPICAL DELIVERY OF INSULIN GROWTH FACTOR 1 (IGF-1), KERATINOCYTE GROWTH FACTOR (KGF) AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

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Dissertation submitted in fulfilment of the requirements for the degree

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All honour to **our Lord God**, my Saviour. Without His love, mercy and guidance I would not have been able to complete this study and chapter in my life.

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ABSTRACT

The biotechnological era is here and many peptides and proteins are being used as part of drug therapy. They are, however, not very stable and do not have the physicochemical properties that allow for oral or topical delivery, and currently the most suitable method of delivery is *via* very invasive subcutaneous or intravenous injection.

Growth factors are the peptides this study was aimed at exploring; in this case insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF) and vascular endothelial growth factor (VEGF). These growth factors in particular have a stimulating effect on hair growth and strong hair shaft production. In addition, KGF has a protective effect on cells against radiation-induced cytotoxicity.

The aim of this study was to investigate the *in vitro* topical diffusion of the growth factors IGF-1, KGF and VEGF with the assistance of Pheroid™ drug delivery technology. In general, peptides such as growth factors are presumed to be unsuitable for topical delivery. It was, however, thought to be sensible to explore the drug delivery with Pheroid™ technology after significant success was achieved with a variety of drugs in previous studies. In order to prevent growth factor degradation by the skin, bestatin, a potent selective aminopeptidase inhibitor was utilized after its proven success in previous studies.

The diffusion studies were done with the use of vertical Franz diffusion cells and human female abdominal skin and were conducted over a period of 6 hours. A solution of each of the growth factors, IGF-1, KGF and VEGF, in the Pheroid™ delivery system was used as the donor solution and placed in the donor compartment of 6 diffusion cells for each growth factor. As a control, a solution of each of the growth factors, IGF-1, KGF and VEGF in phosphate buffered solution (PBS) was placed in the donor compartment of 6 diffusion cells. PBS (pH 7.4) was also utilized as the receptor solution in all of the diffusion studies. Samples were taken after the completion of the 6 hours diffusion study and analysed with the help of ELISA immunoassays.

ELISA immunoassays showed that the Pheroid™ drug delivery system was successful in the improvement of the delivery and stability of both IGF-1 and VEGF. In the case of IGF-1 in Pheroid™, an improvement of 6 % in diffusion and an increase in instability were observed when compared to the data of PBS. In the case of VEGF the Pheroid™ drug delivery system showed a 31 % improvement in diffusion; it does not, however, have a significant effect on the stability thereof when compared with the VEGF in PBS. The adjusted Pheroid™ drug delivery system values for the epidermis, showed a decrease of 43 % for IGF-1, a 97 % decrease for KGF and a 18 % decrease in the tape stripping concentration values obtained

in comparison with PBS. In the dermis case, the adjusted Pheroid™ drug delivery system showed a decrease of 44 % for IGF-1, a decrease of 97 % for KGF and a decrease of 19 % for VEGF in the dermis concentration values obtained in comparison with PBS. It was, however, found that the Pheroid™ drug delivery system does not show an improvement in the delivery or the stability of KGF and is to the contrary detrimental to it, with a 97 % decrease in diffusion and an approximate 51 % degradation of KGF at both 25 and 37 °C in comparison to the KGF in PBS. Therefore, the suitability of the Pheroid™ drug delivery system needs to be ascertained in order to ensure the delivery and stability of the active entrapped within the Pheroid™ drug delivery system.

The Pheroid™ drug delivery system therefore improves the transdermal delivery as well as the stability of IGF-1. It also improves the transdermal delivery of VEGF considerably. This particular Pheroid™ form is, however, not suited for the delivery or maintained stability of KGF, since both the transdermal delivery and stability of KGF have been impaired in this study.

By ascertaining whether the Pheroid™ drug delivery system is able to facilitate the topical delivery of the growth factors, the possible use thereof in the treatment of alopecia in the future can be determined.

UITTREKSEL

Die biotegnologiese era is hier en talle peptiede en proteïene word tans as deel van geneesmiddelbehandeling gebruik. Hulle is ongelukkig nie baie stabiel nie en beskik ook nie oor die nodige fisies-chemiese eienskappe wat orale of topikale aanwending moontlik maak nie, en die mees gepaste toedieningsmetode is *via* indringende subkutane of intraveneuse inspuiting.

In die studie is drie peptiede naamlik: insulienagtige groeifaktor 1 (IGF-1), keratinosiet groeifaktor (KGF) en vaskulêre endoteliële groeifaktor (VEGF) bestudeer. Hierdie groeifaktore beskik oor die vemoë om haargroei te stimuleer en sterk haarskagte te vervaardig. KGF bied boonop 'n beskermende effek teen bestralingsgeïnduseerde sitotoksisiteit.

Die doel van hierdie studie was om die *in vitro* topikale diffusie van die groeifaktore IGF-1, KGF en VEGF met behulp van die Pheroid™-geneesmiddelafleweringstegnologie te bestudeer. Daar word algemeen aanvaar dat peptiede (in hierdie geval, groeifaktore) nie geskik vir topikale aflewering is omrede hulle hoë molekulêre massa molekules is wat moeilik die stratum corneum kan oorsteek. Weens beduidende sukses met die aflewering van 'n verskeidenheid middels met behulp van die Pheroid™-tegnologie in vorige studies behaal is, is besluit om dit ook met die groeifaktore te ondersoek. Om die afbraak van groeifaktore deur die vel te verhoed, is bestatien, 'n potente selektiewe aminopeptidase-inhibeerder gebruik, na sukses daarmee in vorige studies bewys is.

Die diffusiestudies is oor 'n periode van 6 ure met behulp van vertikale Franz-diffusieselle en menslike vroulike abdominale vel gedoen. 'n Oplossing van elk van die groeifaktore, IGF-1, KGF en VEGF, in die Pheroid™-afleweringstelsel is as donor-oplossing gebruik en in die donor-kompartement van 6 diffusieselle per groeifaktor geplaas. Die kontrole het bestaan uit 'n oplossing van elk van die groeifaktore in PBS in die donor-kompartement van 6 diffusieselle. PBS (pH 7.4) is in al die diffusiestudies as die reseptor-oplossing gebruik. Na die verloop van die 6 ure is monsters geneem en met behulp van ELISA-immuuntoetse ontleed.

Die Pheroid™-geneesmiddelafleweringsisteem het die aflewering en stabiliteit vir sowel IGF-1 as VEGF verbeter. In die geval van IGF-1 het die Pheroid™-geneesmiddelafleweringsisteem 'n verbetering van 6 % in diffusie en 'n afname in die afbraak invergeleke met PBS getoon. VEGF in die Pheroid™-geneesmiddelafleweringsisteem het in vergelyking met PBS 'n verhoging van 31 % in diffusie getoon, alhoewel Pheroid™ nie 'n

noemenswaardige effek op die stabiliteit van VEGF gehad het nie. Die konsentrasie van IGF-1 in die epidermis was 43 % laer as dié afgelewer deur PBS. Dié van KGF in die epidermis was 97 % laer met 'n afname van 18 % in die konsentrasie afgelewer in vergelyking met dié afgelewer deur PBS. Die konsentrasie van IGF-1 in die dermis was 44 % laer as dié afgelewer deur PBS. Dié van KGF was 97 % laer en dié van VEGF 19 % laer in vergelyking met dié in afgelewer deur PBS. Daar is egter gevind dat die Pheroid™-geneesmiddelafleweringstelsel nie die aflewering of stabiliteit van KGF verbeter nie, maar dit in teendeel benadeel met 'n afname van 97 % in diffusie en 'n afbraak van ongeveer 51 % by sowel 25 as 37 °C vergeleke met PBS . Die toepaslikheid van die Pheroid™-geneesmiddelafleweringstelsel moet dus vooraf bepaal word, om die aflewering asook die stabiliteit van die aktiewe middel wat daarin vasgevang word te verseker.

Dit kan dus gestel word dat die Pheroid™-geneesmiddelafleweringsisteem transdermale aflewering en stabiliteit van IGF-1 en VEGF verbeter. Verbeterde KGF-stabililteit en –aflewering kon egter nie verkry word deur hierdie spesifieke sisteem nie. 'n Rede daarvoor mag wees dat die spesifieke Pheroid™-vorm nie geskik was om KGF af te lewer nie.

Deur te bepaal of die Pheroid™-geneesmiddelafleweringstelsel daartoe in staat is om die topikale aflewering van die groeifaktore te fasiliteer; kan die moontlik in die toekoms gebruik word in die behandeling van alopesie.

FOREWORD

During the study we aimed at investigating the topical delivery of peptides as drugs, in this case growth factors, with the aid of the novel Pheroid™ therapeutic drug delivery system. The unit for Drug Research and Development at the North-West University has had much success with the delivery of medication and especially the encapsulation of large molecules that have shown difficulty in bioavailability, efficacy and drug delivery *via* traditional methods. Encapsulation thereof in Pheroid™ has shown remarkable improvement in the delivery of such molecules and medication. The growth factors used were easily obtained from Sigma-Aldrich (Sigma, St Louis, USA).

It was decided that this dissertation was to be written in article format, which includes an introductory chapter with sub-chapters and a full-length article for publication in a pharmaceutical journal. The article in this dissertation is to be submitted for publication in The European Journal of Pharmaceutical Sciences and therefore the guide of authors has been included.

In spite of it being a long arduous process, I am glad that I finally have the opportunity to conclude this study. I am certain that my qualification will unquestionably open a variety of doors for me in the pharmacy arena and I am looking forward to the new challenges and opportunities that lie ahead with the benefit thereof.

Lize van Niekerk

2 March 2009

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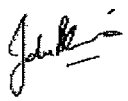
TO WHOM IT MAY CONCERN

I hereby declare that I acted as the supervisor for the study of Ms Lize van Niekerk. My role was in a supervision and advisory capacity. I also played a role during the planning and writing of this publication, but the actual experimental work as well as the writing of the publication was solely done by Ms Lize van Niekerk.

I thus hereby grant permission that she includes this publication as part of her dissertation in order to fulfil the requirements for the MSc qualification.



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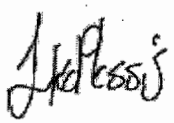
Authors contribution

L.H. du Plessis
Assistant supervisor
Supervised collection of data.

I declare that I have approved the above-mentioned manuscript, that my role in the study, as indicated above, is representative of my actual contribution and that I hereby give my consent that it may be published as part of the M.Sc. dissertation of Lize van Niekerk.

LH du Plessis
07/08/2009



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Yours sincerely

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CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

The skin is the largest organ of the body and is made up of an array of different cell types and organellar bodies, each with a distinct and particular function (Mukhtar, 1992:4). It covers the entire outside of the body, encapsulates the organs, protects them from the environment, protects the body from water loss and acts as a homeostatic barrier. It also serves as protection against chemical, physical and microbial attacks, therefore limiting molecular transport across itself into as well as out of the body (Suhonen *et al.*, 1999:1).

Transport across this barrier poses a big problem for a majority of drugs since significant plasma concentrations are hard to achieve with limited drug absorption. For substantial drug absorption to be obtained a drug needs to be low in molecular weight, must be lipophilic and be in a unionised state at physiological pH (Alexander-Williams & Rowbotham, 1998:3).

Hair follicles are formed by the dermis and epidermis and are found over the entire surface of the body except for some small areas on the body, for instance the soles of the feet, palms, portions of the fingers, toes, etc. Hair growth exhibits a cyclical pattern, with a anagen (growth) phase, telogen (rest) phase and the transition phase between the anagen and telogen phase which is the catagen phase (Revis, 2006:6).

Hair follicles are complex skin appendages and any perturbations or trouble thereof has an impact on human health and emotional welfare. Hair growth disorders that lead to hair loss (alopecia) are common and frequently cause the affected individuals significant mental anguish. Changes in their size, numbers per area of skin and duration of the growth cycle establish the hair coverage and underlie the diagnosis of alopecia or hypertrichosis in an individual. The mechanisms of the majority of these disorders are, however, unknown. Recently the importance of growth factors and cytokines in hair follicle development and cycling has come to the forefront. Certain growth factors and cytokines are actually able to prevent alopecia due to cancer treatment by chemotherapeutic agents (Danilenko *et al.*, 1996:460; McElwee *et al.*, 2008:1). Factors that may improve patient wellbeing and reduce this hair loss are likely to improve patient compliance with the treatment (Booth & Potten, 2000:667).

Growth factors have a stimulating effect on skin and hair follicle proliferation, prevent hair loss and vitalise skin and hair cells (Caregen, 2002:6). In particular insulin-like growth factor

1 (IGF-1), keratinocyte growth factor (KGF) and vascular endothelial growth factor (VEGF) are of interest to us in this study. IGF-1, KGF and VEGF strengthens hair and stimulates the production of strong hair shafts while KGF also protects the hair follicle against cytotoxic assault (Caregen, 2002:9 - 15).

Growth factors do, however, pose a problem when crossing the skin barrier since it has a high molecular weight compared to the optimal molecular weight for diffusion across the skin barrier in the order of 100 - 500 Dalton.

The objectives of the study were the following:

- Investigate the possible *in vitro* transdermal permeation of IGF-1 with the assistance of Pheroid™ technology.
- Investigate the possible *in vitro* transdermal permeation of KGF with the assistance of Pheroid™ technology.
- Investigate the possible *in vitro* transdermal permeation of VEGF with the assistance of Pheroid™ technology.

The journal article included in this dissertation gives account of our findings in regards to the objectives as mentioned above.

CHAPTER 2: TRANSDERMAL DELIVERY OF PEPTIDE DRUGS

2.1 CHALLENGES OF THE TRANSDERMAL DELIVERY OF PEPTIDE DRUGS

2.1.1 DELIVERY OF PROTEIN AND PEPTIDE PHARMACEUTICALS

2.1.1.1. PHARMACEUTICAL PROTEIN SOURCES

Many pharmaceutical peptides and proteins are nowadays being manufactured by means of recombinant DNA- or hybridoma technology. Most pharmaceutical proteins are endogenous, however, some of the proteins used in biotech products are not precisely identical to the endogenous protein or peptide. These biotech-derived products make up a majority of the products available on the market. In some instances therapeutically important proteins still need to be isolated from human or animal sources, for example albumin, blood clotting factors and anti-sera (Crommelin *et al.*, 2002:545).

2.1.1.2. PEPTIDES AS DRUGS

Proteins and peptides are more and more being used as part of drug therapy. Most physiological processes are regulated by peptides, endocrine or paracrine signals and others by neurotransmitters or growth factors. Protein pharmaceuticals have been utilized in areas such as neurology, endocrinology and haematology (Edward *et al.*, 1994:1).

The ideal therapeutic agent would be an inexpensive, small molecular mass chemical mimic of the receptor or ligand and would reach its site of action after oral administration. However, receptors have many binding sites and are large peptides. These receptors have complex tertiary structures which improve both the specificity and provide protection from simple invading molecules, consequently making the production of peptide mimicking unsuccessful (Edward *et al.*, 1994:1).

2.1.2 GROWTH FACTORS FOR TRANSDERMAL DELIVERY

In the field of skin biology, the importance of growth factors and cytokines in the cellular metabolism and regulation of gene expression is well established (Caregen, 2002:6).

Some of the functions of growth factors in skin biology are:

- It removes damaged skin cells,
- stimulates skin and hair proliferation,
- repairs and prevents wrinkle formation and minimises formation of scars,
- sooths acne and pimples,
- reduces and removes cellulite,
- acts as anti-microbial agent,
- prevents hair loss and
- vitalises skin and hair cells (Caregen, 2002:6).

In this study the treatment of alopecia is of particular interest for which a localised effect is required and by topical application of growth factors their proliferating effect on hair growth can be utilized.

2.1.2.1. PHYSIOLOGY OF ENDOGENOUS GROWTH FACTORS

Growth factors are classified into families that act through specific receptors (Sizonenko *et al.*, 2007:242).

2.1.2.1.1. Insulin like growth factor 1 (IGF-1)

In 1957, IGF-1 and IGF-2 were identified and designated "sulphation factor" due to its ability to stimulate sulphate incorporation into rat cartilage. In 1972, it was relabelled as "somatomedin", denoting a substance under control and meditating effect of growth hormone (GH). Finally, in 1976 two active substances, which structurally resemble pro-insulin, were isolated from human serum and was therefore renamed "insulin-like growth factor 1 and 2" (Laron, 2001:311).

Primarily IGF-1 is produced in the liver as an endocrine hormone and it targets tissues in a paracrine or autocrine fashion (PDR health, 2001). GH stimulates IGF-1 production. Under nutrition, GH insensitivity, lack of GH receptors or failure of the downstream signalling pathways after the GH receptor can retard IGF-1 production (Velcheti & Govindan, 2006:607).

Approximately 98 % of IGF-1 is always bound to one of 6 binding proteins (IGF-BP), with IGF-BP-3 being the most abundant protein, accounting for 80 % of all the IGF binding (Velcheti & Govindan, 2006:607).

2.1.2.1.2. Keratinocyte growth factor (KGF)

KGF is a member of the fibroblast growth factor (FGF) family of proteins that was initially isolated from a human embryonic lung fibroblast cell line (Osslund *et al.*, 1998:1681). KGF has distinctive target cell specificity (Zimonjic, 1997:11461). It is produced by a variety of fibroblasts and micro-vascular endothelium from several tissues (e.g. lung, skin, stomach and bladder) with specificity for epithelial tissues expressing KGF receptors (MacDonald & Hill, 2002:395). Binding of KGF to its receptor results in proliferation, differentiation and migration of epithelial cells (Zheng *et al.*, 2005:335).

2.1.2.1.3. Vascular endothelial growth factor (VEGF)

VEGF is a homodimeric, heparin-binding glycoprotein occurring in at least four isoforms of 121, 165, 189 and 201 amino acids, due to alternative splicing. VEGF binds to two type III tyrosine kinase receptors on vascular endothelial cells (Yano *et al.*, 2001:409). It is an endothelial cell-specific mutagen for micro- and macro-vascular endothelial cells derived from arteries, veins and lymphatics (Ferrara & Davis-Smith, 1997:4). VEGF is produced by several cell types, including tumour cells, macrophages, T-cells, smooth muscle cells, kidney cells, mesangial cells, keratinocytes, astrocytes and osteoblasts (Klagsbrun & D'Amore, 1996:259).

2.1.2.2. PHARMACOLOGY AND INDICATIONS FOR USE

2.1.2.2.1. Insulin like growth factor 1 (IGF-1)

Under normal circumstances, GH binds to its receptors in the liver and other tissues; and stimulates the synthesis and / or secretion of IGF-1. The IGF-1 receptor activation leads to intracellular signalling which stimulates numerous processes that lead to statural growth. Metabolic actions of IGF-1 are in part directed at stimulating the uptake of glucose, fatty acids and amino acids with the intention that metabolism supports growing tissue (Tercica Inc., 2005:2). Paediatric patients who are IGF-1 deficient are currently given IGF-1 as supplement, thereby promoting normalised statural growth (Drugbank, 2008).

2.1.2.2.2. Keratinocyte growth factor (KGF)

KGF receptors are present in many tissues; however, it is not present on cells of the haematopoietic lineage though (Amgen Inc., 2007:1). Kepivance™ (Palifermin / KGF) is indicated to increase the incidence and duration of severe mucositis in patients with haematological malignancies receiving myelotoxic therapy requiring haematopoietic stem cell support (Amgen Inc., 2007:5).

2.1.2.2.3. Vascular endothelial growth factor (VEGF)

Topical VEGF administration induces the development of fenestrations in the endothelium of small venules and capillaries. Furthermore, it increases vascular permeability (Ferrara & Davis-Smyth, 1997:5). VEGF may also inhibit the progression of amyotrophic lateral sclerosis (ALS) by delaying its onset and increasing the survival of its victims (Bruijn, 2004).

2.1.2.3. FUNCTION IN THE HUMAN BODY

2.1.2.3.1. Insulin like growth factor 1 (IGF-1)

IGF-1 and IGF-2 circulate in plasma and play central roles in the growth and development of many tissues through endocrine, paracrine and autocrine mechanisms (Danielpour & Song, 2005:60). IGF-1 in plasma is primarily derived from the liver and binds with IGF-BPs in order to circulate. Since the liver is the primary source of IGF-1 in circulation and hepatic production is highly influenced by nutritional factors, IGF-1 deficiencies are therefore modified by nutritional status (Tìryakíoğlu *et al.*, 2003:544). IGF-1 is very important throughout development and continues to regulate cellular survival, growth, differentiation, metabolism and migration in adults (Danielpour & Song, 2005:60). It has been shown to have potent anti-apoptotic activity, has an anabolic effect and is critically involved in promoting hair growth by regulating cellular proliferation and migration during the development of hair follicles (Harada *et al.*, 2007:409). IGF-1 inhibits the hair follicle from entering the catagen / regressive phase of the hair's growth cycle (Platz *et al.*, 2000:1006). IGF-1 also has a hair strengthening effect whilst stimulating hair follicles to form strong hair shafts. It actively generates new skin cells, increases collagen and elastin levels, refines texture and has a fat burning effect (Ceregen, 2002:9). IGF-1 acts as a paracrine modulator of tissue repair due to the synthesis at extra-hepatic sites in non-circulating, local acting forms (Beckert *et al.*, 2007:217). Additionally, the deregulation of IGF has been implicated in a variety of pathophysiological conditions including the development and malignant progression of many cancers. IGFs enter the circulation associated with IGF-BPs, which are produced by numerous tissues. IGF-BPs are known IGF modulators that prolong the half-life

of IGFs, whilst inhibiting their availability and bioactivity (Danielpour & Song, 2005:60). The IGF-1 complexed with IGF-BP is biologically inactive. The controlled release of IGF-1 from the IGF-BPs activates IGF-1 and is crucial for the stimulation of healing. A similar mechanism is of importance for other growth factors as well (Beckert *et al.*, 2007:218). IGF-1 deficiency may lead to severe growth and mental retardation (Denley *et al.*, 2005:423).

2.1.2.3.2. Keratinocyte growth factor (KGF)

KGF is a heparin-binding molecule. Heparin is essential for the induction of maximal KGF stimulation through its receptor. It is a paracrine mediator of cell-cell communication. Inflammatory cytokines induce KGF production from local mesenchymal cells to promote epithelial proliferation. Glucocorticoids inhibits KGF's production, whereas oestrogen and progesterone increase KGF production in the mammary gland and endometrium, respectively (MacDonald & Hill, 2002:395). This growth factor stimulates proliferation of keratinocytes in the epidermis and stimulates the proliferation and differentiation of keratinocytes in hair follicles and sebaceous glands and acts as an important paracrine mediator of normal hair follicle growth, development and differentiation. It also plays an important role in wound healing, stimulating the repair and healing process of wounds. In mouse models it has also been proven successful as pre-treatment before exposure to radiation therapy and preventing damage to gastrointestinal epithelial cells. This cytoprotective effect suggests possible therapeutic use of KGF in patients receiving cancer treatment (Werner, 1998:155). KGF actively generates new skin cells and refines skin texture. Delivery of KGF to the base of the follicle helps strengthens the hair and stimulates the follicles to produce hair shafts (Caregen, 2002:15). The pre-treatment with KGF protects mature and growing hair follicles from radiation damage and increases hair follicle survival after radiation assault, giving KGF large clinical potential for reducing chemotherapy and / or radiation induced alopecia (Booth & Potten, 2000:669 - 672).

