Formulation, in vitro release and transdermal diffusion of Vitamin A and Zinc for the treatment of acne

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This dissertation is presented in the so-called article format, which includes an introductory chapter with sub-chapters, a full length article for publication in a pharmaceutical journal and appendixes containing experimental results and discussion. The article in this dissertation is to be submitted for publication in Skin Pharmacology and Physiology, of which the complete guide for authors is included in Appendix E.

ABSTRACT

Acne vulgaris is the single, most common disease that presents a significant challenge to dermatologists, due to its complexity, prevalence and range of clinical expressions. This condition can be found in 85% of teenage boys and 80% of girls (Gollnick, 2003:1580). Acne can cause serious psychological consequences (low self-esteem, social inhibition, depression, etc.), if left untreated, and should therefore be recognised as a serious disorder (Webster, 2001:15). The pathogenesis of acne is varied, with factors that include plugging of the follicle, accumulation of sebum, growth of *Propionibacterium acnes* (*P. acnes*), and inflammatory tissue responses (Wyatt *et al.*, 2001:1809).

Acne treatment focuses on the reduction of inflammatory and non-inflammatory acne lesions, and thus halts the scarring process (Railan & Alster, 2008:285). Non-inflammatory acne lesions can be expressed as open and closed comedones, whereas inflammatory lesions comprise of papules, pustules, nodules and cysts (Gollnick, 2003:1581). Acne treatment may be topical, or oral. Topical treatment is the most suitable first-line therapy for non-inflammatory comedones, or mildly inflammatory disease states, with the advantage of avoiding the possible systemic effects of oral medications (Federman & Kirsner, 2000:80).

Topical retinoids were very successfully used for the treatment of acne in the 1980s. Their effectiveness in long-term therapies was limited though, due to local skin irritations that occurred in some individuals (Julie & Harper, 2004:S36). Vitamin A acetate presented a new approach in the treatment of acne, showing less side effects (Cheng & Depetris, 1998:7).

In this study, vitamin A acetate and zinc acetate were formulated into semisolid, combination formulations for the possible treatment of acne. Whilst vitamin A controls the development of microcomedones, reduces existing comedones, diminishes sebum production and moderately reduces inflammation (Verschoore *et al.*, 1993:107), zinc normalises hormone imbalances (Nutritional-supplements-health-guide.com, 2005:2) and normalises the secretion of sebum (Hostýnek & Maibach, 2002:35).

Although the skin presents many advantages to the delivery of drugs, it unfortunately has some limitations. The biggest challenge in the transdermal delivery of drugs is to overcome the natural skin barrier. Its physicochemical properties are a good indication(s) of the transdermal behaviour of a drug. The ideal drug to be used in transdermal delivery would