2.1.2.3.3. Vascular endothelial growth factor (VEGF)

VEGF promotes angiogenesis in *in vitro* models by inducing confluent micro-vascular endothelial cells to invade collagen gels and form capillary-like structures (Ferrara & Davis-Smyth, 1997:4). The expression of VEGF is potentiated in response to hypoxia. VEGF induces endothelial cell proliferation, promotes cell migration and inhibits apoptosis. It stimulates angiogenesis and permeabilisation of blood vessels *in vivo* and plays an essential role in the regulation of vasculogenesis. Deregulated VEGF expression contributes to the development of tumours by promoting tumour angiogenesis. The aetiology of a number of additional diseases is characterized by abnormal angiogenesis. Subsequently the inhibition

of VEGF signalling abolishes the development of a wide variety of tumours (Nuefeld *et al.*, 1999:9). VEGF encourages the expression of serine proteases, urokinase-type and tissue-type plasminogen activators and increases expression of metalloproteinase interstitial collagenase in human umbilical vein endothelial cells but not in dermal fibroblasts (Ferrara & Davis-Smyth, 1997:5). Angiogenesis due to VEGF stimulation facilitates the nutrient feeding to the hair follicle, thereby stimulating hair growth (Caregen, 2002:12).

2.1.2.4. BASIC STRUCTURE AND CHARACTERISTICS

2.1.2.4.1. Insulin like growth factor 1 (IGF-1)

Insulin growth factors are members of a family of insulin related peptides that include relaxin and several peptides, isolated from lower invertebrates. IGF-1 is a small peptide that consists of 70 amino acids and has a molecular weight of 7649 Dalton. It has an A and B chain that is connected by three disulphide bonds - much like insulin. The C-peptide region has 12 amino acids and the structural similarity of IGF-1 to insulin explains its ability to bind, with low affinity, to the insulin receptor (Laron, 2001:311). Stability is a problem for IGF-1 though. It is unable to maintain its disulphide bonds in the absence of IGF-1-BP and many problems have been reported with the thermodynamic folding of IGF-1 *in vitro* to form the native sulphide bonds. Under redox conditions, the formation of these sulphide bonds is altered in the absence of IGF-BP and leads to the formulation of several different forms of IGF-1, differing in the arrangements of the disulfide bonds alone. The mismatched disulphide bonds result in an IGF-1 isoform that lacks affinity for the IGF-1 receptor (Hober *et al.*, 1999:271 - 276).

The following physicochemical properties of IGF-1 were obtained from the Sigma-Aldrich Corporation (Sigma-Aldrich Corporation, 2007a):

Molecular mass	~7.6 kDa
Solubility	>100 µg/ml
Amino acids	70 amino acids
Storage	-20 °C
Half-life	2 h
pH	6.5 ± 1.0
Appearance	A white lyophilized powder

Figure 2.1 propose the molecular structure of IGF-1

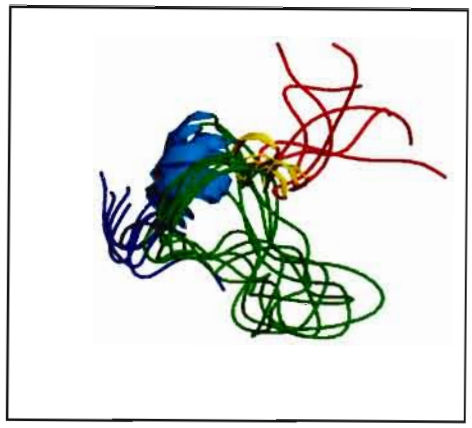

Figure 2.1: Molecular structure of IGF-1 (Cognitive labs, 2008).

2.1.2.4.2. Keratinocyte growth factor (KGF)

KGF has 10 well-defined beta-strands, which form five double stranded, anti-parallel beta-sheets. Contained within the loop between residues 133 and 144 is a sixth poorly defined beta-strand pair, defined only by a single hydrogen bond between the two strands. The sequence alignment of KGF against FGF structures indicates that KGF has a 40 % identity with FGF (Osslund *et al.*, 1998:1681). KGF is quite unstable though and when stored at moderate temperatures unfolding of the structure and aggregation take place due to disulphide scrambling and the formation of dimers. (Hsu *et al.*, 2006:147 - 152). Under redox conditions, the formation of these sulphide bonds is altered and it forms an inactive isoform of the growth factor (Hober *et al.*, 1999:271 - 276).

Figure 2.2 propose the molecular structure of KGF

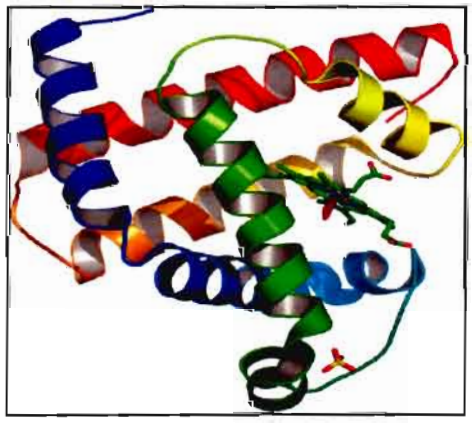

Figure 2.2: Ribbon structure of KGF (Drugbank, 2008).

The following physicochemical properties of KGF were obtained from the Sigma-Aldrich Corporation (Sigma-Aldrich Corporation, 2007b):

Molecular Mass	38.2 kDa
Solubility	0.1 mg/ml
Amino acids	121 - 206 amino acids
Storage	20 °C
pH	6.5 ± 1.0
Appearance	A white lyophilized powder

2.1.2.4.3. Vascular endothelial growth factor (VEGF)

VEGF is a disulfide-linked homodimer glycoprotein (Muller *et al.*, 1997:1326). The human VEGF gene is organised in 8 exons, separated by 7 introns (Ferrara & Davis-Smyth, 1997:5). An interesting attribute of VEGF is that multiple species of VEGF mRNA are generated by alternative splicing from a single gene (Klagsbrun & D'Amore, 1996:259). It may exist in one of four different molecular species, having 121, 165, 189 and 206 amino acids (Ferrara & Davis-Smyth, 1997:5). The dominant isoform is $VEGF_{165}$. Both the $VEGF_{165}$ and $VEGF_{189}$ isoforms bind to heparin with high affinity (Muller *et al.*, 1997:1326). The crystal structure of VEGF shows a triclinic crystal form containing 8 independent monomers in the asymmetric unit. The structure shows that the 8 monomers resemble a typical solution ensemble of protein structures, as determined by nuclear magnetic resonance (NMR) spectroscopy (Muller *et al.*, 1997:1326). The VEGF molecule is instable though, and the disulphide bridges within the molecule contribute to the thermal stability and structural integrity of the protein (Muller *et al.*, 2002:43415). Under redox conditions, the formation of these sulphide bonds is altered and it forms an inactive isoform of the growth factor (Hober *et al.*, 1999:271 - 276).

The following physicochemical properties of VEGF were obtained from the Sigma-Aldrich Corporation (Sigma-Aldrich Corporation, 2007c):

Molecular mass	18.9 kDa
Solubility	>0.5 mg/ml
Amino acids	163 amino acids
Storage	-20 °C
pH	6.5 ± 1.0
Appearance	A white lyophilized powder

Figure 2.3 propose the molecular structure of VEGF

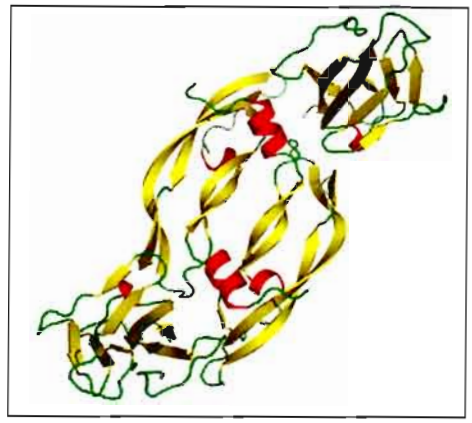

Figure 2.3: Ribbon structure of VEGF (3DChem, 2001).

2.1.3 TRANSDERMAL DRUG DELIVERY

2.1.3.1. INTRODUCTION

The skin is a multifunctional membrane that covers the outside of the body, it encapsulates the organs and protects them from the environment, protects the body from water loss and acts as a homeostatic barrier that also protects the body against chemical, physical and microbial assault. It therefore limits the molecular transport across itself into and out of the body (Suhonen *et al.*, 1999:149).

Skin consists of two mutually dependent layers, the dermis and epidermis, which lie on a subcutaneous fatty layer, the panniculus adiposus. The epidermis is colonised by melanocytes, antigen-processing Langerhans cells and pressure-sensing Merkel cells. The dermis contains collagen, elastic fibres, blood vessels, sensory structures and fibroblasts (Revis, 2006:2).

The skin is a very dynamic organ, undergoing continual changes throughout life as outer layers are shed and replaced by inner layers. Skin thickness varies with the anatomical location, age and sex of the individual. This varying thickness is primarily representative of a difference in dermal thickness, since epidermal thickness is rather constant throughout life from one anatomical location to another. Male skin is characteristically thicker than female skin in all anatomical locations. Skin is thickest on the palms and soles of the feet and thinnest on the eyelids and in the post-auricular region. It progressively thickens from birth until the fourth or fifth decade of life. Thereafter it begins to thin due to dermal change, with a loss of ground substance, epithelial appendages and elastic fibres (Revis, 2006:2).

Growth factors possess positive stimulating effects on skin and hair growth (Caregen, 2002:6). Both IGF-1 and KGF actively induce the production of new skin cells (Caregen, 2002:9). However, these peptides undergo digestion in the small intestine where it is degraded into their constituent amino acids, making it impossible for example, to treat alopecia using oral preparations. An alternative administration route is needed (PDR health, 2001). Transdermal delivery might be the ideal alternative route in administering growth factors.

The barrier properties of skin are determined by the stratum corneum, which varies from 5 - 20μm in thickness and consists of stacks of keratinized cells (corneocytes) (Cevc, 2004).

2.1.3.2. STRUCTURE OF THE SKIN

2.1.3.2.1. Subcutaneous fat layer / Hypodermis / Subcutis

The hypodermis is the layer of tissue just below the dermis of the skin. It consists of loose connective tissue and fat; and serves as a bridge between the dermis and the underlying body constituents. It is a relatively thick layer that varies from a few mm up to 3 cm. The blood and nerve supply is also carried to the skin in this layer (Williams, 2003:2). Adiposcyte tissue (fat cells) provides energy storage, insulation and serves as mechanical protection against physical shock (Lund, 1994:137).

2.1.3.2.2. Dermis or Corium

The dermis or corium is approximately 3 - 5 mm thick and is the main component of skin (Williams 2003:2). It is found directly below the epidermis where its function is to support and sustain. The epidermis is roughly dividable into the upper papillary and lower reticular portion, which is reflective of their respective composition of connective tissue components, cell number and blood vessel and nerve supply (Tobin 2005:7; Revis, 2006:4). Extensive skin vasculature is essential for regulating body temperature, delivery of oxygen and nutrients to the surrounding tissue, removal of toxins, and is of importance in wound repair (Williams, 2003:3). Skins flexibility and strength is provided by the dermis (Berkow *et al.*, 2002). Reticular dermis is made up of a thick layer of dense connective tissue that contains larger blood vessels, interlaced elastic fibres and coarse bundles of collagen fibres arranged in layers (Revis, 2006:4). IGF-1 increases elastin and collagen levels increasing its flexibility and firmness (Caregen, 2002;9). Neural structures such as nerve endings, for example Merkel cells (located in the epidermis), and free nerve endings, for instance Meissner's corpuscles, and Pacinian corpuscles are all found deep in the dermis (and hypodermis). The Meissner's corpuscles perceive soft touch and Pacinian corpuscles perceive pressure

(Horwitz, 2003). Viewed essentially as gelled water in terms of transdermal drug delivery, the dermis provides almost no barrier to the delivery of most drugs, however, in the delivery of highly lipophilic molecules the dermal barrier may be of significance (Williams, 2003:2). During transdermal drug delivery the blood supply maintains a concentration gradient between the applied compound on the surface of the skin and the vasculature across the skin barrier (Williams 2003:4).

2.1.3.2.3. Epidermis

The epidermis is stratified squamous epithelium that consists mainly of keratinocytes responsible for synthesising keratin (Gawkrodger, 2002:2). The epidermis consists of the stratum corneum and the viable epidermis (Walters, 1989:198). Desmosomes are protein bridges that connect the keratinocytes (Gawkrodger, 2002:2). Keratinocytes constantly divide and differentiate: They slowly migrate *via* differentiation from the innermost superficial layer, the stratum basale, until they reach the stratum corneum. These fully differentiated keratinocytes are subsequently shed in the epidermal turnover process (Revis 2006:3). The epidermis has four separate layers that form during different stages of keratin maturation (Gawkrodger, 2002:2). The viable epidermis consists of three distinct layers: the stratum basale, the stratum spinosum and the stratum granulosum. However, the viable tissue does not act as a substantial diffusion barrier and drugs pass through by means of gradient diffusion towards the closest capillary to reach systemic circulation (Flynn & Weiner, 1993:42).

2.1.3.2.3.1. The stratum basale or basal layer

The stratum basale is the deepest layer, sitting directly on the dermis (Horwitz, 2003). It consists mainly of dividing and non-dividing keratinocytes, which are attached to the dermo-epidermal membrane by hemidesmosomes (Gawkrodger, 2002:3). Hemidesmosomes act like an anchor for the lower cells. The stratum basale is the only layer in the epidermis that contains cells that undergo cell division (Williams, 2003:7). A small proportion of the basal cell population is melanin that produces melanocytes. Melanin provides protection against ultraviolet radiation. Merkel cells are closely associated with cutaneous nerves and perceive soft touch (Gawkrodger, 2002:3). Epidermal appendages like sebaceous glands, sweat glands, apocrine glands and hair follicles often lie deep within the dermis. Hair follicles are found over the entire external surface of the body except for some small areas such as the palms of the hands, soles of the feet, portions of the fingers and toes, etc. (Revis, 2006:6). They are remarkable in the fact that they can respond to most hormones. The hair follicles in different regions respond to different hormones, but even more surprisingly hair follicles are

capable of producing hormones for itself, e.g. sex steroid hormones, prolactin, etc. (Tobin, 2006:61).

Growth factors such as IGF-1, KGF and VEGF affect the dermal papilla of the hair follicle, which subsequently produces signals that inhibit or stimulate the growth of follicular epithelium (Roh *et al.*, 2002:44). IGF-1 is necessary for normal anagen growth; in its absence, the follicle cycles into a catagen-like state and the production of any new hair shafts ceases (Kealy *et al.*, 1997:214 - 215). Both IGF-1 and KGF stimulate hair follicles to produce strong hair shafts and strengthen hair (Caregen, 2002:9 - 15). IGF-1 stimulates the cellular proliferation and migration during hair follicle development and since IGF-1 mRNA has been detected in keratinocytes, the possibility exists that IGF-1 acts on keratinocytes and thereby promotes keratinocytes in hair follicles (Harada *et al.*, 2007:409 - 413). KGF receptor stimulation results in epithelial cell proliferation, differentiation and migration. Epithelial cells are present in many tissues such as hair follicles and sebaceous glands (Amgen Inc., 2007:1). VEGF stimulates endothelial cells in the hair follicle. Perifollicular vascularization is consequently induced, which in turn facilitates nutrient feeding to the hair follicle, promotes hair growth rates and increases the follicle quantity as well as the follicle and hair size (Yano *et al.*, 2001:413; Caregen, 2002:12).

2.1.3.2.3.2. The stratum spinosum

This layer contains sheets of keratinocytes, with a limited capacity for cell division. Langerhans cells, the bone marrow derived sentinel cells of the immune system, scour the skin for entry of foreign entities, plays a significant role in skin immune reactions and act as antigen-presenting cells (Tobin 2005:2; Gawkrodger, 2002:4).

2.1.3.2.3.3. The stratum granulosum

The stratum granulosum is a 3 - 5 sheet granular layer of non-dividing keratinocytes that produce keratinohyalin protein granules. These cells flatten, their cell contents break down and cell membranes become increasingly less permeable as the dividing cells below progressively push toward the skin surface (Tobin 2005:2).

2.1.3.2.3.4. The stratum lucidum

The stratum lucidum is the layer in which the cell nucleus disintegrates and an increase in other morphological changes, such as cell flattening, takes place (Williams, 2003:9). This layer is a clear layer of dermis only present in thick skin, such as the palms and soles of the feet where it reduces friction and shear forces between the stratum corneum and the stratum granulosum (Brannon, 2007).

2.1.3.2.3.5. The stratum corneum

Stratum corneum cells are the largest and most abundant of the epidermis (Revis 2006:3). It is the outermost layer of the epidermis and consists of a cornified layer of up to 30 sheets of non-viable, biochemically-active cells known as corneocytes (Tobin 2005:2). The corneocyte layer can absorb up to three times its weight in water. However, if the water content drops below 10 % it is no longer pliable and cracks (Grawkrodger, 2002:4). Orientation of the keratin proteins and the cellular shape add strength to the stratum corneum. In the extra cellular spaces, surrounding the cells, the cornified envelope is stacked in layers of lipid bilayers. The resulting structure provides the water-retaining barrier of the skin and prevents the entry of harmful materials and micro-organisms into the body. Cell envelope protein is highly insoluble and very resistant to chemical attack (Grawkrodger, 2002:4; Williams 2003:9). Human stratum corneum contains a unique mixture of lipids and continuous multiplying of the bilayered lipid component in the stratum corneum plays a key role in regulating the flux through tissue for most permeants (Williams 2003:10). These layers in a layered cross section of the skin are illustrated in figure 2.4.

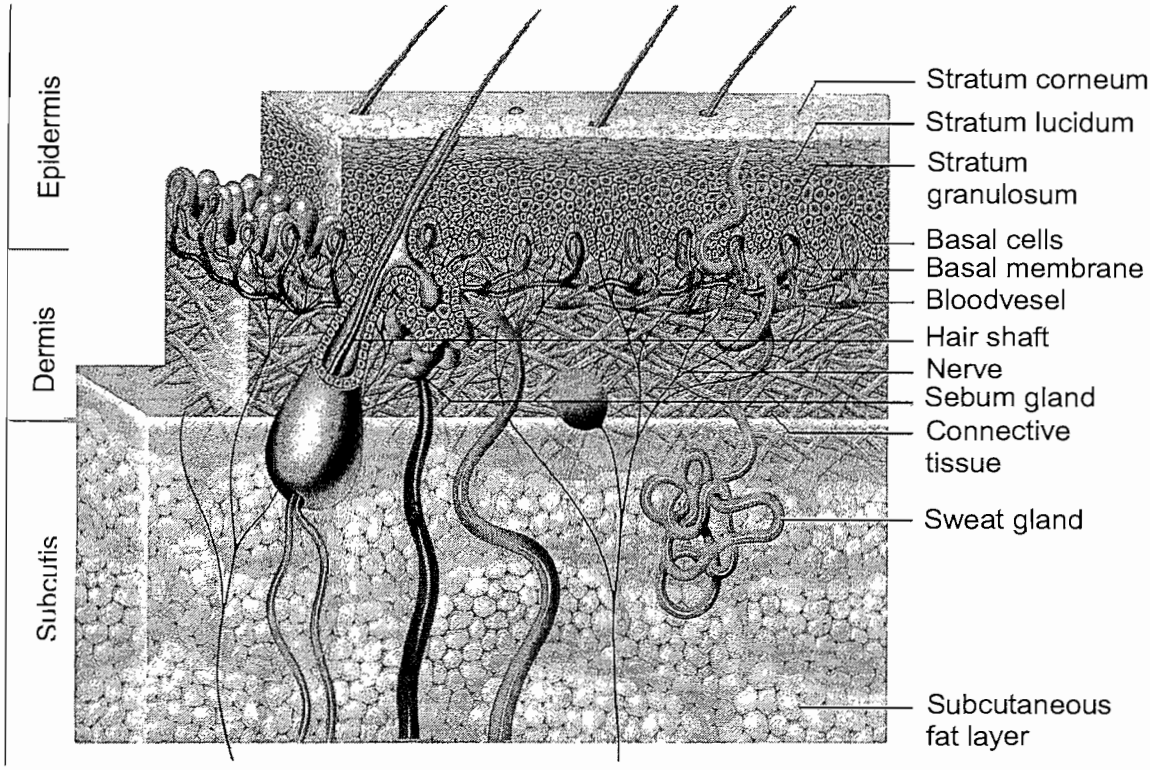

Figure 2.4: Structure of human skin. The stratum corneum, the viable epidermis including the basal membrane, the dermis and the subcutaneous fat. Local skin structures are blood vessels, hair follicles, nerves, sebaceous glands and the sweat glands (Grams, 2005:13).

2.1.4 FUNCTIONS OF THE SKIN

In table 2.1 the main functions of the skin is illustrated.

Table 2.1: The main functions of the skin

The main functions of the skin:
Contain body fluids and tissues – the mechanical function
Protect from potentially harmful external stimuli – the protective or barrier function
Receive external stimuli, that is, to mediate sensation: tactile (pressure), pain or heat
Regulate body temperature
Synthesise and to metabolise compounds
Dispose of chemical wastes (glandular secretions)
Provide identifications by skin variations

Attract the opposite sex (apocrine secretions are defunct in this role)
Regulate blood pressure (Barry, 1983:14).

2.1.4.1. ADVANTAGES AND LIMITATIONS OF TRANSDERMAL DRUG DELIVERY

2.1.4.1.1. Advantages

- Delivery of therapeutic drug levels is painless.
- Few or no gastrointestinal effects from the drug itself.
- Peak plasma levels of the drug are reduced and thus, a decrease in side effects occur.
- Eliminates the first pass effect and is useful in drugs that have a high first pass effect and undergo hepatic metabolism, or has poor oral bioavailability or instability in acids.
- Higher levels of drug absorption are attained by avoiding hepatic metabolism.
- Delivers more consistent serum drug levels due to the steady drug permeation.
- Toxicity from a transdermally administered drug could be limited by removing the patch or washing it off.
- Simplicity and safety of the dosing regimen can aid in patient adherence and tolerance to drug therapy.
- Useful in patients that are unable to take other oral drugs, for instance comatose patients (Wilkosz & Bogner, 2003a; Morrow, 2004).

2.1.4.1.2. Disadvantages and limitations

One of the greatest disadvantages of transdermal delivery is the possibility of local irritation developing at the application site, for example erythema, itching and local oedema caused by the drug, adhesive or other excipients (Wilkosz & Bogner, 2003b).

The skin's low permeability limits the number of drugs that can be delivered transdermally. Hydrophilic structured drugs permeate the skin too slowly to be of therapeutic benefit. Lipophilic drugs on the other hand are better suited for transdermal delivery (Wilkosz & Bogner, 2003a).

2.1.5 PROPERTIES FOR EFFECTIVE PENETRATION OF THE STRATUM CORNEUM

Very few substances are suitable for transdermal delivery and therefore, have to be chosen specifically for the purpose. To ensure efficient penetration of the stratum corneum the chosen substance should have the following properties:

- The daily systemic dose should be ≤ 20 mg.
- Molecular weight of the drug should be below 500 Daltons.
- Drug's log P (water-octanol partition coefficient) should be in the range of 1 - 3.
- The melting point should be < 200 °C, correlating with good ideal solubility.
- Hydrogen bonding groups should be ≤ 2.
- The drug should not be directly irritating to the skin.
- No immune reaction should be stimulated in the skin
- Adequate solubility in oil and water – in order to provide a high membrane concentration-gradient and thus, provide maximum flux.
- A high, yet balanced, partition coefficient is neccesary (Finnin & Morgan 1999:1).

2.1.6 ROUTES OF TRANSDERMAL DRUG PERMEATION AND PENETRATION

Drugs can cross the skin barrier to reach systemic circulation *via* three pathways: directly across the stratum corneum, through the sweat ducts or *via* the hair follicles and sebaceous glands (collectively called the appendageal or shunt route) (Flynn 1985:19).

The transcellular pathway is a more direct route and drugs cross the skin by passing directly through the phospholipid membranes and the cytoplasm of the dead keratinocytes that constitutes the stratum corneum. Even though it is the shortest distance for the drug to travel, significant resistance to permeation will be encountered in this route. The drug needs to cross the lipophilic cell membranes and the hydrophilic cellular contents, after which it has to cross the phospholipid bilayer of the cell again (Wilkosz & Bogner, 2003a).

Intercellular route is regarded as the polar route, due to the aqueous environment it creates with the hydrated keratin in keratinocytes (Williams, 2003:32). The hydrophilic and hydrophobic areas within the stratum corneum make this route unfavourable for delivery of hydrophilic molecules such peptides and proteins (Roberts *et al.*, 2002:94). Growth factors will, therefore, also experience difficulty in transdermal delivery *via* this route.

The transappendageal (intracellular) pathway is important for large polar molecules and ions that cross the stratum corneum poorly. Polar molecules are carried through the stratum corneum *via* the route of least resistance (Williams, 2003:32). Appendages provide a shunt route that is important during short times prior to attaining steady state diffusion. Polymers and colloidal particles can also target the follicles for passing through the skin barrier (Barry, 2001:101). It is possible that growth factors will make use of this route for some of the transdermal delivery.

The various pathways as suggested in the brick and mortar model is illustrated in figure 2.5.

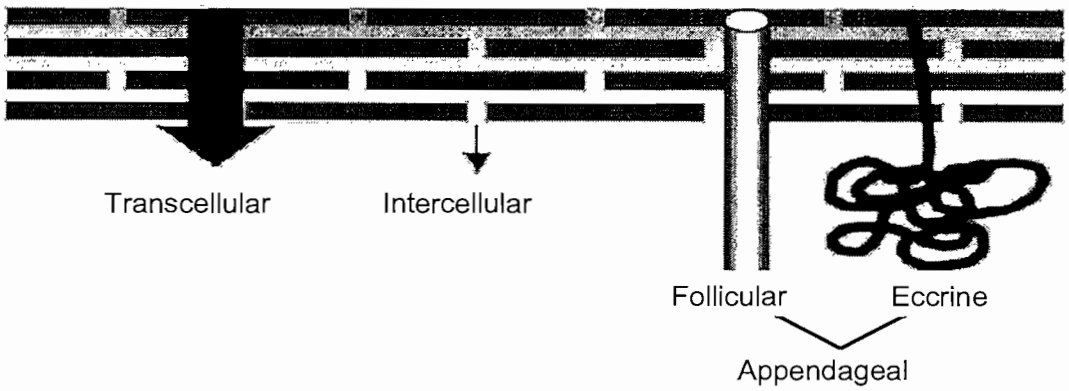

Figure 2.5: Brick and mortar model with the illustration of the major routes of permeation, namely: the transcellular, intercellular and appendageal route (Hadgraft, 2001:2).

2.1.7 INFLUENCIAL FACTORS IN TOPICAL PENETRATION

Topical penetration refers to the absorption rate of topically applied compounds through the skin barrier. The absorption rate is of importance in determining the potential for toxicity and effectiveness. Skin permeability may vary in diseased skin due to variation in the rate of diffusion through the stratum corneum, thus causing dose absorption variation.

2.1.7.1. PHYSICOCHEMICAL FACTORS

2.1.7.1.1. Diffusion coefficient (D)

Diffusion through skin is the transport of molecules from one medium to another through a restricting, partially permeable membrane. The ability to diffuse depends on the capability of the substance to enter a particular skin layer or reach a specific site; this process is called penetration (Rieger, 1993:34).

The diffusion coefficient or diffusivity (cm^2/s), D, is a rough measurement of the ease with which a molecule moves within a medium (Smith 1990:24). The diffusion coefficient across the stratum corneum is influenced by various factors, such as viscosity of the surrounding medium, the molecular size and molecular structure of the drug, penetration enhancers and additives (Tojo, 1997:116). The large molecular masses of IGF-1, KGF and VEGF, that vary from 7.6 - 38.2 kDa, will influence the diffusion coefficient. The smaller diffusion coefficient indicates to slower diffusion of the growth factors across the stratum corneum.

2.1.7.1.2. Concentration difference or Diffusant solubility (C_s - C)

Diffusion is a passive movement along a concentration gradient across a rate limiting membrane, from a region of high diffusant concentration to a region of low diffusant concentration. The diffusant solubility is the determining rate at which the diffusant transverses the rate-limiting membrane, the stratum corneum in this case (Nielloud & Marti-Mestres, 2000:264). The concentration difference provides the driving force for the net movement of the drug molecules between the donor and receptor environment, consequently, the higher the drug concentration in the applied solution, the greater the diffusion gradient (Nielloud & Marti-Mestres, 2000:265; Smith & Surber, 2000:25).

Drugs need to be in solution in order to influence the concentration gradient. The selected growth factors: IGF-1, KGF and VEGF, respectively have relatively high diffusant solubility values and are able to create a large concentration difference across the stratum corneum, therefore, providing a good driving force for diffusion. However, the large molecular masses may negate the increased diffusability of the growth factor in spite of the good solubility. Drugs in suspension do not affect the concentration gradient; however, drug crystals in suspension may act as a drug reservoir by maintaining saturation for an extended period. Emulsions may also influence the concentration gradient depending on the O/W dispersion of the drug (Nielloud & Marti-Mestres, 2000:265). Sink conditions are created *in vivo* and *in vitro*, where the drug concentration on the distal side of the membrane is swept away instantaneously by the receptor environment, therefore, the value of (C_s - C) approximates to C_s (Smith & Surber, 2000:25).

2.1.7.1.3. Drug concentration (C)

The higher the drug concentration or solubility, the better the penetration due to the higher concentration gradient created across the stratum corneum (Dalby, 2001:8). Drug concentration influences the thermodynamic activity of the permeant and provides the driving force for permeation (Williams, 2003:73). IGF-1, KGF and VEGF, have relatively high solubility values that may provide a high concentration gradient across the stratum corneum if the applied concentration was large, creating a high driving force for diffusion. An increase in the concentration usually increases the thermodynamic activity resulting in an increase in the quantity of the drug absorbed The applied drug concentration is not very large, however, suggesting a smaller quantity diffusing across the stratum corneum. (Lund, 1994:141). Drug flux is relative to the concentration gradient across the stratum corneum. A saturated permeant concentration in solution would ensure the highest flux in thermodynamically stable conditions (Barry, 2002:512). By modifying the vehicle constituents, the thermodynamic activity of the same drug concentration can change radically (Williams, 2003:73).

2.1.7.1.4. Drug solubility and melting point

The solubility of a substance influences its ability to penetrate biological membranes such as the stratum corneum. During formulation of preparations for topical applications, substances are selected or prepared to have specific solubility characteristic before attempting to improve penetration by pharmaceutical manipulation.

Compounds that are more aqueous soluble and less lipophilic are less active after topical application (Idson, 1975:912). Growth factors have relatively good aqueous solubility. The growth factors, IGF-1, KGF and VEGF have relatively high solubility values that may influence the diffusion across the stratum corneum negatively and reduce the amount to cross the skin.

For optimal permeation, some lipophilicity is desired to allow intercellular permeation, however, some aqueous solubility is also desired to allow enough permeation within an aqueous formulation and to minimise donor depletion over time after application (Williams, 2003:38). There is a clear relationship between the melting point and solubility of a substance (Williams 2003:37). Molecules with low melting points penetrate the skin barrier more readily than molecules with high melting points and therefore compounds with lower melting points exhibit higher permeability coefficients (Hadgraft & Wolff, 1993:44; Roy, 1997:147).

2.1.7.1.5. Molecular weight, size, volume or shape

Molecular weight influences the diffusion coefficient of a molecule. Larger molecules show a decrease in diffusivity (Williams, 2003:36). Idson (1975:919) suggested an inverse relationship exists between molecular weight and transdermal flux of the permeant. Increasing the molecular weight or volume increases the hydrophobic surface area resulting in enhanced partitioning however, larger molecules diffuse more slowly because they require a larger volume to be displaced in the medium, leading to diminished permeability (Potts & Guy, 1995:1632).

Most therapeutic agents selected for transdermal delivery have molecular weights within the range of 100 - 500 Dalton. When larger molecules such as peptides or proteins are selected as therapeutic agents, the transdermal flux dependency on molecular weight becomes more apparent (Williams, 2003:37). Growth factors have very large molecular masses that vary from 7.6 - 38.2 kDa, making transdermal penetration and delivery more problematic. Many typical topical delivery formulations are unable to delivery these active molecules, which are why ionotophoresis and lipid based vesicles are the most popular delivery methods being used for their delivery.

2.1.7.1.6. Ionization and pH

The pH-partition theory states that most drugs are weak acids or bases, which dissociate to varying degrees, depending on the pH of the transdermal formulation (Williams, 2003:38). The non-polar, lipid rich nature of the stratum corneum suggests that ionisable drugs will encounter high resistance to permeation through it (Zats, 1993:28). Transport across the skin barrier takes place *via* various pathways; the shunt route provides a transport route for aqueous molecules and ionised permeants, though the amount of ionised permeant to cross may be less than the non-ionised and crosses *via* the lipoidal intercellular route (Williams, 2003:38).

Drug flux is a result of the effective drug concentration in the vehicle and the permeability coefficient. The permeability coefficient of unionised species that pass through the lipid membrane may be high, even though its aqueous solubility will be low. In contrast, for ionised species, the permeability coefficient may be low, but the solubility may be high, and the resulting fluxes may be comparable in these two situations (Williams, 2003:39). Due to the many amino acids that growth factors consist out of, there are many sites that can undergo dissociation, which will take place at many different pHs.

It is, therefore, difficult to classify the growth factors as ionised or unionised.

2.1.7.1.7. Vehicle formulation

Vehicle formulation may have an effect on permeant solubility and on the skin's barrier properties (Hostynek, 2003:4). The concentration, diffusion coefficient and partition coefficient of the drug are all influenced by the nature of the vehicle. If a substance has a high affinity for the vehicle, the substance will be released more slowly due to its low thermodynamic activity. In the case where the substance solubility is reduced in the vehicle, more favourable conditions for drug release will be obtained (Lund, 1994:141).

The diffusion coefficient of a drug lowers with an increase in vehicle viscosity. Solvent properties may also alter the penetration of the permeant. For example: lipophilic solvents may increase the penetration of permeants (Dalby, 2001:8).

The Pheroid™ drug delivery system consists of fatty acids that provide a more lipophilic vehicle that improves the diffusivity of molecules that are physicochemically unsuited for delivery and have shown marked improvement in the transdermal delivery of peptides, proteins and other molecules.

2.1.7.2. BIOLOGICAL-BIOMEDICAL FACTORS

2.1.7.2.1. Skin age

Skin shows the most obvious outward signs of ageing; this damage is cumulative environmental damage to the skin as barrier and not due to the ageing of skin itself (Buck, 2004:4). Damage may be due to repeated chemical assault from soap or cosmetics, or from a lifetime of UV radiation exposure to the sun (Williams, 2003:14). Corneocytes undergo an increase in surface area, resulting in implications for the stratum corneum functionality due to the decrease in inter-corneocyte space, important for transdermal penetration, movement of water vapour (TEWL) and cell-to-cell cohesion due to changes in structure (Levin & Maibach, 2004:292). Decreased topically reaction to applied irritants in aged skin suggests a decrease in transdermal penetration (Buck, 2004:4). Topical drug delivery is influenced by the hydration of the tissue and can alter drug permeation to a minor extent. Other factors that alter the hydration level of the skin will, however, have a larger influence on the drug permeation than the hydration loss due to advancement in age (Potts *et al.*, 1984:99).

2.1.7.2.2. Body site

Skin structure and thickness varies to some degree over the entire body surface. Permeability is not just influenced by the thickness of the stratum corneum since different permeants demonstrated varying degrees of penetration through different skin sites.

According to Wester & Maybach (1989) variations in drug absorption were observed in a site-to-site comparison with similar stratum corneum thickness sites and similar drug absorption levels were observed in areas with differing stratum corneum thickness. Skin on genital tissue is most permeable for topical drug administration, skin on the neck and head is slightly less permeable, the trunk area of the body has intermediate permeability and the arms and legs are the least permeable sites on the body (Williams, 2003:16). Factors, such as stratum corneum cell size, lipid composition, the number of layers, the associated stacking pattern and depth of distribution of the appendages all influence the skin's permeability (Lund, 1994:140). There is also significant variation in permeation, of up to 40 %, between identical sites on different individuals; this is due to the permeability coefficient (k_p) of a penetrant, since the permeability coefficient is inversely proportional to the diffusion path length (h) (Mukhtar, 1992:23; Williams, 2003:17). This variability due to regional differences can be surpassed by using tissue from similar areas, where the regional factor is small, for instance, arm and leg tissue (Williams, 2003:17).

2.1.7.2.3. Race

Racial differences between black and white skin have been shown in anatomical and physiological functions, although data on the subject is relatively meagre. Black skin has increased intracellular cohesion, higher lipid content and higher electrical skin resistance levels in comparison to white skin. It also appears to have decreased susceptibility to cutaneous irritants that is due to the stratum corneum that modulates the different racial responses to topical irritants (Buck, 2004:4). There are also significant differences in the water content of the stratum corneum between different races, suggesting that there would be differences in drug absorption interracially. However, with very limited data about the racial variation, the delivery of topically applied drugs cannot be assumed (Williams, 2003:17).

2.1.7.2.4. Other factors

Transdermal drug delivery can be influenced by other physiological factors as well. The hydration level of the stratum corneum has a remarkable effect on drug permeation and increasing hydration increases transdermal delivery of most drugs (Williams, 2003:17). The diffusion coefficient of a permeant can increase by increasing the temperature. By largely elevating the skin temperature, alterations in the structure within the stratum corneum can be induced, resulting in increased diffusion through the tissue. However, for most situations in experimental application of topical medication, a small temperature increase of the skin or the environment had negligible effect on transdermal delivery (Williams, 2003:18).

2.1.7.2.5. Pathological disorders

Diseased skin is damaged skin and the potential for transdermal penetration is increased due to inflammation of the skin. It is more penetrable by environmental, occupational or topical substance exposure, whether the skin is diseased by a transient or chronic infection or condition (Jackson, 1993:178).

Skin disorders can alter the barrier properties of skin and will consequently influence the topical and transdermal drug delivery. Many skin conditions compromise the stratum corneum integrity; however, as treatment of the condition progress, skin condition improves and results in improved skin integrity with a consequential decrease in the drug delivery (Williams, 2003:18). Eruptions, infections, ichthyoses and tumours all cause variability in the transdermal and topical transport of drugs and should be kept in consideration (Williams, 2003:20). Skin which has been breached by an epidermal break that may penetrate into the dermis, subcutaneous layer or muscle tissue pose a risk of instantaneous exposure to substances that has come in contact with the skin. Any scratch, crack, cut or split due to excessive dryness also poses this risk (Jackson, 1993:178).

2.1.8 PENETRATION ENHANCEMENT

The main goal of permeability enhancement is to reduce the barrier function of the stratum corneum. Safety should remain of importance when making use of penetration enhancers and the higher drug penetration and greater risk for toxicity should be kept in mind (Hsieh, 1994:9). In principle, enhancers modify either skin or drug diffusibility, drug solubility. In some cases both are modified resulting in an increase in transport (Hsieh, 1994:11).

Drug solubility parameters, such as the partition coefficient and permeability coefficient, can be modified by enhancers, which do not damage the skin structure and integrity. Examples of such enhancers are ethanol and macro-cyclic ketones and lactones. Their effect on the skin is reversible and transient (Hsieh, 1994:11).

2.1.8.1. CHEMICAL PENETRATION ENHANCERS

Chemicals that promote skin penetration of topically applied drugs are referred to as absorption enhancers, penetration enhancers or accelerants.

2.1.8.1.1. Penetration enhancer properties

Permeability can be enhanced by altering the skin structure and therefore modifying the diffusion coefficient, or by increasing the drug solubility in the skin (Hsieh, 1994:11).

Permeation enhancers include alcohols, polyalcohols, pyrrolidones, amines, fatty acids, sulphoxides, esters, alkanes, terpenes, surfactants, phospholipids, etc. The structural diversity of these penetration enhancers listed rule out the formulation of a singular mechanistic hypothesis (Naik *et al.*, 2000:321).

There are hundreds of chemical penetration enhancers with shown penetration improvement; however, there is no universal penetration enhancer. Each different drug formulation requires a specific enhancer or combination thereof to obtain the maximum penetration possible and only a small percentage of the multiple enhancers are approved for use in pharmaceutical products (Ghosh, 1997:442).

The ideal penetration enhancer should boast with the following properties:

- non-irritating,
- not damaging to the skin,
- non-toxic and safe,
- pharmacologically inert,
- non-allergic,
- skin should regain normal integrity and barrier properties upon removal of the penetration enhancer from the skin (Hsieh, 1994:20),
- should have a rapid mode of action,
- generate predictable and reproducible penetration upon topical application,
- it should also work uni-directionally, enhancing drug penetration whilst preventing loss of endogenous material from the body,
- it should finally be cosmetically acceptable with appropriate 'feel' on the skin, with ideally no taste, colour or odour (Williams, 2003:86).

2.1.8.2. PHYSICAL AND TECHNOLOGICAL PENETRATION ENHANCERS

Physical and technological methods of penetration enhancement have the main purpose to try to disturb or circumvent the barrier function of the stratum corneum. Examples of physical penetration enhancers are microneedles, laser ablation, electroporation or physically enhanced flux such as ionotophoresis. Other strategies considered are sonophoresis, magnetophoresis, needleless injection and vesicular carriers (Williams, 2003:123). Therapeutic agents that have been emerging recently are molecules that are particularly prone to difficulty, such as peptides, proteins and oligonucleotides. These are particularly labile, and therefore, difficult to deliver by conservative methods. Their large molecular masses, polarity and charge exclude effective transdermal penetration and delivery. Physical

enhancement technologies recently offered powerful and thrilling approaches to resolve the delivery of these molecules (Naik *et al.*, 2000:322). These technologies show much promise for transdermal drug delivery systems that incorporate a large drug reservoir on the surface of the skin for the delivery of very large molecular weight substances that reach into the kiloDalton range (Finnin & Morgan, 1999:956).

2.1.8.2.1. Vesicles

The encapsulation of drugs into vesicular systems in order to deliver them through multiple delivery routes, has been very popular (Williams, 2003:123). There are a variety of encapsulating systems including liposomes, deformable liposomes or transfersomes, ethosomes and niosomes (Benson, 2005:27).

2.1.8.3. ELECTRICAL METHODS OF PENETRATION ENHANCEMENT

A number of electrical methods for penetration enhancement have been developed. These include iontophoresis, phonophoresis, electroporation (Benson, 2005:31). The most evolved and most studied method of these is iontophoresis (Naik *et al.*, 2000:322).

2.1.8.4. USE OF PHEROID™ TECHNOLOGY AS A THERAPEUTIC DRUG DELIVERY SYSTEM

Pheroid™ technology consists mainly of modified essential fatty acids in a vesicular structure with sizes that range from 200 - 440 nm. The vesicle membranes contain pores formed by the packing of the fatty acids. The technology is particularly versatile and can be manipulated to meet required entrapment volumes and efficiencies (Grobler *et al.*, 2008:283).

2.1.8.4.1. Introduction

MeyerZall Laboratories developed Pheroid™, formerly known as Emzaloids™. It is capable of enhancing the absorption and efficacy of a variety of drugs. In 2003, the North-West University of South Africa purchased the intellectual property from MeyerZall (Pty) Ltd.

Pheroid™ technology shows major improvements in the control of lipophilic-hydrophilic characteristics, charge and size of therapies in comparison to other delivery systems. It promotes the absorption and increases the efficacy of dermatological, biological and oral medicines (Grobler *et al.*, 2008:284).

2.1.8.4.2. Structural characteristics of Pheroid™

Pheroid™ is a colloidal system consisting of a unique and stable lipid-based submicron- and micron-sized structures that are uniformly distributed in a dispersion medium that is adapted to suit its indication (Grobler *et al.*, 2008:285). Pheroid™ mainly consists of plant and essential fatty acids that are designed to encapsulate, transport and deliver pharmacological compounds (Grobler, 2004:4). It consists of pegylated and ethylated polyunsaturated fatty acids, including the omega-3 and -6 fatty acids. The fatty acids are the correct stereochemical isoform to ensure compatibility with the fatty acids in the human body. These vesicles are formulated to have a diameter ranging from 200 nm - 2 µm and can be manufactured to provide a specific capacity, rate of delivery and route of administration (Grobler *et al.*, 2008:285). The Pheroid™ formulation also contains nitrous oxide (N_2O) that is distributed in association with the dispersed phase throughout the continuous phase. This association of N_2O with the dispersed phase contributes to the miscibility of the fatty acids, the self-assembly process of the Pheroid™, and the stability of the formed Pheroid™ (Grobler *et al.*, 2008:289).

As colloidal dosage form, Pheroid™ is commonly used as liposomes, emulsions and microemulsions, polymeric microspheres and macromolecular microspheres (Grobler *et al.*, 2008:286).

This technology entraps pharmacological drugs with high efficiency and deliver them at exceptional speeds. They can penetrate keratinized cells, skin, intestinal lining, vascular system, bacteria, fungi and parasites. Pheroid™ is unique due to the manipulatetive ability, specifically to ensure high entrapment capabilities, fast transport rate, delivery and stability. Additionally, the absorption capability and drug release characteristics can be controlled. It is capable to act in synergy with some drugs or compounds resulting in enhancement of the therapeutic action, which in return results in safer, more effective preparations with additional, specifically formulated, characteristics (Grobler, 2004:4). Figure 2.6 demonstrates how versatile and pliable Pheroid™ technology is. The system is so adaptable that it can be manipulated to produce the best penetration for a specific drug.

2.1.8.4.3. The key advantages with the use of Pheroid™ technology:

- Increased delivery of actives,
- decreased onset time of action,
- reduced minimum effective concentration,
- increased therapeutic efficacy,
- reduced cytotoxicity,
- penetration of most cellular barriers of the body,
- targeting of treatment areas,
- lack of immunological response,
- able to transfer genes to cell nuclei and
- reduced drug resistance (Grobler, 2004:3).

2.1.8.4.4. Functions of Pheroid™

The fatty acids Pheroid™ consists of are essential and necessary for a variety of normal cell functions, however, human cells are unable to manufacture these fatty acids, and therefore, it needs to be absorbed *via* the intestinal tract or oral route. The modern Western diet is, however, very limited in the supply of these essential fatty acids (Grobler, 2004:4).

Functions of the essential fatty acids that are contained in Pheroid™ technology:

- Maintenance of cellular membrane integrity.
- Energy homeostasis.
- Modulation of the immune system (Grobler, 2004:4).

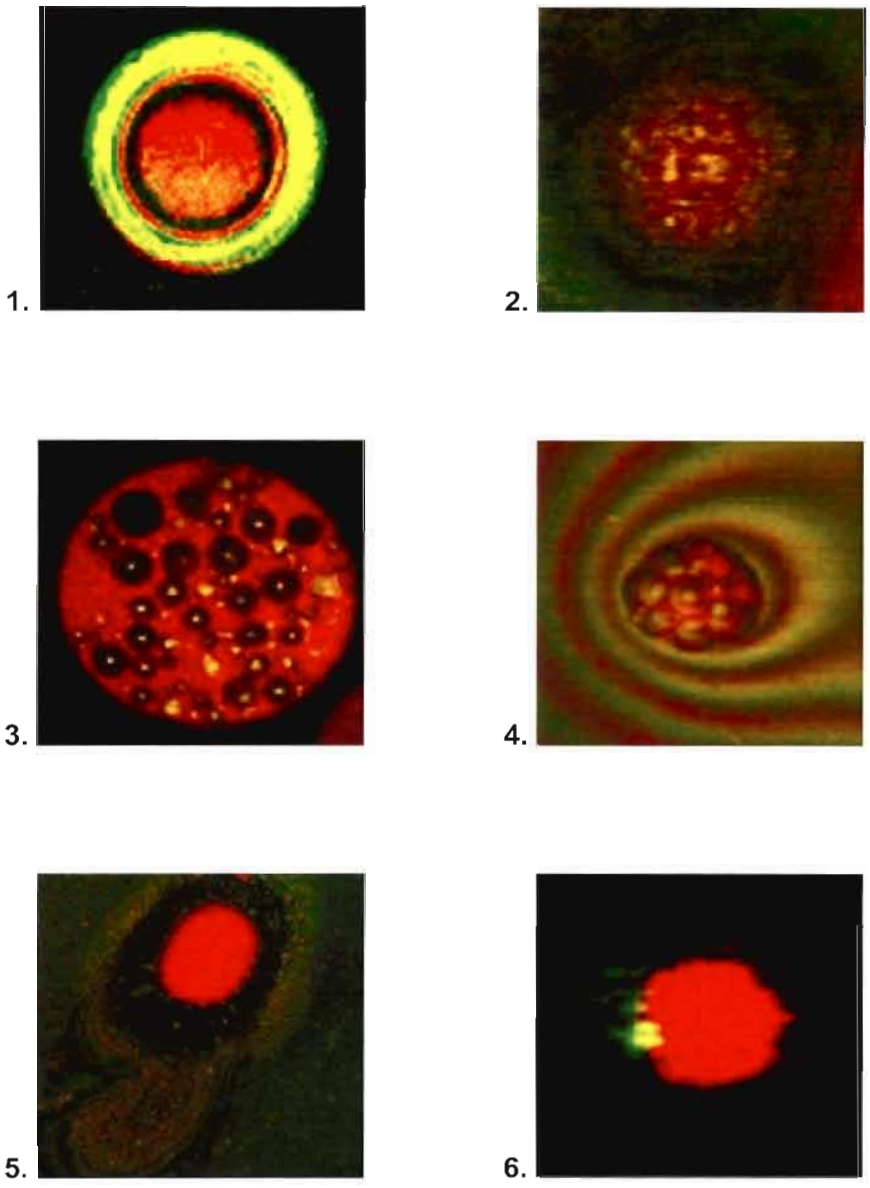

Figure 2.6: A few of the basic Pheroid™ types. **1.** A bilayer membrane vesicle, with a diameter of 100 nm containing rifampicin in this case. **2.** A highly elastic or fluid bilayered vesicle with loose packing, which contains rifampicin. **3.** Pheroid™ with a general size of 1 µm and with multiple particles of coal tar contained within. **4.** A pro-Pheroid™ is used in some oral formulations. **5.** Pro-Pheroid™ with a hydrophobic core, in a surrounding hydrophilic area and outer vesicle containing area and which range in size from 5 - 100 µm **6.** Depiction of the process of entrapment of a fluorescently labelled diclofenac. Sizes of the Pheroid™ depicted above are not to scale (Grobler, 2004:5).

2.1.8.4.5. Therapeutic efficacy

Even though the efficiency of entrapment and the rate of transport and release of actives into, and across the skin or stratum corneum can be indicative of the performance of a delivery system. Ideally, the effect of the delivery system should be determined by its contribution to the therapeutic efficacy of the active compound. The formulation of active compounds within Pheroid™ has shown an increase in the efficacy of several compounds (Grobler *et al.*, 2008:300).

Pheroid™ is able to:

- decrease the time to onset of action,
- increase the delivery of actives,
- reduce the minimum effective concentration,
- increase therapeutic efficacy and
- reduce cytotoxicity (Grobler, 2004:9 - 11).

2.1.8.4.6. Cellular uptake and metabolism of Pheroid™; and release of entrapped actives

The Pheroid™ vesicles are sterically stabilized by electro-chemical interactions and not cholesterol unlike most lipid-based delivery systems, resulting in a very elastic vesicular structure. It is able to cross capillary walls and the stratum corneum, which both offer similar challenges to cross. Pheroid™ uptake into the cells is influenced by the method of absorption by the cells of the Pheroid™ as well as the Pheroid™ formulation. Permeation of the Pheroid™ formulation is determined by one or more of the following factors:

- size of the Pheroid™,
- morphology of the Pheroid™,
- molecular geometry of the fatty acids,
- concentration and ratios of the different fatty acids,
- hydration medium, ionic strength, etc,
- pH of the preparation,
- presence of molecules able to change its charge,
- presence of molecules that alter the electrostatic environment,
- concentration and character of the drug or active and
- state of the Pheroid™, i.e. in either the fluid, gel or in transition between either (Grobler *et al.*, 2008:297 - 299).

The uptake mechanism of Pheroid™ is largely speculative and the suggested cellular uptake mechanism is actively facilitated by the fatty acid membrane-binding proteins (FABs) contained within the cell membrane lipids. Whether this is an active or passive process has not been determined yet. However, in high concentrations, the free fatty acids diffuse across membranes at rates that are sufficient to support metabolism. Pheroid™ is hypothesized to be transported through protein-mediated transport, subsequently being an active process. Intracellular FABs have been identified to be proteins that efficiently mediate the trans-bilayer movement of fatty acids. These proteins bind hydrophobic ligands non-covalently and in a reversible manner. The FABs have specific functions in fatty acid uptake, oxidation and metabolic homeostasis thereof (Grobler *et al.*, 2008:299).

The cellular uptake of Pheroid™ takes place *via* binding between fatty acids and FABs, and between fatty acids and the cell membrane lipid rafts. Pheroid™ metabolism is dependent on their composition and takes place in either the mitochondria or the peroxisomes of the cell that result in the release of the active compound entrapped in the Pheroid™ vesicles (Grobler *et al.*, 2008:300).

2.1.8.4.7. Peptide drug delivery

Topical delivery of peptides such as growth factors is a difficult feat. This is due to their large molecular mass which makes them unsuitable to cross the skin *via* the transepidermal route. In physicochemical terms, they are too polar, hydrophilic and additionally they are chemically too instable, therefore, making them poor candidates for topical delivery.

Recent studies on the delivery of peptide drugs were done with the use of Pheroid™ technology in order to improve its transport across the skin. The Pheroid™ technology proved to be very important technology in the delivery of peptide and protein actives (Strauss, 2005:96; Steyn, 2006:94). The most important advantages of the use of this delivery system in peptide and protein delivery are the increase in the penetration of most known barriers in the body and an increased delivery of the active resulting in an increase in therapeutic efficiency (Strauss, 2005:96).

2.1.8.4.8. Pheroid™ versus other lipid based delivery systems

From table 2.2 it is obvious that Pheroid™ technology differs substantially from conventional lipid based delivery systems (Grobler, 2004:6).

Table 2.2: Differences and similarities between Pheroid™ and other lipid based delivery systems (Grobler, 2004:6 - 8).

Pheroid™	**Other lipid based delivery systems**
Consists of fatty acids essential to the body.	Contains substances foreign to the body.
Pheroid™ elicits no immune response.	Some liposomal formulations elicit immune responses.
A variety of Pheroid™ is formulated, depending on the composition and manufacturing method.	A variety of liposomes have been described.
Pheroid™ can be manipulated in terms of size, charge, lipid composition and membrane packing. Desired types and repeatability can be obtained.	Problems with the degree of repeatability of liposomal systems, types and sizes.
Affinity between the Pheroid™ and cell membranes, due to the fatty acids within the Pheroid™, results in a high and fast penetration and delivery rate.	Specific binding and uptake mechanisms have not been described for other lipid-based delivery systems.
Pheroid™ can, by varying its composition, be designed to target at a subcellular level to a certain extent.	Phospholipids are metabolised in the cell membrane making it difficult to envisage subcellular targeting of organelles by this system.
Pheroid™ causes no cytotoxicity and assists with the maintenance of cell membranes.	Cytotoxicity and impaired cell integrity are common.
Drug resistance is reduced or eliminated.	Some delivery systems are prone to drug resistance or adverse immune responses.
Pheroid™ is polyphillic and drugs with different solubilities, as well as insoluble drugs, can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
Pheroid™ passively targets the reticulo-endothelial system (RES). Distribution can be adjusted to prevent phagocytosis.	Liposomal systems generally target RES. Development of stealth liposomes aim to adjust the redistribution of liposomes.
Entrapment of actives in Pheroid™ reduces the volume of distribution, thus increasing the concentration of the active at the target site, resulting in a narrow, but enhanced therapeutic index, with a decrease in toxicity.	Liposomes encapsulating small molecules also reduce the volume of distribution and enhance the concentration of the active resulting in a decrease of aspecific toxicity.

Pheroid™	Other lipid based delivery systems
Pheroid™ protects the active from metabolism, opsonization and inactivation in the plasma / other bodily fluids.	Some liposomal systems act as protection against metabolism and opsonization in the plasma after IV administration.
Sterically stabilized without the disadvantages of increased size or decreased elasticity.	Delivery systems generally need to be sterically stabilized.
Pheroid™ has a stable interior volume yet is able to maintain a high degree of elasticity and fluidity.	Lipid-based delivery systems contain cholesterol for stabilising and maintaining the interior of the vesicle; however, this leads to a loss in fluidity and elasticity.
Pheroid™ is able to inhibit drug efflux due to its composition and thereby enhances the bioavailability.	A separate compound needs to be administered to inhibit drug efflux
Pheroid™ enhances the bioavailability of oral, topical and buccal administration of actives.	Some delivery systems enhance absorption, whereas others decrease absorption.
Pheroid™ changes the pharmacokinetics of actives, resulting in a decrease in time needed to achieve maximum concentration levels (t_{max}).	Liposomes also change the pharmacokinetics of actives.
Entrapment efficiency in all compounds tested is high.	Entrapment efficiencies may be problematic due to charge and sterical limitations.
The type of Pheroid™ formulated for a specific compound determines the loading capacity of the Pheroid™.	The loading capacity of most lipid-based delivery systems is limited due to its dependence on the interior or intra-membrane volume.
Can be formulated as pro-Pheroid™.	Can be formulated as pro-liposomes.
Micro-sponges are ideal for combination therapies: one drug can be entrapped in the interior volume and the other in the sponge spaces, therefore minimizing interaction between the two actives.	Combination treatment is problematic for most delivery systems.
Pheroid™ with entrapped antibodies and small peptides have been shown to interact with specific micro-domains and drug targeting is therefore possible.	Antibody-containing liposomes for drug targeting have been described.

Pheroid™	Other lipid based delivery systems
Batch to batch reproducibility and stability have been proven.	Low batch-to-batch reproducibility and problems with size control sometimes occur in large-scale manufacturing.
Pheroid™ showed *in vivo* stability	Both product and *in vivo* chemical and physical instability are problematic in some lipid-based delivery systems.

Table 2.2 illustrates the multiple advantages of Pheroid™ technology above that of other lipid based penetration enhancers. It is obvious that this technology is a phenomenal breakthrough in penetration enhancement and has the potential to cause a revolution in drug administration with improved efficacy and reduced side effects as additional benefits.

2.1.8.4.9. Conclusions of Pheroid™ as a therapeutic drug delivery system

It is very apparent that Pheroid™ technology is an exceptional breakthrough in the pharmaceutical industry as it:

- compares well with and in many cases exceed current delivery systems,
- increases drug delivery,
- decreases the time to onset of drug action,
- decreases drug related toxicity,
- in some cases, improves drug therapy affectivity in lower concentrations when entrapped in Pheroid™ than when the drug is administered alone.

It can also be therapeutically used, without the addition of any active compounds for delivery.

Pheroid™ technology is one of the most effective, versatile and inexpensive delivery systems currently commercially used. All of its constituents are pharmaceutically safe and the system is based on the natural occurring molecules of the body. Furthermore, Pheroid™ stability has been proven for Pheroid-based commercial products (Grobler *et al.*, 2008:309).

Pheroid™ technology holds many advantages above current delivery systems and has many possibilities for future drug and disease therapies. It may possess the key to multiple pharmaceutical breakthroughs in the improved treatment and management of diseases and conditions that current delivery systems have not been able to do.

Peptides, for example growth factors, are therefore, good candidates for delivery with Pheroid™ technology.

2.1.9 TRANSDERMAL KINETICS

The complexity of human skin as multilayered and heterogeneous biological tissue is very apparent, with enormous variation from species to species, individual to individual and from site to site. Therefore, to suggest that any attempt to describe the behaviour of such a system with an ideal mathematical model, would be impossible. However, Fick's second law of diffusion can be applied to data obtained from experiments done with human skin. Drug absorption across human skin is a passive process and can thus be described in physical terms. The following description of the mathematical treatments applicable to transdermal drug delivery studies is intended to provide an overview. Only two situations will be considered:

- Where the drug is applied at an infinite dose – the dose does not deplete over the time of application.
- Where a small finite dose is administered, a pseudo-steady state permeation would therefore not be encountered (Williams, 2003:41).

2.1.9.1. FICK'S LAWS OF DIFFUSION

Molecules move due to a concentration gradient that results in a thermodynamic force response (Williams, 2003:41).

Fick's first law states: The rate of transfer of a diffusing substance through a unit area of a section (flux, J) is proportional to the concentration gradient (ΔC) measured normal to the section and is inversely proportional to the membrane thickness (h). The distribution coefficient (K) of the drug between the solvent or vehicle, and the stratum corneum and the average membrane diffusion coefficient (D) for the solute in the stratum corneum, is also proportionate to the flux (J) of the drug (Equation 1) (Smith & Surber, 2000:25):

$$J = \frac{K \cdot D}{h} \Delta C \qquad \text{(Equation 1)}$$

Where J = Flux (μg/cm^2.h)

K = Partition coefficient

D = Diffusion coefficient (cm^2/h)

ΔC = Concentration gradient e.g. ($C_1 - C_2$) (μg/cm^3)

h = Membrane thickness (cm)

According to Tojo (1997), Fick's second law of diffusion can mathematically describe diffusion of a topically applied permeant through the skin, given that the skin contains no drug molecules prior to the application of the drug. Steady-state permeation is attained in diffusion studies with Franz cells, where sink conditions are created after the sufficient passing of time (Equation 2) (Williams, 2003:43):

$$\frac{dM}{dt} = \frac{DKC_v}{h} \qquad \text{(Equation 2)}$$

Where dM = Cumulative mass of permeant through a unit area of the membrane (μg/cm^2)

t = Unit of time (h)

K = Partition coefficient

C_v = Concentration of the permeant in the vehicle

h = Membrane thickness (cm)

In order to obtain measurable flux, the permeant molecule must penetrate the stratum corneum first, concentrate within the stratum corneum, and begin its time dependent diffusion until the permeant reaches the border of the stratum corneum and the viable dermis. Subsequently, it will give a measurable flux value (Rieger, 1993:39).

Lag time is the time before steady-state diffusion is reached and is characteristic for the diffusivity of the permeant in the membrane. The lag time, t_{lag}, can be obtained from extrapolation of the steady-state portion of the graph to the intercept on the time axis and is illustrated in figure 2.7 (see equation 3):

$$t_{lag} = \frac{h^2}{6D} \qquad \text{(Equation 3)}$$

Where t_{lag} = Lag time

h = Membrane thickness (cm)

D = Diffusivity (cm^2/s)

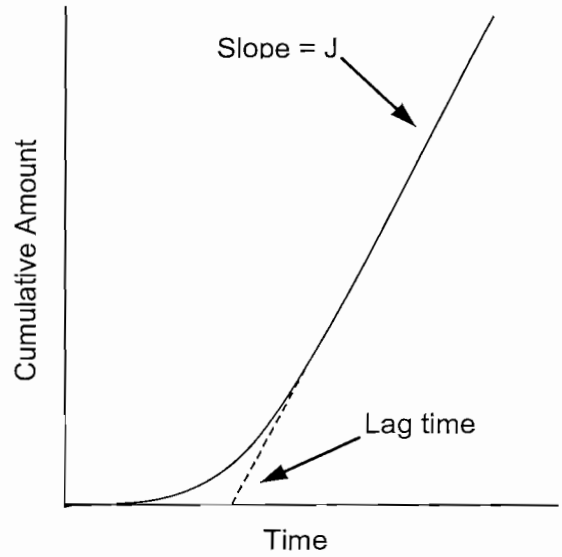

Figure 2.7: Typical cumulative amount of drug ($\mu g/cm^2$) permeated through the skin versus time plot. The slope of the linear portion of the curve is the flux (J), whilst the x-axis intercept of the slope provides the lag time (t_{lag}) (Roy, 1997:145).

2.1.9.2. TRANSDERMAL DELIVERY CHALLENGES OF PEPTIDES

There has been considerable interest in the transdermal delivery of peptides and proteins in recent years (Hadgraft, 2001:13). Many proteins and peptide drugs can be produced relatively economically: However, there are a number of physicochemical and biological barriers that limit their use as therapeutic agents. There are basically three barriers that account for the low bioavailabilities of protein and peptide drugs: low permeability of absorbing tissue to the drug; short residence time of the dosage form at the absorption site; and degradation of compounds (Malik *et al.*, 2007:141). Due to their large size and their electric charge, the transdermal delivery of peptides and proteins have been problematic to achieve; with ionotophoresis being the predominant method of transdermal delivery of these molecules (Hadgraft, 2001:13).

The most important of the challenges of peptides and proteins in transdermal delivery are the following:

- They are delicate molecules that are large in size and have various functional groups.
- Their structures are readily and irreversibly changed, due to the weak physical bonds that stabilize their structures.
- They are relatively instable molecules and environments with extreme temperatures and pH, lead to denaturization of the secondary and tertiary structures.
- Proteolytic enzymes pose a considerable threat to the feasibility of peptide delivery.
- Protein epithelial penetration capacity is very low except in the case where proper transfer molecules are available.
- Protein membrane transport is diminished in general, due to its electric charge and hydrophilic nature (Crommelin *et al.*, 2002:545).

2.1.9.2.1. Administration routes for peptides

Most peptides cannot be administered orally, since they are rapidly inactivated by gastrointestinal enzymes, and therefore, subcutaneous or intravenous administration is required. Research is consequently focussing on alternative routes for the delivery of peptides and proteins, including inhaled, buccal, intranasal and transdermal routes. Novel delivery systems, such as the use of protective liposomes, have also been researched (Edwards *et al.*, 1999:1).

Due to (1) the inability of proteins and peptide drugs to cross the mucosal tissue, (2) poor transdermal delivery; and (3) poor oral stability; most protein and peptide products are only formulated in an injectable dosage form. Considerable effort has been put into the development of non-invasive delivery methods for proteins (Malik *et al.*, 2007:141).

Novel methods that have been developed for the enhanced delivery of proteins and peptides are:

- micro-sphere drug delivery system,
- prolease drug delivery system,
- double walled (polylactide-*co*-glycolide) PLGA micro-spheres,
- nano-particles and micro-particles for protein and peptide delivery,
- muco-adhesive nano-particulate system for peptide drug delivery,
- gelatine nano-particle poly(lactic-*co*-glycolic acid) PLGA micro-sphere composites,
- polymer-alloy method,
- polymer coated liposomes for the delivery of protein or peptide drugs,

- pulmonary delivery of proteins and peptides,
- nasal delivery of proteins and peptides,
- chimeric peptide strategy to deliver proteins and peptides to the brain,
- covered-rod-type formulation for protein or peptides,
- colon targeted delivery of proteins and peptides,
- macro-flux transdermal technology,
- xenoport's transcytosis; as well as
- polymeric micelles (Malik, 2007:141 - 150).

Non-conventional delivery systems for proteins that are biodegradable and non-biodegradable have also been found suitable for peptide delivery. In addition some other novel approaches for protein and peptide delivery, (vector mediated delivery, by making use of adenovirus, macro-flux transdermal patches, pulmonary delivery of proteins; the delivery of proteins or peptides across the blood brain barrier; and polymeric micelles) have been utilised (Malik, 2007:150).

In passive delivery strategies, small amounts of oligonucleotides can be delivered with the use of the appropriate solvents. DNA has been delivered when using a complex with 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP). The resulting complexes are quite large and the mechanism for transdermal delivery is unclear. Delivery *via* the use of flexible liposomes (transfersomes) has also been reported, and makes the delivery of sufficient amounts of insulin to invoke a lowering of blood glucose levels, possible. This mechanism of skin penetration is also unclear, however, it is possible that they pass through the defects in the intercellular lipids (Hadgraft, 2001:13).

Thus, the various routes that are open to peptide delivery are the following, which include the invasive and non-invasive delivery methods:

Invasive protein and peptide delivery:

- Parenteral delivery (subcutaneous or intravenous) (Edwards *et al.*, 1999:1).

Options for non-invasive protein and peptide delivery are:

- transdermal,
- pulmonary,
- buccal,
- nasal,
- vaginal,
- ocular,
- rectal and
- oral (DeFelippis *et al.*, 2006).

With the advancement in technology and formulation knowledge, many approaches have been used to overcome the limitations with proteins and peptides. This advancement in technology, therefore, makes proteins and peptide viable drugs (Malik, 2007:150).

2.1.10 POSSIBLE EXPEDIENTS TO FACILITATE TOPICAL PERMEATION OF PEPTIDE DRUGS

2.1.10.1. Use of bestatin hydrochloride as enzyme inhibitor

2.1.10.1.1. Introduction

Aminopeptidase B (APB) is an exopeptidase that is able to remove basic amino acid residues from the NH_2-terminus of peptide substrates (Pham *et al.*, 2007:2). Aminopeptidases are widely distributed throughout plants and animals. They are found in subcellular organelles and cytoplasm, and form part of membrane components (Taylor, 1993:290). Bestatin is a natural product of *Actinomycetes* that inhibits most metallo-aminopeptidase (MAP) families (Harbut *et al.*, 2008:3). It is a specific and potent inhibitor of APB, leucine aminopeptidase (LAP) and tri-aminopeptidase. The surface of mammalian cells, contain LAP and APB, additionally mammalian cells also contain specific binding sites for bestatin (Petrov *et al.*, 2000:538; Umezawa *et al.*, 1975:99).

Bestatin was used in this study to inhibit the aminopeptidases that are present in, and on the skin to prevent the potential degradation of the studied polypeptides: IGF-1, KGF and VEGF. All experimental work was done with Fluka (Sigma-Aldrich, St. Louis, USA) bestatin hydrochloride (MW = 344.83).

2.1.10.1.2. Basic structure and characteristics

Bestatin is a dipeptide immunologic, anti-bacterial, anti-HIV agent, anti-biotic, anti-neoplastic factor, and protease inhibitor.

Solubility testing was done in water and a clear solution was obtained at 25 mg/ml (Sigma-Aldrich Corporation, 2008) in PBS at pH 7.2 and quantities of >4 mg/ml at 25 °C. It is also soluble in ethanol (EtOH), dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) (Cayman, 1999:2). Bestatin is stable as a solid for 2 years when stored at -20 °C (Cayman, 1999:2). A 1.0 mM solution is expected to be stable for at least one month if stored at -20 °C (Sigma-Aldrich Corporation, 2008).

2.1.10.1.3. Mechanism of inhibition

Bestatin inhibition is obtained through the binding of it to the catalytic site of the enzyme and thus, competing with the substrate. Its inhibitory effect is, however, delayed. With low inhibitor concentrations, a lag time of up to an hour can occur before full inhibition is established. The delay is due to slow binding to the catalytic site. In contrast, higher concentrations of bestatin exhibits competitive kinetics with the substrate. In order to attain maximum inhibition, concentrations much lower than the substrate are required (Scornik & Botbol, 2001:71).

2.1.11 SUMMARY

During this study the peptides IGF-1, KGF and VEGF were used, which are currently mainly administered *via* the parenteral route. Though this route offers very good bioavailability, it remains an invasive procedure, which leads to poor patient compliance. The aim is to explore the transdermal applications of these peptides to improve the ease of application as well as possible alternative uses for the peptides. These uses include the promotion of hair growth in alopecia sufferers, whether due to androgen stimuli or cytotoxic assault by chemotherapy in cancer patients.

The transdermal delivery of peptides poses a problem due to its large molecular weight, resulting in poor permeation and topical penetration and thus its inability to transverse the skin through the transepidermal route. Additionally its physicochemical properties such as its polarity, hydrophilic nature and relative instability due to the weak physical bonds, are all contributing factors to the difficulty with which peptide drugs are delivered into systemic circulation.

Pheroid™ technology is therefore a possible solution to the various complexities of transdermal delivery of peptides. The Pheroid™ technology makes use of submicron-sized vesicles or sponges, which capture and transport the drug molecules across the skin barrier, and therefore, carries it to the desired destination. Pheroid™ is formulated from modified ethylated and pegylated polyunsaturated fatty acids, essential fatty acids and N_2O. The essential fatty acids are not produced by the human body, however, it is of great importance for normal cellular function of the human body, and therefore, needs to be ingested. Oleic acid is but one of these essential fatty acids contained within the Pheroid™ and has been proven an effective penetration enhancer. The hypothesis is that the Pheroid™ system would be able to transport the peptide across the skin barrier due to the dual advantage of microspheres with the incorporation of a penetration enhancer.

In an effort to prevent degradation of the active peptide by proteolytic enzymes that are present in the skin, bestatin a proteolytic enzyme inhibitor, was added to the donor solution prior to administration in the Franz cell during the diffusion studies. Bestatin is a selective aminopeptidase inhibitor that has been proven to discourage the degradation of peptides on the surface of the skin as well as within it.

The objective of this study was to investigate the *in vitro* topical permeation of IGF-1, KGF and VEGF with the assistance of Pheroid™ technology.

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CHAPTER 3: ARTICLE FOR PUBLICATION IN THE EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

PHEROID™ TECHNOLOGY FOR THE TOPICAL DELIVERY OF INSULIN GROWTH FACTOR 1 (IGF-1), KERATINOCYTE GROWTH FACTOR (KGF) AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

PHEROID™ TECHNOLOGY FOR THE TOPICAL DELIVERY OF GROWTH FACTORS: IGF-1, KGF AND VEGF

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Abstract

The aim of this study was to investigate the *in vitro* topical delivery of Insulin-like growth factor 1 (IGF-1), Keratinocyte growth factor (KGF) and Vascular endothelial growth factor (VEGF), (growth factors) with the aid of the Pheroid™ drug delivery system in order to possibly treat alopecia, by stimulating hair growth and protecting the hair follicles. Generally, peptides are unsuited for topical delivery, but due to success in the delivery of peptides through the Pheroid™ delivery system; topical delivery of the growth factors, was explored. Bestatin was employed as an excipient to negate skin mediated growth factor degradation. Vertical Franz diffusion cells were used in the diffusion studies performed on human female abdominal skin. Each growth factor was put into a PBS or Pheroid™ solution at predetermined concentrations. PBS (pH 7.4) was used as receptor solution for the 6 h diffusion study. The data showed that the Pheroid™ drug delivery system, when compared to PBS, successfully improved diffusion for IGF-1 (5%) and VEGF (31%). However, the diffusion of KGF decreased by 97%. Interestingly, the Pheroid™ system increased IGF-1 stability, but VEGF stability was negligible and KGF degradation was increased.

Insulin-like growth factor 1 (IGF-1)

Keratinocyte growth factor (KGF)

Vascular endothelial growth factor (VEGF)

Topical delivery

Pheroid™

Alopecia

1. Introduction

The use of peptides in drug therapy has become more popular in recent years due to its role in the regulation of most physiological processes and for being very therapeutically diverse. It is already being used in neurology, endocrinology and haematology. Research for alternative routes to deliver peptides is performed due to the inability to deliver them orally. Since peptides undergo rapid inactivation by gastrointestinal enzymes, subcutaneous and intravenous administration are required for delivery thereof (Edwards et al., 1999). Transdermal delivery would be a non-invasive method that is able to avoid hepatic metabolism. Therefore, topical administration will possibly be able to improve the bioavailability of the growth factors (Malik et al., 2007).

The stratum corneum is a very selective membrane to cross and the physicochemical properties of the growth factors make it more difficult to penetrate this barrier. Transdermal delivery of peptides is, however, not an easy feat due to their large molecular mass and poor stability. Peptides, like growth factors, have large molecular masses ranging from 7.6 - 38.2 kDa. Most therapeutic agents selected for transdermal delivery have a molecular mass within the range of 100 - 500 Dalton, which is the best for transdermal delivery. Therefore, the molecular mass of a growth factor is too large to ensure maximum drug delivery. When larger molecules such as peptides or proteins are selected as therapeutic agents, the transdermal flux dependency on molecular mass becomes more apparent (Williams, 2003). Bioavailability is currently also a big challenge in transdermal delivery of peptides as the skin also serves as a proteolytic barrier that is a potential threat to protein drugs (Banga, 2007). Most proteins have short half-lives *in vivo*, are unstable in biological fluids and are not fully absorbed due to their high molecular mass (Malik et al., 2007). Due to all these obstacles in the delivery of peptides, ionotophoresis and vesicles are the most used methods for their topical delivery (Hadgraft, 2001).

Pheroid™ technology has shown promising results in the delivery of peptides in previous studies (Strauss, 2005:96; Steyn, 2006:94), making it a logical choice for delivery of the growth factors. It is a novel drug delivery system that consists mainly of fatty acids in a submicron oil or water emulsion type formulation that forms spherical structures, which can be manipulated to produce specific sizes, functions, structures and morphology. It entraps active compounds, improve their delivery and has other positive effects for example: improved efficacy. This technology has been patented and the intellectual property thereof belongs to the North-West University, South Africa (Grobler et al., 2008).

Bestatin is a natural product that inhibits most metallo-aminopeptides (MAPs) (Harbut *et al.,* 2008). It is a specific and potent inhibitor of aminopeptidase B (APB), leucine aminopeptidase (LAP) and tri-aminopeptidase of which LAP and APB are contained on the surface of mammalian cells (Petrov et al., 2000; Umezawa et al., 1975). In this study, bestatin was employed in order to inhibit aminopeptidase-mediated degradation of the growth factors. Phosphate buffered solution (PBS) was used as the receptor phase for the diffusion study and was employed in the manufacturing of the Pheroid™ delivery system. PBS with a pH of 7.4 was used to ensure that it was close to the physiological pH and would therefore ensure maximum stability of the growth factors.

2. MATERIALS AND METHODS

2.1. MATERIALS

Human IGF-1 (MW 7.6 kDa), KGF (MW 38.2 kDa) and VEGF (MW 18.9 kDa), expressed in *Escherichia coli*, as well as bestatin hydrochloride (MW 344.83 Da) were obtained from Sigma-Aldrich (Sigma, St Louis, USA). Pheroid™ vesicles were freshly prepared at the North-West University. The Pheroid™ drug delivery system consists mainly of modified essential fatty acids in a vesicular structure. For the control groups, PBS was freshly prepared in the laboratory and buffered at a pH of 7.4. The Quantikine Human Immunoassay, also known as ELISA immunoassays, that were used for the quantification of the growth factors were obtained from Whitehead Scientific (Pty) Ltd. (WhiteSci, Brackenfell, RSA).

2.2. EXPERIMENTAL METHODS

2.2.1. Encapsulation of the actives in Pheroid™ vesicles

4 % (m/m) Pheroid™ in a PBS solution was used. The growth factors were reconstituted to the recommended concentrations with PBS, as per instruction from the Sigma-Aldrich product information leaflet on each growth factor. It was subsequently diluted to the desired concentration in the Pheroid™ or PBS solution, depending on whether it was the control or experimental solution. For each growth factor, a growth factor / Pheroid™ mixture and a growth factor / PBS mixture were placed in volumetric flasks together with a magnetic stirring bar. It was placed on a magnetic stirrer in the refrigerator at 2 - 8 °C in order to ensure maximum stability of the growth factors. The mixtures stirred in the fridge for the duration of 14 hours, in order to ensure full encapsulation and entrapment into the Pheroid™ vesicles.

2.2.2. Procedure for stability testing

Equal volumes of the growth factor / Pheroid™ mixtures as well as the growth factor / PBS mixtures were placed in respective volumetric flasks. One flask for each mixture was filled and marked for testing at room temperature (25 °C), whereas the other was filled and marked for testing at 37 °C, in a heated water bath. The flasks were placed into their respective testing conditions and an initial concentration sample of 500 µl was taken from each volumetric flask. Samples of 500 µl from each volumetric flask were collected after 1 h and for every hour thereafter for the duration of 12 hours during stability testing. Each collected sample was immediately stored at 2 - 8 °C in a marked and sealed high pressure liquid chromatography (HPLC) vial to ensure minimal cross contamination between samples. The last samples were taken 12 hours after commencement of the stability experiment. All the samples were stored overnight in the fridge (2 - 8 °C) and subsequently the ELISA analyses were conducted.

2.2.3. Preparation of donor solutions

For each of the selected growth factors (IGF-1, KGF and VEGF), solutions were prepared by diluting the selected growth factor in the Pheroid™ or PBS solutions. The growth factor / Pheroid™ mixture as well as the growth factor / PBS mixture were placed in respective volumetric flasks in equal volumes.

2.2.4. Preparation of skin

The Research Ethics Committee of the North-West University (Reference nr. 04D08) provided Ethical approval for the procurement and utilization of the tissue. Female skin obtained from plastic surgeon practices, after cosmetic abdominoplasty, was used. The skin was stored frozen at -20 °C and thawed at room temperature prior to preparation for use in the diffusion study. Any excess blood was wiped off with tissue paper and the subcutaneous

fat layer carefully removed with forceps and a scalpel. Any residual adipose tissue was scraped of with a dull scalpel. The skin was placed on a piece of Whatman® filter paper, left to air dry, carefully wrapped in aluminium foil, sealed, and frozen again at -20 °C until needed for the diffusion study. Full thickness skin was thawed at room temperature for the diffusion study and visually examined for any flaws. To minimise the variation between the skin samples, a single skin source was employed for each of the diffusion studies.

2.2.5. Skin permeation studies

Growth factor / Pheroid™ mixture as well as growth factor / PBS mixture were placed in the heating bath for approximately an hour and a half prior to the start of the diffusion study. This was to ensure that skin temperature (32 °C) (at which the diffusion study was to take place) was reached. The diffusion study was limited to 6 hours at 32 °C to ensure maximum stability of the growth factors due to unwanted degradation at high temperatures. The stability of KGF was of particular concern in the Pheroid™ drug delivery system at both 25 and 37 °C. Vertical Franz diffusion cells were used in the permeation studies. The skin was carefully cut into skin discs with a diameter of approximately 10 mm. It was then mounted, with the stratum corneum side facing upwards, over the lower half of the Franz cell (Steenekamp, 2003) in order to separate the donor and receptor compartments. The donor compartment was placed on the lower receptor compartment, sealed with the help of Dow Corning® high-pressure vacuum grease, and fastened together with a specially designed metal clamp. The receptor compartment has a volume of 2.3 ml. A small stirring magnet was placed in the receptor compartment and was filled with 2.0 ml PBS (pH 7.4) 1 hour prior to commencing of the diffusion study and left to equilibrate at 32 °C. Special care was taken not to trap any bubbles in the receptor compartment under the skin. The Franz cells were placed on a plastic Franz cell stand, in the heating bath, upon a Variomag® stirring plate: For the duration of the experiment it was left to rotate at 750 rpm. Special care was taken to ensure that only

the receptor compartment was submerged in the water of the heating bath. The donor compartments of 6 Franz cells were each filled with 1.0 ml of the donor solution containing the growth factor / Pheroid™ mixture. The remaining 6 Franz cells' donor compartments were used as the control group and were filled with 1.0 ml of donor solution containing the growth factor / PBS mixture. Vertical Franz diffusion cells were left in the heating bath at 32 °C for the duration of 6 hours allowing diffusion and permeation to take place. After the completion of the diffusion at 6 hours, samples were analysed with ELISA immunoassays. The skin discs were removed from the Franz cells and subjected to tape stripping.

2.2.6. Tape stripping

At 6 hours the system was dismounted and the donor and receptor phases were removed. All diffusion cells were dismantled and the skin pieces removed for the tape stripping analysis in order to determine the amount of growth factor that had remained trapped in the skin after diffusion. The skin was affixed to a piece of Whatman® filter paper, which in turn was stapled to a solid surface. It was carefully wiped with tissue paper to remove any excess donor solution. Imprints made by the flanges from the diffusion cells clearly marked the skin exposed to the donor solution. Pieces of Sellotape® clear tape with a width of approximately 1.9 cm were used for tape stripping. The tape was pressed onto the diffusion area imprint and removed with a quick even movement. The first strip was discarded as a part of the cleansing procedure. After the cleansing strip, 15 more strippings were conducted with a constant amount of pressure applied to the tape onto the diffusion imprint each time. These strips were snipped into a small vial and 2.0 ml PBS (pH 7.4) added - this was done for all the pieces of skin used in the diffusion study.

For the determination of the concentration of growth factor that had been present in the remainder of the skin (epidermis and dermis), the excess skin around the flange imprint was

trimmed and discarded. Each piece of the remaining skin was snipped into individually marked vials and 1.0 ml PBS (pH 7.4) added to each.

All the vials were subsequently placed in the fridge at 4 °C to ensure minimal degradation of the growth factors and left to stand overnight allowing the dissolution of the growth factors into the PBS. The PBS in each vial was carefully removed and analysed with the use of Quantikine® ELISA kits to quantify the amount of growth factor present in each sample.

2.2.7. Sample analysis by ELISA immunoassay

The collected samples were all analysed with Quantikine® immunoassay kits. The immunoassay kit can be used for *in vitro* quantitative measurement of human IGF-1 / KGF / VEGF in both serum and plasma samples.

2.2.7.1. General information on the IGF-1 / KGF / VEGF kit

- Proprietary name : Quantikine human IGF-1 / KGF / VEGF immunoassay
- Catalogue number : DG100 / DKG00 / DVE00
- Manufactured by : R & D Systems, Inc., 614 McKinley Place NE, Minneapolis, MN 55413, United States of America.

2.2.7.2. Principle of the assay for IGF-1, KGF and VEGF

The assays employ the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody that is specific for IGF-1 / KGF / VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IGF-1, KGF or VEGF present then binds to the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IGF-1, KGF or VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate

solution is added to the wells and colour develops in proportion to the amount of IGF-1 / KGF / VEGF bound in the initial step. The colour development is stopped and the colour intensity is measured with a microplate reader (R & D Systems Inc., 2008).

2.2.7.3. Reagent preparation for IGF-1 / KGF / VEGF (R & D Systems Inc., 2008)

- Bring all the reagents (except Conjugate for IGF-1) to room temperature before use.
- Wash buffer – Dilute 20.0 ml of the wash buffer concentrate into deionised or distilled water to prepare 500.0 ml wash buffer.
- Substrate solution – mix colour reagents A and B in equal volumes, 15 min prior to use. Protect from light (200 µl of the mixture is required per well).
- IGF-1 / KGF Standard – reconstitute the IGF-1 / KGF standard with 1.0 ml of deionized or distilled water. The reconstitution produces a stock solution of 60 pg/ml respectively 20 000 pg/ml. Allow the standard to sit for a minimum of 15 min with gentle agitation prior to making dilutions.
- VEGF Standard – reconstitute the VEGF standard with 1.0 ml of calibrator diluent RD5K. For cell culture supernatant samples, use calibrator diluent RD6U for serum / plasma samples. The reconstitution produces a stock solution of 2 000 pg/ml. Allow the standard to sit for a minimum of 15 min with gentle agitation prior to making dilutions.
- Use polypropylene tubes.
- IGF-1: Pipette 360 µl of calibrator diluent RD5-22 into the 6 ng/ml tube. Pipette 200 µl of calibrator diluent RD5-22 into the remaining tubes. Use the stock solution to produce a dilution series as shown figure 1. Mix each tube thoroughly before the next transfer. The 6 ng/ml standard serves as the high standard. Calibrator diluent RD5-22 serves as the zero standard (0 pg/ml).

Figure 1: Dilution series for IGF-1 from stock solution.

- KGF: Pipette 900 µl of calibrator diluent RD5R into the 2 000 pg/ml tube. Pipette 500 µl of calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series as shown in figure 2. Mix each tube thoroughly before the next transfer. The 2 000 pg/ml dilution serves as the high standard. Calibrator diluent RD5K serves as the zero standard (0 pg/ml).

Figure 2: Dilution series for KGF from stock solution.

- VEGF: Pipette 500 µl of calibrator diluent RD5K into each tube. Use the stock solution to produce a dilution series as shown in figure 3. Mix each tube thoroughly before the next transfer. The 1 000 pg/ml dilution serves as the high standard. Calibrator diluent RD5K serves as the zero standard (0 pg/ml).

Figure 3: Dilution series for VEGF from stock solution.

2.2.7.4. Assay procedure for IGF-1 / KGF / VEGF (R & D Systems Inc., 2008)

IGF-1: Conjugate should remain at 2 - 8 °C until use.

Bring all reagents and samples to room temperature before use.

Step 1: Prepare all reagents and standards as directed in the previous section

Step 2: Remove excess microplate strips from the plate frame, return the excess wells to the foil pouch containing the desiccant pack and reseal.

Step 3: Add 150.0 ml of assay diluent RD1-53 to each well for IGF-1; 100.0 ml of diluent RD1-25 to each well for KGF and 50.0 ml of diluent RD1W to each well for VEGF.

Step 4: Add 50.0 ml / 100.0 ml / 200.0 ml standard, control or sample per well for IGF-1 / KGF / VEGF, respectively. Cover with adhesive strip provided and incubate IGF-1 for 2 hours at 2 - 8 °C; KGF for 3 hours at 25 °C and VEGF for 2 h at 25 °C,

respectively. Record the standards and samples assayed on the provided plate layout.

Step 5: Aspirate each well and wash, repeating the process three times for a total of four washes for IGF-1 or three times for a total of four washes for KGF,or twice for a total of three washes for VEGF, respectively. Wash by filling each well with wash buffer (400 µl) using a squirting bottle, manifold dispenser or auto washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Step 6: Add 200 µl of cold IGF-1 conjugate, to each well, add 200 µl of room temperature KGF conjugate, to each well and add 200 µl of room temperature VEGF conjugate, to each well. Cover with a new adhesive strip. Incubate IGF for 1 hour at 2 - 8 °C, KGF for 1.75 hour at room temperature, and VEGF for 2 hours at room temperature, respectively.

Step 7: Repeat the aspiration / wash as in step 5.

Step 8: Add 200 µl of IGF-1 / KGF / VEGF substrate solution to each well. Protect from light. Incubate for 30 min / 30 min / 20 min at room temperature for IGF-1 / KGF / VEGF, respectively.

Step 9: Add 50 µl stop solution to each well. The colour in the well should change from blue to yellow. If the colour in the wells is green or the change in colour does not appear uniform, gently tap the plate to ensure thorough mixing.

Step 10: Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm. If wavelength correction is available, set to 450 nm or 570 nm. If wavelength correction is not available, subtract readings at 450 nm or 570 nm from the readings at 450 nm. The subtraction will correct for optical imperfections

in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

2.2.7.5. Handling notes for all ELISA analysis (R & D Systems Inc., 2008)

- Bring all reagents to room temperature prior to use.
- Record standards and samples assayed by noting it on the provided plate layout.
- Thoroughly mix all the reagents and samples by means of gentle agitation or swirling.
- Avoid cross-contamination by using clean disposable micropipette tips for the addition of each sample and reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Adhere to all the prescribed incubation times.

2.3. DATA ANALYSIS

2.3.1. Analysis of stability data

The concentration of each growth factor that remained intact in the PBS and Pheroid™, was plotted against time. The amount of intact growth factor was depicted as a percentage of the initial concentration at time 0.

2.3.2. Statistical analysis of transdermal data

The concentration per skin area of growth factor that permeated the skin as a function of time was plotted for IGF-1, KGF and VEGF in both vehicles (PBS and Pheroid™). Six replicate skin samples in Franz cells were taken after 6 h for each vehicle and growth factor

combination; the average of the aforementioned is the average flux at 6 h. The yield of each cell was depicted as a percentage of the applied initial concentration.

An independent samples t-test and the non-parametric Mann-Whitney test were performed in order to compare the differences between the PBS and Pheroid™ average and median values (UCLA, 2008). The Shapiro-Wilk test was used to test the normality of the data, and the Levene test for equality of variances was also performed on the data. These are both assumptions of the t-test (Arsham, 2008; Kritsonis, 2008).

Data were graphically represented in box plots and descriptive measures were given.

With no major violations of the normality assumption (tested with Shapiro-Wilk) and equal variances assumed (tested with the Levene test) the results of the t-tests (comparing PBS with adjusted Pheroid™) are shown in table 1.

Table 1: T-test comparison of PBS and adjusted Pheroid™ p-values using the Levene test.

A p-value is a measure of how much evidence there is against the null hypothesis, there by measuring the probability that random variation is the only reason for sample differences in the data (Arsham, 2008). If the p-value for the Levene test were more than 0.05, then equal variances are assumed. However, if the p-value is less than 0.05, it demonstrates significant statistical differences between the groups' averages.

The same comparison between PBS and the adjusted Pheroid™ values' p-values was performed with the Mann-Whitney non-parametric test, (the normality assumption is not required) in this test and is presented in table 2. The Mann-Whitney non-parametric test has p-values of less than 0.05 indicating that there is a significant statistical difference between the data groups.

Table 2: Comparison of the PBS with the adjusted Pheroid™ p-values using the Mann-Whitney non-parametric test.

3. RESULTS AND DISCUSSION

3.1 STABILITY STUDY RESULTS

3.1.1 IGF-1

Stability data (figure 4) indicated that IGF-1 was least stable at 25 °C in a PBS medium. This resulted in an approximated degradation of 16 % IGF-1 after 12 hours during stability testing. Most degradation took place within the first hour of testing, falling to approximately 91 % intact IGF-1. No significant differences in IGF-1 stability at 37 °C, in both the Pheroid™ and PBS medium ,could be detected. Degradation in both mediums resulted in an approximated 93 % intact IGF-1 after 12 hours stability testing.

The obtained data indicated marked improvement in IGF-1 stability in the Pheroid™ medium at 25 °C (total degradation of approximately 5 %) when compaired to degradation of IGF-1 in a PBS medium (16 %) at the same temperature. This could be due to the Pheroid™ formulation which protects the thermo labile IGF-1, though the actual mechanism of protection is currently unknown. Testing at higher temperatures would naturally cause greater IGF-1 degradation and the Pheroid™ drug delivery system is apparently only able to partially prevent degradation thereof.

Figure 4: Stability of IGF-1 in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

3.1.2 KGF

From figure 5 it could be established that KGF was least stable in the Pheroid™ at 37 °C (degradation of 52 %). Only 48 % was still intact after the 12 hour stability testing. KGF in the Pheroid™ (at 25 °C) also depicted remarkable degradation (51 %) after the 12 hour study. The data suggest that KGF in PBS is more stable at both 25 and 37 °C when compaired to KGF in the Pheroid™ system, with 90 % KGF remaining intact after the 12 hour stability

study. It was therefore evident that the Pheroid™ system is not a suitable delivery medium for the optimization of KGF stability.

Figure 5: Stability of KGF in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

3.1.3 VEGF

The stability data obtained as illustrated in Figure 6 indicated that VEGF is very stable at 25 and 37 °C in both PBS and Pheroid™ mediums. An improvement that delivers more than a 100 % of stability was seen for VEGF in PBS at 37 °C. The most degradation took place in the VEGF in Pheroid™ at both 25 and 37 °C and VEGF in PBS at 25 °C. VEGF in Pheroid™ at 25 °C showed the least degradation of the three. In the aforementioned, it was observed that for all three stability studies only approximately 1 % of VEGF had undergone degradation with 99 % of the VEGF remaining intact after the 12 h stability study.

Figure 6: Stability of VEGF in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

3.2 STATISTICAL ANALYSIS OF TRANSDERMAL DATA

According to Fick's laws on diffusion, the applied concentration can act as a driving force in the diffusion process (Hadgraft, 2001:3). From table 3 it could be seen that the applied concentrations differed substantially in some cases.

Table 3: Comparison of individually applied growth factor concentration values (pg/cm^2) in PBS and Pheroid™.

The obtained diffused quantities could, therefore, need theoretical correction to be able to compare the diffusion values of the growth factors in Pheroid™ and PBS. By applying the ratio of the different applied concentrations to the obtained values, theoretically more likely diffused concentrations were obtained for the growth factors. For example the ratio of the applied concentration IGF-1 in Pheroid™ to that in PBS was 1.818, by taking this ratio into account and applying it to the value obtained for the Pheroid™, an expected value for IGF-1 at the same concentration was obtained and was, therefore, more comparable. This calculation would not have been necessary if the applied concentrations during commencement of the diffusion study were exactly equal. These theoretically corrected values (due to unequal applied concentrations) that were calculated for the tape stripping, dermis and receptor solution will from here on be referred to as the adjusted Pheroid™ values.

3.2.1 Diffusion study results

Normality of the data was comfirmed by the Shapiro-Wilk test. And since the non-parametric Mann-Whitney test and Levene test depicted very small p-values, we conclude that there is statistical significant differences between the data and is of use in this study.

The diffusion studies were carried out and the samples analysed as described in the immunoassay for IGF-1, KGF and VEGF in 2.2.7.4. The following diffusion values for IGF-1, KGF and VEGF in PBS, Pheroid™ and adjusted Pheroid™ were obtained from the receptor compartments of the Franz diffusion cells after the conclusion of the 6 h diffusion study. The obtained values were illustrated in table 4.

Table 4: Average and median concentration values (pg/cm^2) after 6 h diffusion of IGF-1, KGF and VEGF together with the standard deviation (SD).

A comparison was drawn between the individual growth factor diffused concentrations and an average flux depicted in Figure 7

Figure 7: Comparison between skin diffused concentration data of IGF-1, KGF and VEGF average flux after 6 hours.

3.2.1.1 IGF-1

The adjusted diffusion concentration (pg/cm^2) of IGF-1 delivered by Pheroid^{TM} technology did indeed show improved skin permeation compared to that of IGF-1 in PBS. IGF-1 incorporated into the Pheroid^{TM} delivery system (adjusted value: 14172.50 pg/cm^2) depicted an approximated 5 % improvement of diffusion when compared to IGF-1 in PBS (13416.51 pg/cm^2).

As seen in Figure 8, the unadjusted Pheroid^{TM} values of 25764.55 pg/cm^2, had a 45 % higher diffusion concentration (pg/cm^2) when compared to adjusted Pheroid^{TM} values, as well as a diffusion increase of 48 % compared to that of PBS.

The Pheroid^{TM} delivery system possibly resulted in improved diffusion due to the entrapment of the IGF-1 and, therefore, increased the lipophilic nature of the applied compound, resulting in improved permeation across the stratum corneum, possibly through the intercellular route (Williams, 2003).

Figure 8: Box and whisker plot of average flux of IGF-1after 6 hours of diffusion.

3.2.1.2 KGF

Significantly decreased skin permeation (97 %) was observed when the adjusted Pheroid^{TM} concentration value (5.97 pg/cm^2) of KGF was compared to the KGF diffusion concentration value (153.31 pg/cm^2) in PBS.

Figure 9 illustrates that the unadjusted Pheroid™ value (203.50 pg/cm^2) had a 97 % higher diffusion concentration (pg/cm^2) for KGF compared to adjusted Pheroid™ as well as an increase of 25 % compared to that of PBS.

Pheroid™ as medium possibly result in decreased diffusion due to a decrease in the stability of KGF in Pheroid™.

Figure 9: Box and whisker plot of average flux of KGF after 6 hours of diffusion.

3.2.1.3 VEGF

The diffusion concentration of VEGF delivered by the Pheroid™ technology show an increase in skin permeation in comparison with VEGF applied in PBS. The adjusted Pheroid™ VEGF value (634.34 pg/cm^2) showed an average improved diffusion of approximately 31 % in comparison with VEGF in PBS (436.89 pg/cm^2).

In Figure 10 was observed that the unadjusted Pheroid™ value (790.40 pg/cm^2) had a 19 % higher diffusion concentration (pg/cm^2) for VEGF when compared to the adjusted Pheroid™ value, the adjusted Pheroid™ value also showed an increased diffusion of 45 % compared to that of PBS. Pheroid™ technology possibly results in improved diffusion due to the entrapment of the KGF and, therefore, increased the lipophilic nature of the applied compound resulting in improved permeation across the stratum corneum, possibly by the intercellular route (Williams, 2003).

Figure 10: Box and whisker plot of the average flux of VEGF over a 6 h diffusion period.

3.2.2 Tape stripping results

3.2.2.1 IGF-1

The average IGF-1 concentration found in the dermis, epidermis and receptor solution as obtained from the diffusion study after 6 hours, were illustrated in table 5.

Table 5: Average and median IGF-1 concentration in both the epidermis and dermis as well as the SD and % SD in PBS, Pheroid™ and the adjusted Pheroid™ values after 6 h of diffusion.

From figure 11 the tape stripping concentration value for IGF-1 in PBS appears to be higher than the adjusted IGF-1 in Pheroid™ value. It showed a 44 % decreased in the average tape stripping concentration for IGF-1 (1763.83 pg/ml) in comparison to that of IGF-1 (3096.36 pg/ml) in PBS. Additionally the average dermis concentration of the adjusted Pheroid™ delivery values showed a decrease of 44 % for IGF-1 (2515.96 pg/ml) in comparison with IGF-1 (4528.63 pg/ml) in PBS. Therefore, the concentration values for IGF-1 in both tape stripping and dermis in PBS were approximately 44 % higher than the adjusted Pheroid™ values.

The results suggest that the Pheroid™ delivery system improved the transdermal delivery of IGF-1 by decreasing the amount of IGF-1 remaining trapped within the skin during the diffusion process, by improving IGF-1's permeability through skin.

Figure 11: Concentration IGF-1 in PBS, Pheroid™ and the adjusted Pheroid™ values calculated in the epidermis and dermis after a 6 h diffusion.

These results together with the results obtained of diffusion concentration suggested that the Pheroid™ delivery system might have improved the transdermal delivery of IGF-1 by increasing the lipophylic nature of the compound by entrapment and, thereby increasing

penetration through the stratum corneum. It also improved the stability of IGF-1 at 25 °C in comparison to PBS by protecting it from the redox conditions in solution.

3.2.1.2 KGF

The average KGF concentration found in the dermis, epidermis and receptor solution as obtained from the diffusion study after 6 hours, were illustrated in table 6.

Table 6: Average and median KGF concentration in the dermis and epidermis as well as the standard deviation (SD) and percentage standard deviation (% SD) in PBS, Pheroid™ and the adjusted Pheroid™ values after 6 h of diffusion.

The concentration for the tape stripping of the KGF in PBS, as illustrated in Figure 12, appeared to be more than that of the KGF in the adjusted Pheroid™. The average tape stripping concentration for KGF in PBS (10.59 pg/ml) was approximately 97 % higher than that of KGF in the adjusted Pheroid™ values (0.30 pg/ml). The average dermis concentration of KGF in PBS (30.24 pg/ml) also showed a 97 % larger value than that of KGF in the adjusted Pheroid™ values (0.93 pg/ml).

The results acquired for tape stripping together with the diffusion concentration suggested that the Pheroid™ delivery system decreased penetration into skin as well as the transdermal delivery of KGF.

These results together with the results obtained of diffusion concentration suggested that the Pheroid™ delivery system might improve the transdermal delivery for KGF by increasing the lipophylic nature of the compound by entrapment thereby increasing penetration of the stratum corneum during the diffusion process. The Pheroid™ delivery system also showed a decrease in KGF stability at both 25 and 37 °C in comparison to KGF in PBS.

Figure 12: Concentration KGF in PBS, Pheroid™ and the adjusted Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

3.2.1.3 VEGF

The average VEGF concentration found in the dermis, epidermis and receptor solution as obtained from the diffusion study after 6 hours, were illustrated in table 7.

Table 7: Average and median VEGF concentration in both the epidermis and dermis as well as the SD and % SD in PBS, Pheroid™ and the adjusted Pheroid™ values after 6 h of diffusion.

The concentration for the tape stripping of the VEGF in PBS as was illustrated in Figure 13 appeared to be more than that of VEGF in the adjusted Pheroid™. The average tape stripping concentration for VEGF in PBS (30.60 pg/ml) was approximately 17 % higher than that of VEGF in the adjusted Pheroid™ values (25.05 pg/ml). The average dermis concentration of VEGF in PBS (55.83 pg/ml) was found to be 19 % higher than that of VEGF in the adjusted Pheroid™ (45.40 pg/ml).

The diffusion and tape stripping results suggested that the Pheroid™ delivery system increased the transdermal delivery of VEGF and decreased the amount of VEGF remaining trapped within the skin during the diffusion process.

It should be taken into consideration, however, that due to the higher lipophylic nature that the Pheroid™ delivery system exhibited in comparison to PBS, it would show improved penetration of the stratum corneum, therefore the amount of VEGF present in the dermis and epidermis should be equal to or larger than that of VEGF in PBS. The unadjusted Pheroid™ values may therefore be more accurate than the adjusted Pheroid™ values in the case of the dermis and tape stripping values. Therefore, the unadjusted values that show a 3 % lower

tape stripping value and the 1 % higher dermis values of the Pheroid™ drug delivery system in comparison to the PBS may be the most accurate for this study.

These results together with the results obtained of diffusion concentration suggested that the Pheroid™ delivery system may have improved the transdermal delivery of VEGF by increasing the lipophylic nature by entrapping the compound and thereby increasing penetration of the stratum corneum during the diffusion process. The Pheroid™ delivery system also showed a decrease in VEGF stability at 37 °C in comparison to the PBS.

Figure 13: Concentration VEGF in PBS, Pheroid™ and the adjusted Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

3.2.3 Difference in average and median

For the statistical analysis, both the median and average values were scrutinised. Median is calculated to be the centre of a data set and is not influenced by skewed distribution of the data. Average is influenced by skewed distribution of the data.

In the adjusted Pheroid™ delivery system, the median flux after 6 h for IGF-1, KGF and VEGF, was 22.11, 0.04 and 0.58 pg/cm^2 lower than the average flux, respectively.

In PBS, the median flux after 6 h for IGF-1, is 195.61 pg/cm^2 lower than that of the average flux, but for KGF and VEGF, the median flux after 6 h was 1.79 and 1.51 pg/cm^2 higher than that of the average flux, respectively.

The difference between average and median flux in the aforementioned growth factors was an indication that there was a slight skew distribution of the data. The median value was a more accurate representation of the true flux, however, because it is neither affected by skewed distribution of the data nor affected by a distortion in the spread of the data (Gerber et al., 2008), unlike the average value.

4. CONCLUSION

The Pheroid™ vesicles illustrated increased stability of IGF-1 at 25 °C, it however, did not show marked increase in stability of VEGF or KGF. It actually demonstrated a decrease in stability at both 25 and 37 °C for KGF. In the diffusion studies of IGF-1 and VEGF, the Pheroid™ vesicles produced an improvement in the diffusion and lowered the concentration IGF-1 and VEGF remaining trapped within the skin when compared to that of PBS. However, in the case of KGF, the Pheroid™ delivery system neither, decreased the amount remaining trapped in the skin during diffusion nor, increased the amount diffused during the *in vitro* studies.

This showed that even though the Pheroid™ delivery system had shown very promising results with a great variety of drugs and compounds, the miscibility of the drug and the Pheroid™ delivery system has to be ascertained in advance in order to ensure the correct delivery medium for the particular drug.

The following aspects need further investigation:

- The growth factors' route and mechanism of penetration.
- The Pheroid™ vesicles' route and mechanism of penetration.
- The extent to which the applied concentration affects the penetration of the growth factors should be determined, thereby ascertaining the true improvement in penetration of these growth factors in the Pheroid™ delivery system.
- Furthermore, the morphological changes of the Pheroid™ delivery system would improve the penetration and stability of KGF.

AKNOWLEDGEMENTS

The authors would like to express their heartfelt gratitude towards the National Research Foundation (NRF) of South Africa for their generous financial support as well as the Drug Research and Development Unit of the North-West University, Potchefstroom campus, South Africa. A special thank you to Dr Gerhard Koekemoer for the statistical analysis of the data and Silverani Padayachee for the preparation of the Pheroid™ vesicles.

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FIGURE LEGENDS:

Figure 1: Dilution series for IGF-1 from stock solution.

Figure 2: Dilution series for KGF from stock solution.

Figure 3: Dilution series for VEGF from stock solution.

Figure 4: Stability of IGF-1 in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

Figure 5: Stability of KGF in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

Figure 6: Stability of VEGF in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

Figure 7: Comparison between skin diffused concentration of IGF-1, KGF and VEGF average flux after 6 h.

Figure 8: Box and whisker plot of average concentration IGF-1 diffused after 6 h of diffusion.

Figure 9: Box and whisker plot of average concentration KGF diffused over 6 h of diffusion

Figure 10: Box and whisker plot of the average concentration VEGF diffused over a 6 h diffusion period.

Figure 11: Concentration of IGF-1 in PBS, Pheroid™ and the adjusted Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

Figure 12: Concentration of KGF in PBS, Pheroid™ and the adjusted Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

Figure 13: Concentration of VEGF in PBS, Pheroid™ and the adjusted Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

TABLES:

Table 1: T-test comparison of PBS and adjusted Pheroid™ p-values using the Levene test.

		Levene's Test for Equality of Variances (p-values)	T-test p-value
IGF	Equal variances assumed	0.150	0.018
KGF	Equal variances not assumed	0.000	0.000
VEGF	Equal variances assumed	0.355	0.000

Table 2: Comparison of the PBS with the adjusted Pheroid™ p-values using the Mann-Whitney non-parametric test.

	Mann-Whitney p-value
IGF	0.041
KGF	0.002
VEGF	0.002

Table 3: Comparison of individually applied growth factor concentration values in PBS and Pheroid™.

Applied concentrations(pg/cm^2)		
	PBS	Pheroid™
IGF-1	29295.98	53257.93
KGF	1283.98	1599.87
VEGF	105.96	3611.28

Table 4: Average and median concentration values after 6 h diffusion of IGF-1, KGF and VEGF together with the SD.

	PBS	Pheroid™	Adjusted Pheroid™
IGF-1			
Ave	13416.51	25764.55	14172.50
Median	13220.89	25724.35	14150.38
SD	282.04	484.52	266.52
KGF			
Ave	153.11	203.30	5.97
Median	155.11	202.11	5.93
SD	10.27	18.04	0.53
VEGF			
Ave	436.89	790.40	634.33
Median	438.40	789.67	633.75
SD	6.18	3.80	3.05

Table 5: Average and median IGF-1 concentration in both the epidermis and dermis as well as the SD and % SD in PBS, Pheroid™ and the adjusted Pheroid™ values after 6 h of diffusion.

IGF-1	PBS	Pheroid™	Adjusted Pheroid™
	Tape stripping concentration (pg/ml)		
SD	65.99	86.08	47.35
AVE	3096.36	3206.51	1763.83
% SD	2.13	2.69	2.69
Median	3092.74	3204.91	1762.95
	Dermis concentration (pg/ml)		
SD	245.47	104.36	57.40
AVE	4528.63	4573.82	2515.95
% SD	5.42	2.28	2.28
Median	4508.47	4562.91	2509.95

Table 6: Average and median KGF concentration in both the epidermis and dermis as well as the SD and % SD in PBS, Pheroid™ and the adjusted Pheroid™ values after 6 h of diffusion.

KGF	PBS	Pheroid™	Adjusted Pheroid™
	Tape stripping concentration (pg/ml)		
SD	0.25	0.63	0.02
AVE	10.59	10.26	0.30
% SD	2.34	6.10	6.10
Median	10.52	10.52	0.31
	Dermis concentration (pg/ml)		
SD	3.34	4.39	0.13
AVE	30.24	31.83	0.93
% SD	11.05	13.80	13.80
Median	29.57	32.47	0.95

Table 7: Average and median VEGF concentration in both the epidermis and dermis as well as the SD and % SD in PBS, Pheroid™ and the adjusted Pheroid™ values after 6 h of diffusion.

VEGF	PBS	Pheroid™	Adjusted Pheroid™
	Tape stripping concentration (pg/ml)		
SD	1.54	0.92	0.74
AVE	30.60	31.21	25.05
% SD	5.02	2.96	2.96
Median	30.14	31.53	25.31
	Dermis concentration (pg/ml)		
SD	1.96	1.91	1.53
AVE	55.83	56.57	45.40
% SD	3.52	3.38	3.38
Median	55.21	56.60	45.43

FIGURES:

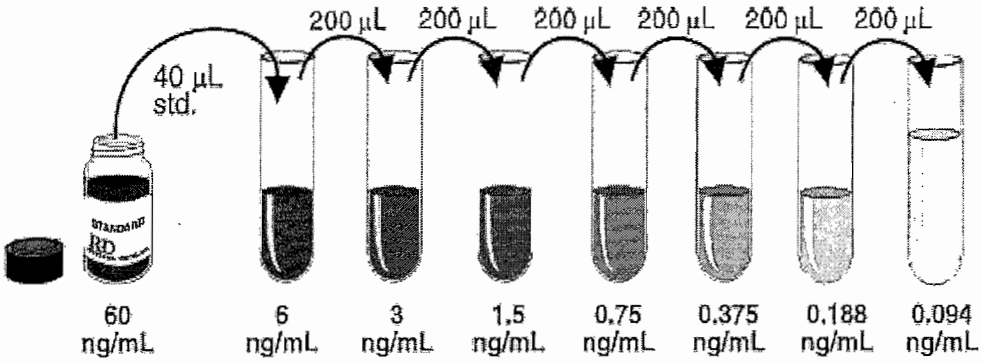

Figure 1: Dilution series for IGF-1 from stock solution.

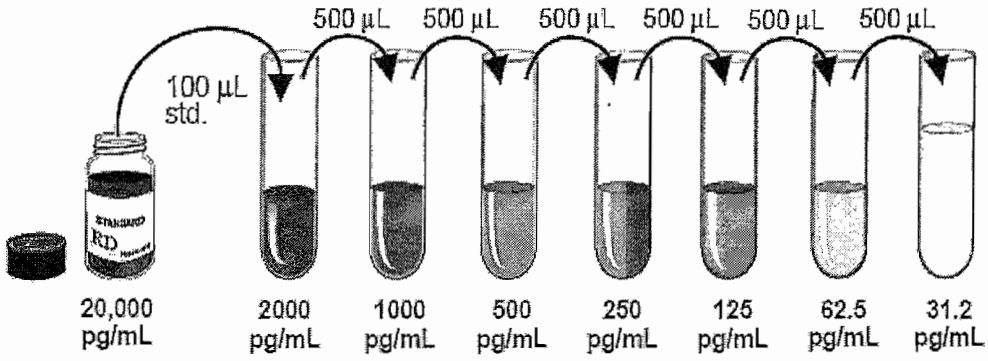

Figure 2: Dilution series for KGF from stock solution.

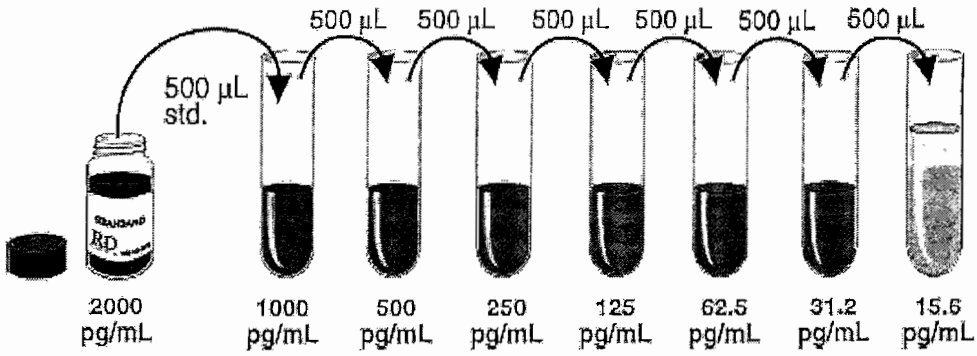

Figure 3: Dilution series for VEGF from stock solution.

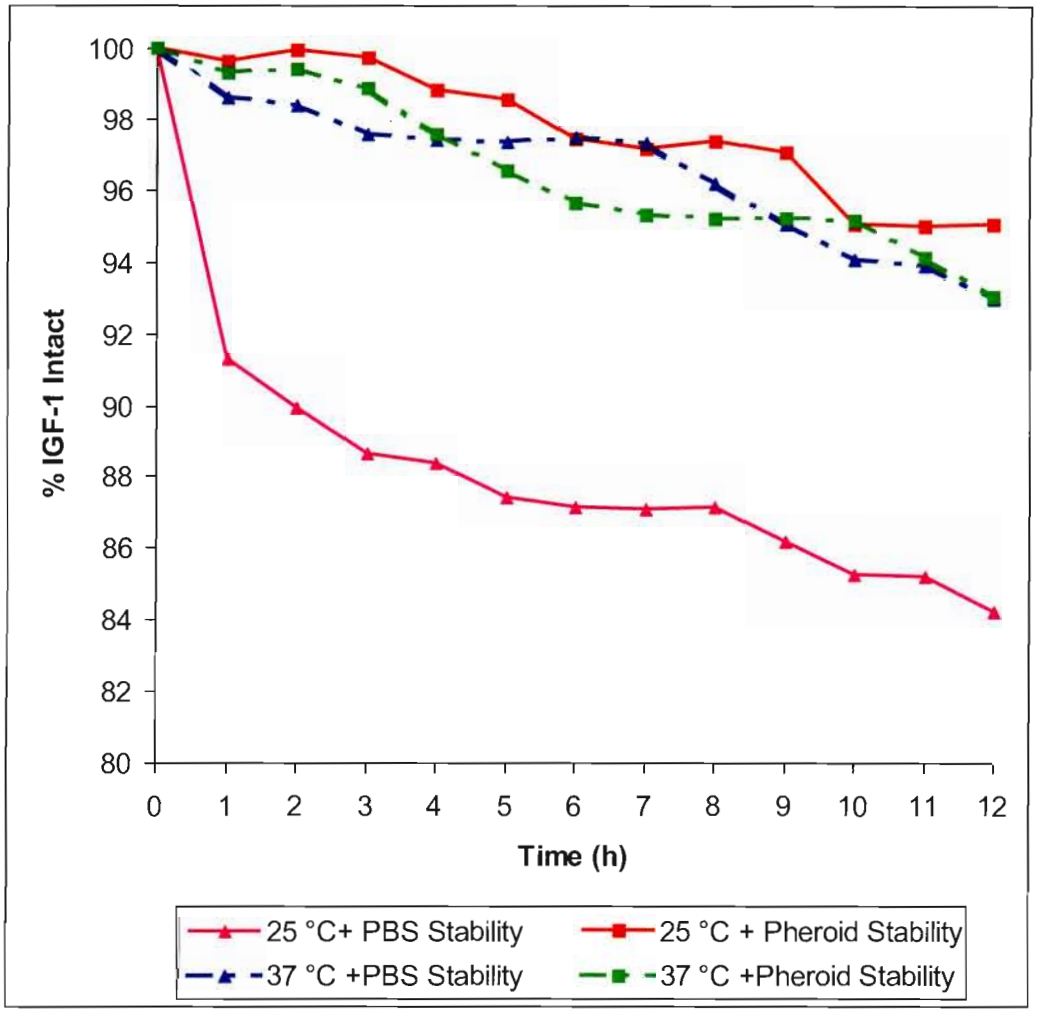

Figure 4: Stability of IGF-1 in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

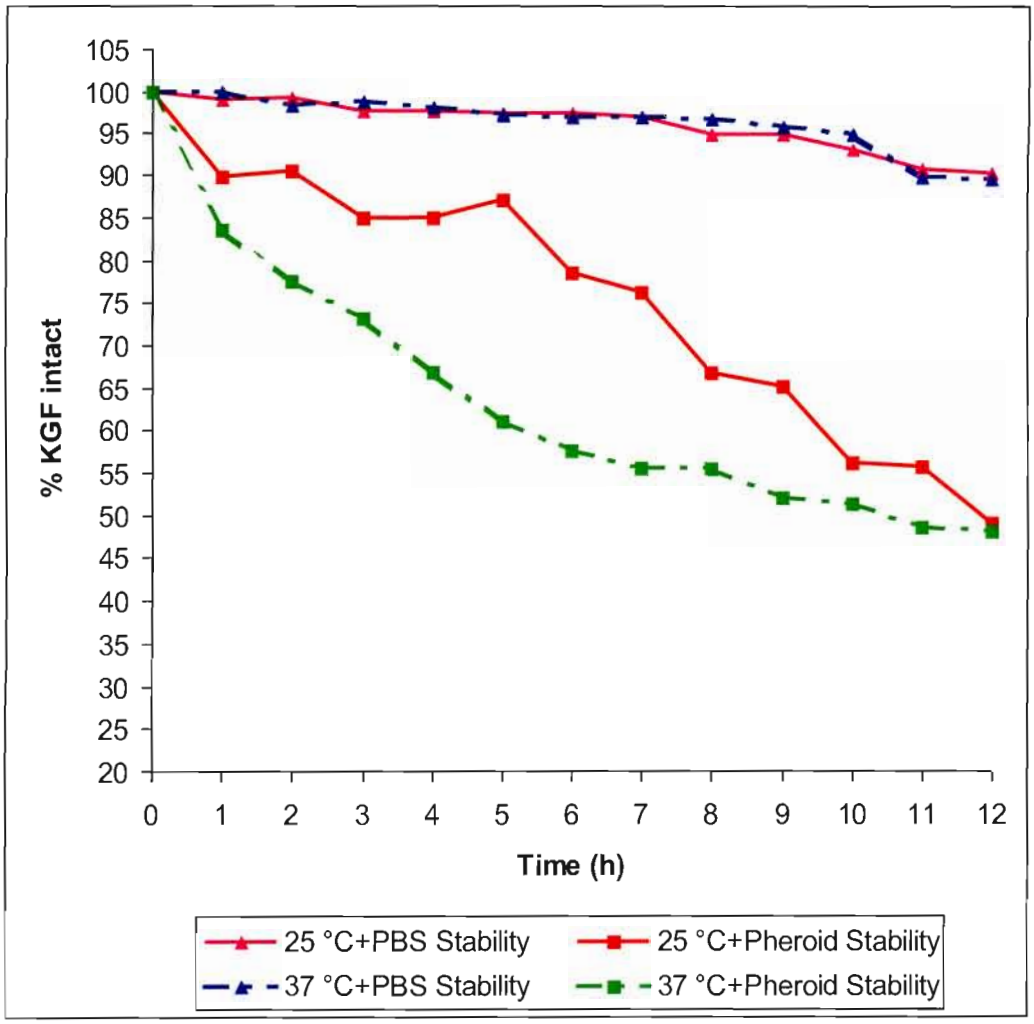

Figure 5: Stability of KGF in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

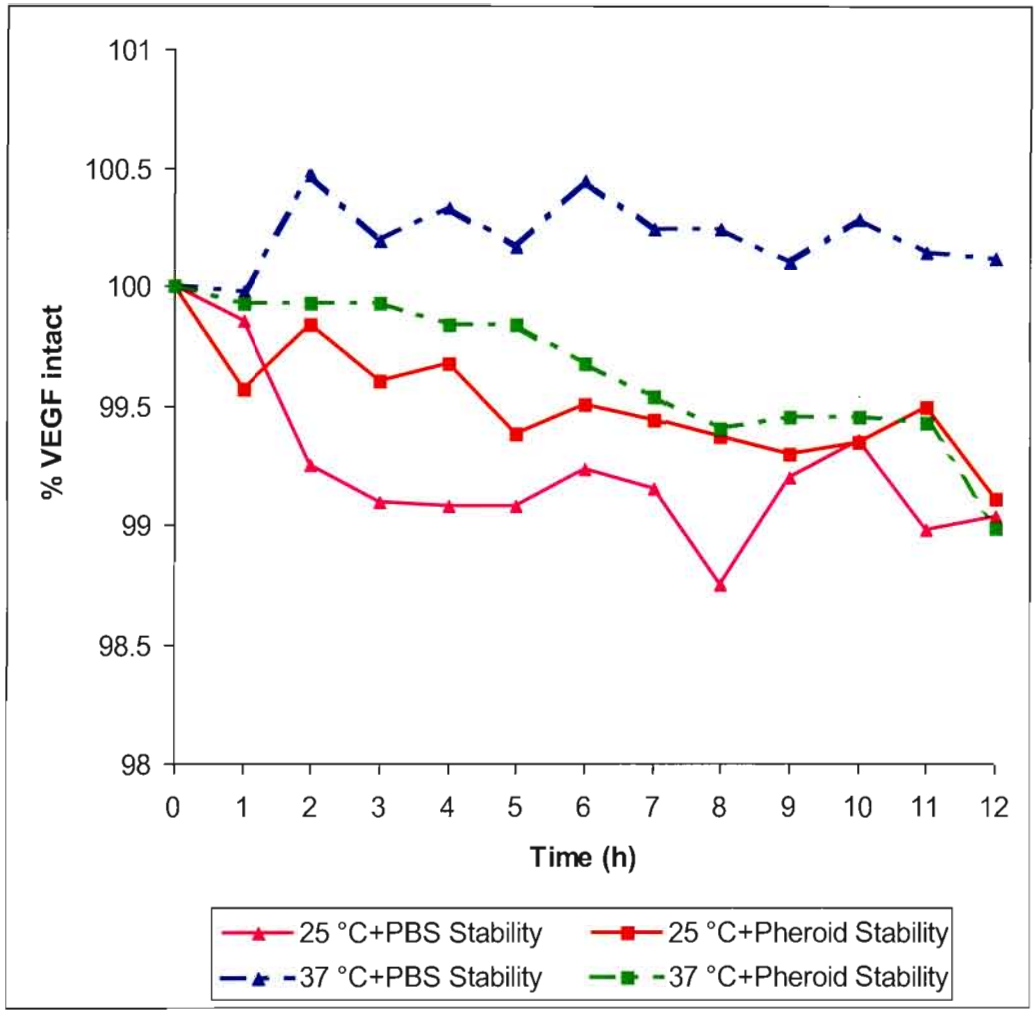

Figure 6: Stability of VEGF in both PBS and Pheroid™ during 12 h stability testing at 25 and 37 °C.

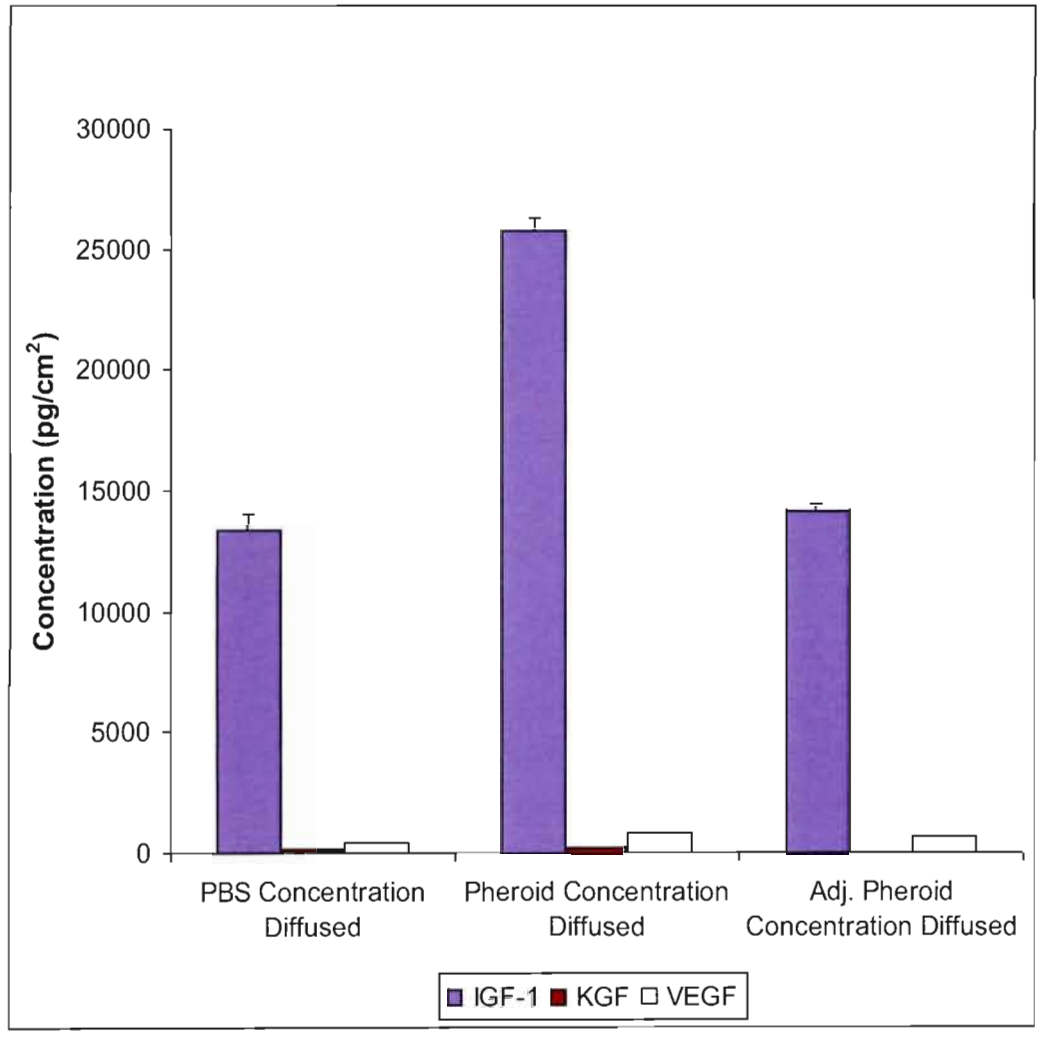

Figure 7: Comparison between skin diffused concentration of IGF-1, KGF and VEGF expressed as the average flux after 6 h.

Adjusted (Adj.)

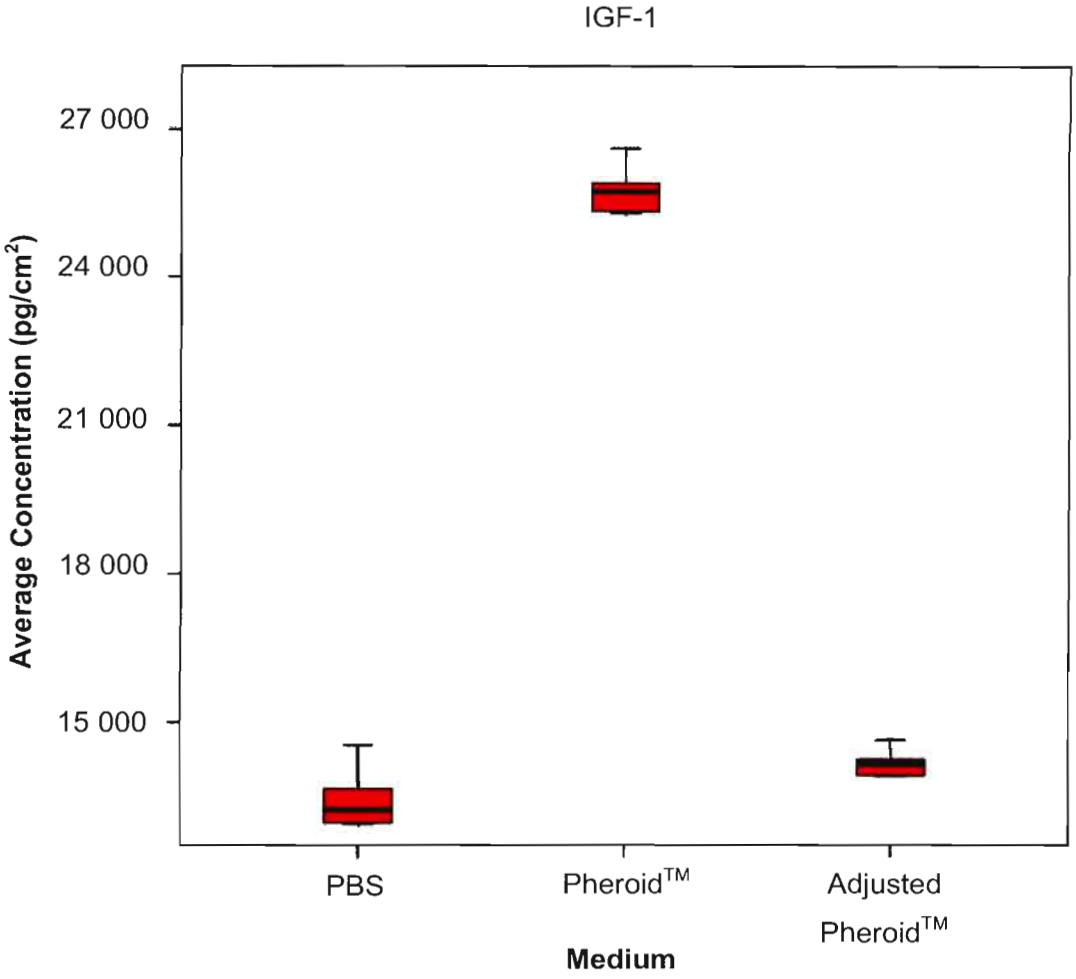

Figure 8: Box and whisker plot of average concentration IGF-1 diffused after 6 h of diffusion.

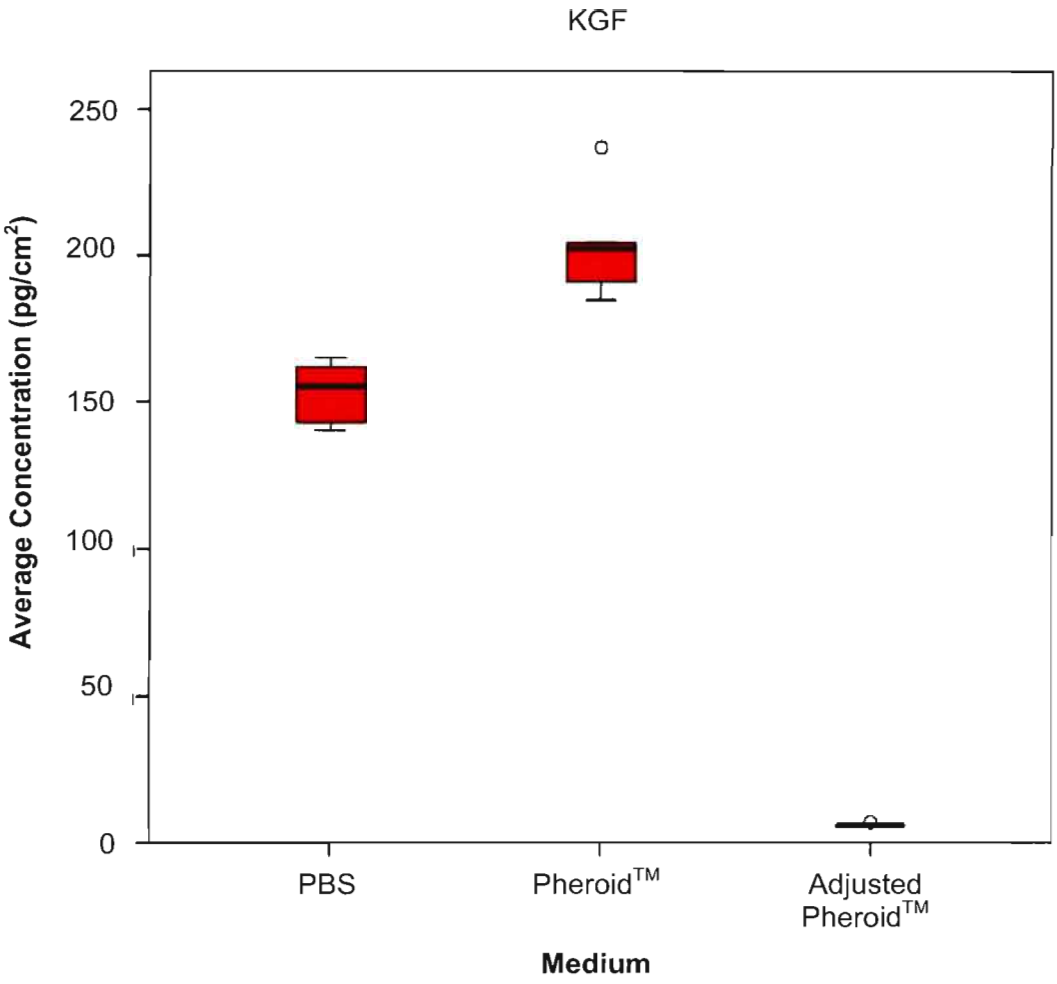

Figure 9: Box and whisker plot of average concentration KGF diffused over 6 h of diffusion.

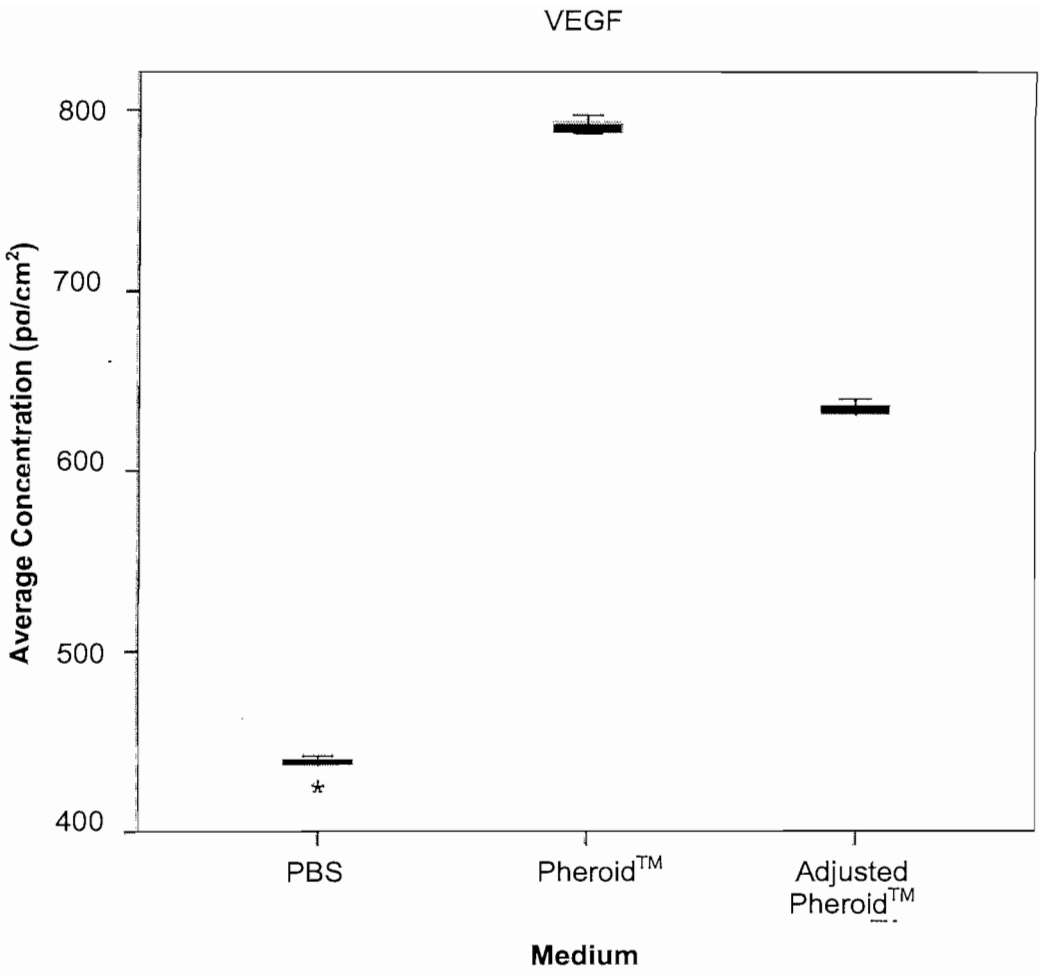

Figure 10: Box and whisker plot of the average concentration VEGF diffused over a 6 h diffusion period.

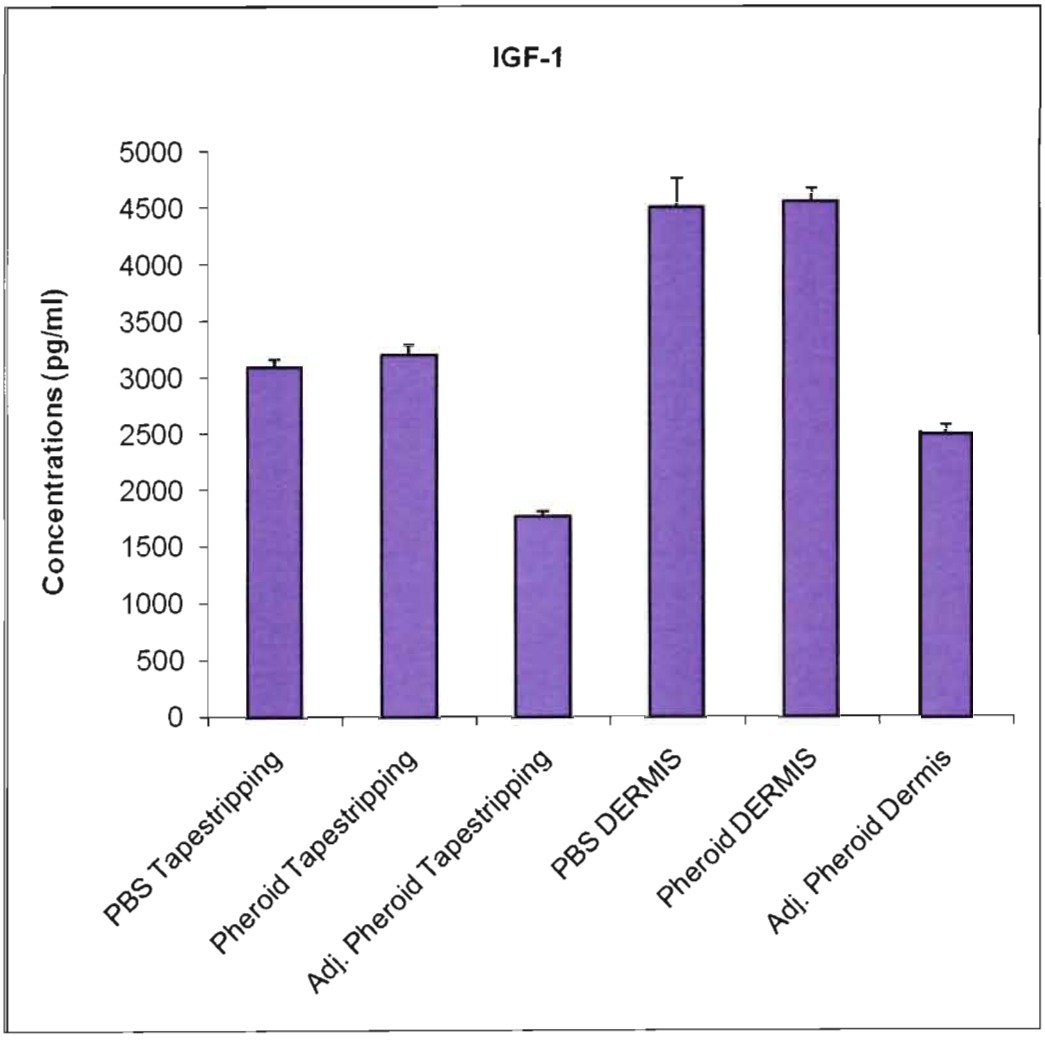

Figure 11: Concentration of IGF-1 in PBS, Pheroid™ and the adjusted (Adj.) Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

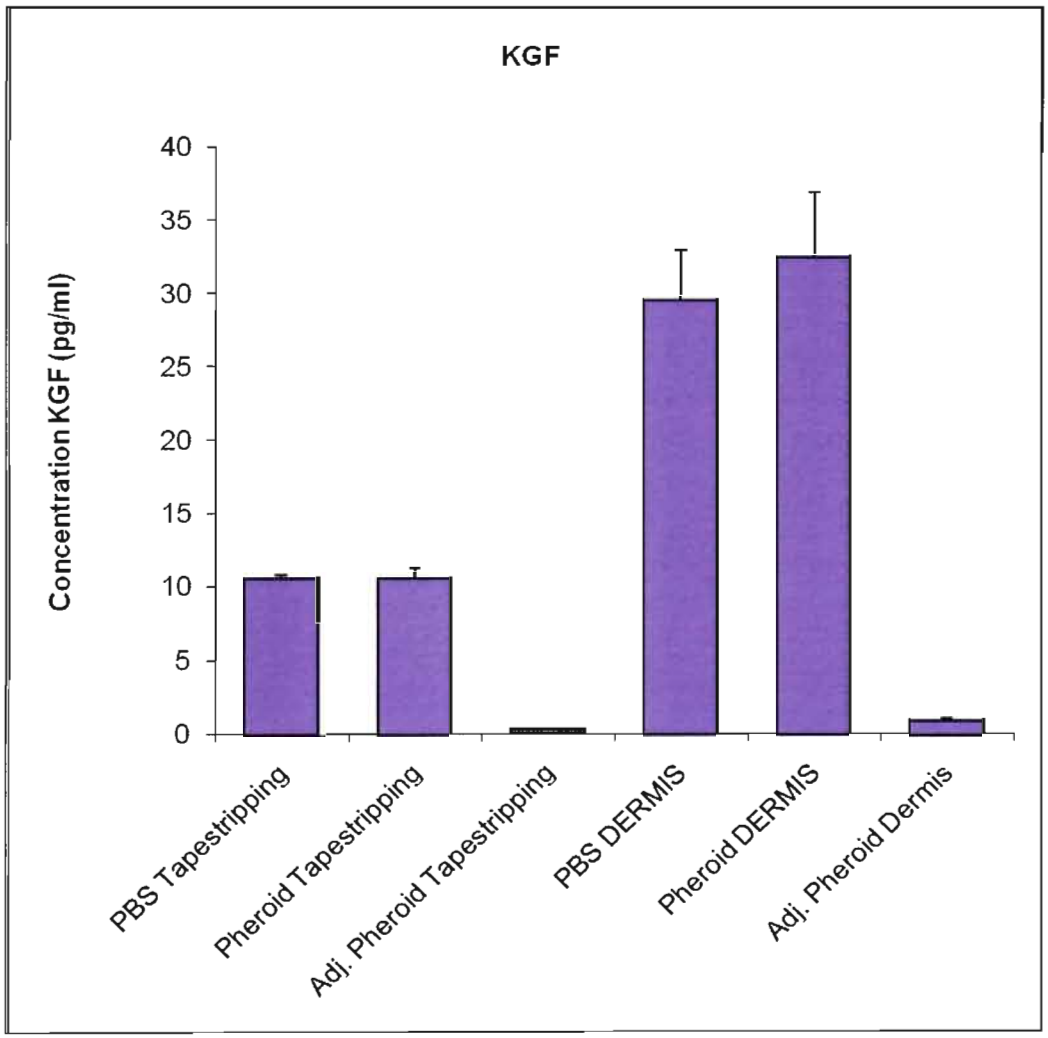

Figure 12: Concentration of KGF in PBS, Pheroid™ and the adjusted (Adj.) Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

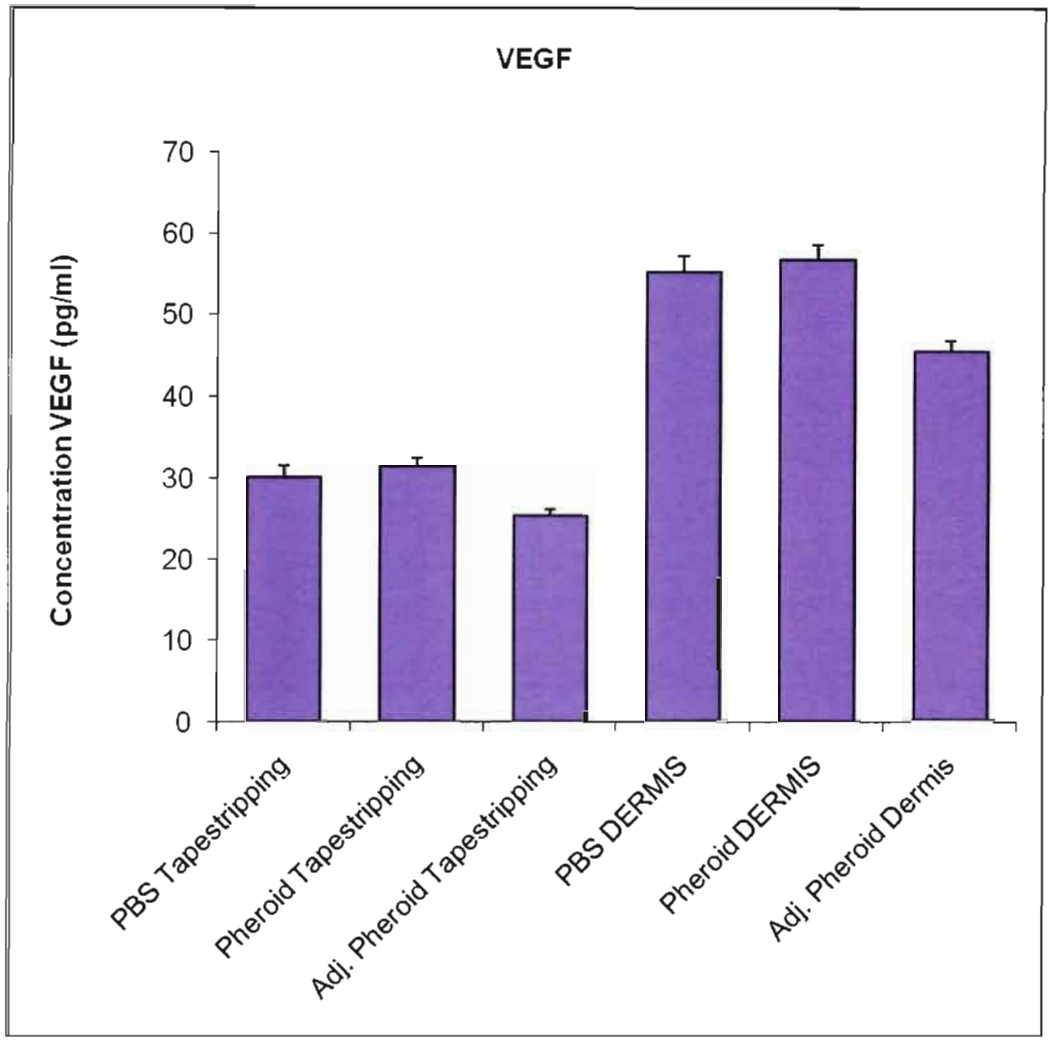

Figure 13: Concentration of VEGF-1 in PBS, Pheroid™ and the adjusted (Adj.) Pheroid™ values calculated in the epidermis and dermis after 6 h of diffusion.

CHAPTER 4: FINAL CONCLUSIONS AND FUTURE PROSPECTS

4.1. INTRODUCTION

The aim of the study was to investigate the possible *in vitro* topical diffusion and delivery of the growth factors IGF-1, KGF and VEGF by means of the novel Pheroid™ delivery system. This delivery system was employed in order to improve topical delivery of growth factors. The physicochemical properties of these growth factors are not ideal for topical delivery.

Bestatin, a potent and selective aminopeptidase inhibitor was added to the topical solutions with the selected growth factor in order to prevent its degradation on the surface skin (Petrov *et al.*, 2000:538; Umezawa *et al.*, 1975:99). This was successfully utilised in the protection of other peptides in a previous transdermal delivery study (Coetzee, 2007:97).

The growth factors were chosen with future prospects of employing them in the treatment of alopecia. IGF-1, KGF and VEGF all have stimulating effects on hair growth and the production of strong hair shafts (Caregen, 2002:6 - 15).

The Pheroid™ delivery system is a novel micro-emulsion-like formulation that has shown great promise in previous studies. It depicted the ability to improve poor diffusivity of drugs or compounds, including peptides and peptide hormones (Steyn, 2006:94). The Pheroid™ delivery system was, therefore, chosen to improve of poor diffusivity of peptides in particular (Coetzee, 2007:97).

The above-mentioned constituents were combined in diffusion studies conducted over a 6 h period in vertical Franz diffusion cells at 32 °C. Due to the general degradation of the growth factors at high temperatures, the diffusion studies were limited to 6 h (32 °C) in order to ensure maximum stability. Female abdominal skin, with PBS as buffer, was employed in the receptor of the Franz cell. Sample analyses were done with ELISA immuno-assays for each of the selected growth factors.

4.2. OBSERVATIONS AND CONCLUSIONS

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

- The Pheroid™ delivery system, when compared to PBS, showed an 11 % improvement in IGF-1 stability after 12 hours at 25 °C. This may be due to the Pheroid™ delivery system serving as protection from the redox conditions that lead to oxidation of the disulfide bonds within the IGF-1 molecule. The Pheroid™ delivery system at 37 °C, however, showed similar stability to that of IGF-1 in PBS at 37 °C, with only a 0.1 % improvement in its stability. The higher temperature accelerates degradation and unfolding of IGF-1. It may also influence the fluidity of the Pheroid™ delivery system, causing greater exposure to redox conditions.
- For VEGF, the Pheroid™ delivery system showed no marked improvement in stability at 25 or 37 °C for the first 12 hours. However, VEGF in PBS at 37 °C showed the best stability after 12 hours in comparison to the other growth factors. This may be due to poorer stability and compatibility with the Pheroid™ formulation when compared to the other growth factors in this study. If the study were performed over a longer period, a larger difference in stability between the Pheroid™ delivery system and PBS might have been more noticeable.
- The Pheroid™ delivery system depicted a decrease in KGF stability at both 25 and 37 °C. Only 49 % of intact KGF was found in the Pheroid™ delivery system at 25 °C in comparison with approximately 90 % intact KGF in PBS after 12 hours. This may need further investigation, since the cause of the decreased stability is unclear, as the Pheroid™ delivery system showed improved stability in the other growth factors in this study.
- The adjusted Pheroid™ values, when compared to PBS, showed improved diffusion for IGF-1 and VEGF after 6 hours. For IGF-1, at 32 °C, Pheroid™ showed a 6 % improvement in permeation compared to that in PBS. In VEGF, it showed a 31 % diffusion improvement compared to delivery in PBS. It also showed decreased concentrations of IGF-1 and VEGF remaining trapped within the dermis and epidermis. The Pheroid™ delivery system might have improved IGF-1 and VEGF penetration by means of protein-mediated transport.
- The adjusted Pheroid™ values for IGF-1 showed a decrease in the average tape stripping (43 %) and average dermis concentrations (44 %) when compared to PBS. Additionally, for VEGF, the adjusted Pheroid™ values showed a decrease in the average tape stripping (17 %) and dermis concentrations (19 %) when compared to PBS. This was due to the unique ability of the Pheroid™ drug delivery system to

improve penetration and permeation of compounds, therefore, crossing the stratum corneum with higher efficiency than when delivered in PBS alone.

- The Pheroid™ delivery system showed a 97 % decrease of KGF in the diffusion and concentration trapped in the dermis and epidermis compared to KGF in PBS. This may need further investigation, since the Pheroid™ delivery system has improved the diffusivity of VEGF and IGF-1. These growth factors are both large molecules. In particular VEGF is almost double the size of KGF. In transdermal delivery, this is a limiting factor, yet it still showed improved diffusion. This may be due to poor suitability of the Pheroid™ drug delivery system to entrap and deliver KGF, due to its negative effect on its stability.
- These results suggested that the Pheroid™ delivery system may be superior in the delivery of all of the chosen growth factors, with improved stability for IGF-1 and VEGF and a decrease in stability for KGF in comparison to PBS.

  Overall, the Pheroid™ delivery system proved superior over the PBS solution for the topical delivery and improved stability in the cases of IGF-1 and VEGF. The use thereof, in the delivery of IGF-1 and VEGF as alopecia treatment in the future, is therefore a definite possibility. For KGF, however, the adjusted values for the Pheroid™ delivery system showed that it did not provide the desired topical delivery and stability improvement, and the PBS proved to be a better delivery medium for KGF in this case.

4.3. FUTURE PROSPECTS

The following aspects were identified that might necessitate further investigation:

- The delivery pathway of IGF-1, KGF and VEGF through the skin should be determined by means of confocal laser scanning microscopy.
- The cause of decreased KGF stability in the Pheroid™ delivery system should be established in order to optimise topical delivery.
- The extent to which the applied concentration effected the topical delivery rate of KGF, with and without the aid of the Pheroid™ delivery system, should be determined.
- The effect manipulation of the morphology of the Pheroid™ delivery system has on KGF stability and transdermal delivery. For example, incorporation of the growth factor into bilayer membrane Pheroid™-vesicles, or into pro-Pheroid™'s.

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

ELSEVIER

EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

Official Journal of the European Federation for Pharmaceutical Sciences (EUFEPS)

Guide for Authors

1.1. Manuscripts

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Manuscripts submitted to the journal are accepted on the understanding that: (1) they are subject to editorial review, (2) they have not been and will not be published in whole or in part in any other journal and (3) the recommendations of the Declarations of Helsinki and Tokyo, for humans, and the European Community guidelines as accepted principles for the use of experimental animals, have been adhered to. *The European Journal of Pharmaceutical Sciences* will, therefore, only consider manuscripts that describe experiments which have been carried out under approval of an institutional or local ethics committee. Only manuscripts written in English should be submitted.

1.2. Format

Manuscripts should be neatly typed, double-spaced throughout, including tables, with at least 2.5 cm margins on all sides. Use one font type and size throughout the manuscript. Author(s)

should not break or hyphenate words. The manuscript should be submitted with a cover letter containing the declaration that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and that the protocol complies with the particular recommendation and that approval of their protocols was obtained.

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

1.3. Electronic manuscripts

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

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

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1.4. *Abbreviations*

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

Some abbreviations may be used without definition:

ADP,CDP, GDP, IDP	5'-pyrophosphates of adenosine
UDP	cytidine, guanosine, inosine, uridine
AMP etc.	adenosine 5'-monophosphate etc.
ADP etc.	adenosine 5'-diphosphate etc.
ATP etc.	adenosine 5'-triphosphate etc.
CM-cellulose	carboxymethylcellulose
CoA and acetyl-CoA	coenzyme A and its acyl derivatives
DEAE-cellulose	*O*-(diethylaminoethyl)-cellulose
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid

FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione, reduced and oxidized
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
Pi, PPi	orthophosphate, pyrophosphate
RNA	ribonucleic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

Two alternative conventions are currently in use in some cases. For example, for the phosphoinositides there are both the abbreviations recommended by the IUPAC-IUB and those of the Chilton Convention (e.g., PtdIns(4,5)P_2 vs. $PIP_2$for phosphatidylinositol 4,5-biphosphate). The journal will accept either of these forms but not their combination.

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

Units of mass

kilogram	kg
gram	g
milligram	mg
microgram	µg
nanogram	ng
mole (gram-molecule)	mol
millimole	mmol

micromole	µmol
nanomole	nmol
picomole	pmol
femtomole	fmol
equivalent	eq

Units of time

hour	h
minute	min
second	s
millisecond	ms
microsecond	µs

Units of volume

litre	l
millilitre	ml
microlitre	µl

Units of length

metre	m
centimetre	cm
millimetre	mm
micrometre	µm

nanometre nm

Units of concentration

molar (mol/l) M

millimolar mM

micromolar µM

nanomolar nM

picomolar pM

Units of heat, energy, electricity

joule J

degree Celsius (centigrade) °C

coulomb C

ampere A

volt V

ohm Ω

siemens S

Units of radiation

curie Ci

counts per minute cpm

disintegrations per minute dpm

becquerel Bq

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

The isotope mass number should appear before the atomic symbol, e.g., [^{3}H]noradrenaline, [^{14}C]choline. Ions should be written: Fe^{3+}, Ca^{2+}, Mg^{2+}. The term absorbance (A) is preferred to extinction or optical density. For abbreviations not included in this list consult: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Authors and Editors*, 1994 (The Royal Society of Medicine, London), ISBN 0-905958-78-0, or *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 6th edn. (Cambridge University Press, Cambridge), ISBN 0-521-47154-0.

1.5. Nomenclature

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

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

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

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

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

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

1.6. Editorial review

All manuscripts are generally submitted to 2-3 referees who are chosen for their ability to evaluate the work. Supplementary material may be included to facilitate the review process. Authors may request that certain referees should not be chosen. Members of the editorial board will usually be called upon for advice when there is disagreement among the referees or between referees and authors, or when the editors believe that the manuscript has not received adequate consideration by the referees.

All referees' comments must be responded to, and suggested changes be made. The author should detail the changes made in response to the referees' comments and suggestions in an accompanying letter. If the author disagrees with some changes, the reason, supported

by data, should be given. The editors may refuse to publish manuscripts from authors who persistently ignore referees' comments. Handwritten additions or corrections will not be accepted. Only complete retyping of the pages affected by revision is acceptable. A revised manuscript should be received by the editorial office no later than 4 months after the editorial decision was sent to the author(s); otherwise it will be processed as a new manuscript.

2. Organization and style of manuscripts

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

2.1. Research articles

2.1.1. General

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

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

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

2.1.2. Abstract and keywords

The abstract with keywords should be typed on a separate sheet. The abstract should

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

2.1.3. Introduction, Materials and Methods, Results, Discussion

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

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

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

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

2.2. References

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

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

year indicating the alphabetical order of the second or third author, etc.

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

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

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

Journals:

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

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

Books:

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

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

Unpublished observations, personal communications and manuscripts in preparation or submitted for publication may be referred to in the text but should not appear in the list of references. Manuscripts in press (i.e., accepted for publication) may be included in the references citing the DOI article identifier, which enables the citation of a paper before

volume, issue and page numbers are allocated. The name of the journal in which they are to appear must be given.

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

2.3. Illustrations

The number of illustrations should be limited to the essential.

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

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

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

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

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

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

(g) If, together with your accepted article, you submit usable colour figures then Elsevier will

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2.4. Tables

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

(a) Each table should have a brief explanatory heading and sufficient experimental detail (following the table body as a footnote) so as to be intelligible without reference to the text.

(b) Tables should not duplicate material in text or illustrations.

(c) Short or abbreviated column headings should be used and, if necessary, explained in footnotes, and indicated as [a], [b], [c], etc.

(d) Statistical measures of variation, S.D., S.E., etc. should be identified.

(e) Tables should be numbered separately in Arabic numerals (Table 1, 2, etc.).

2.5. Formulas and equations

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

2.6. GenBank accession numbers

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

For each and every accession number cited in an article, authors should type the accession number in **bold, underlined text.** Letters in the accession number should always be capitalised. (See Example 1 below.) This combination of letters and format will enable Elsevier's typesetters to recognize the relevant texts as accession numbers and add the required link to GenBank's sequences.

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

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

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

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

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2.8. Review articles

One page suggestions for comprehensive reviews should be sent to the Editor-in-Chief at ejps-journal@helsinki.fi for consideration.

The manuscript of a review article should be arranged as described for research articles (see sections 2.1 - 2.5) but according to the following sections: title page, abstract and keywords (indexing terms, normally 3-6 items), Introduction, Specific sections determined by the author, Conclusions, Acknowledgements, References, Figure legends and Figures, Tables. Sections ranging from the Introduction to the Conclusions should be numbered. Subdivisions within a section should also be numbered within that section: 2.1., 2.2., 2.3. etc. All pages should be numbered consecutively, the title page being p.1.

2.9. Commentaries and Mini-reviews

One page suggestions for commentaries and mini-reviews should be sent directly to the Editor-in-Chief at ejps-journal@helsinki.fi for consideration. Please see detailed information on commentaries and mini-reviews below.

2.9.1 Commentaries (Guidance)

The definition of a Commentary for EJPS is three-fold. Firstly, it can be an argued piece of provocative scientific writing purporting to take a balanced position on a controversial pharmaceutical science topic. A second option is for the author to approach the topic from a

particular viewpoint on one side of an argument. A third option is to provide a topical update on a hot topic in Pharmaceutical Sciences and this can be more informative than controversial.

Commentaries will be commissioned by the editors in advance or invited from non-commissioned authors if they wish to initially submit a one page summary of the intended Commentary to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The journal is looking for a stimulating and provoking essay, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to prefereably recent peer-reviewed material. The word count should be approximately 2,000 words maximum.
The commentary should have a short abstract summary of 150 to 200 words and 4-5 key words should be included. The text should be broken down into 4-5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes

2.9.2 Mini-review (Guidance)

Mini-reviews are thought provoking reviews of contemporary pharmaceutical research. Themes are as described in the Scope of the Journal section.

Mini-reviews will usually be commissioned by the editors in advance, but contributions are invited from non-commissioned authors if they wish to initially submit a one page summary of the intended review to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The structure of the mini-review is as follows: a title page followed by a 200-300 word abstract with 4-5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-reviewed material. There is a combined maximum of 5 figures / tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

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APPENDIX B: LIST OF USED ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
APB	Aminopeptidase B
Da	Dalton
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
ER	Enhancement ratio
EtOH	Ethanol
FAB	Fatty acid membrane-binding proteins
FGF	Fibroblast growth factor
GH	Growth hormone
HPLC	High-performance liquid chromatography
IGF	Insulin-like growth factor
IGF-1	Insulin-like growth factor 1
IGF-BP	Insulin-like growth factor binding proteins
KGF	Keratinocyte growth factor
LAP	Leucine aminopeptidase
MAP	Metallo-aminopeptidases
N_2O	Nitrous oxide
NMR	Nuclear magnetic resonance
NRF	National research foundation

PBS	Phosphate buffered solution
PGLA	Poly(lactide-*co*-glycolide)
SD	Standard deviation
TEWL	Transdermal water loss, is the outward diffusion of water through skin
TS	Tape stripping
VEGF	Vascular endothelial growth factor

APPENDIX C: PHOTOS OF THE APPARATUS USED DURING DIFFUSION STUDIES

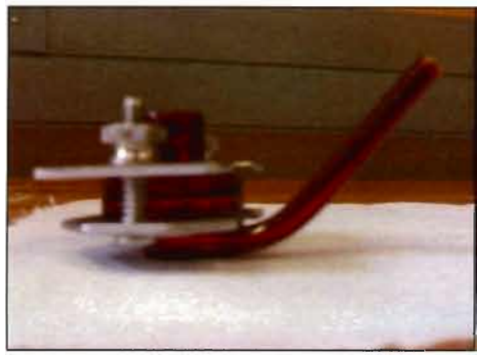

Photo C.1: Amber Franz diffusion cell, with the donor and receptor compartments clamped together with a metal clamp.

Photo C.2: Grant water bath.

Photo C.3: Vaariomag® magnetic stirring plate

Photo C.4: Milli-Q water purifying system.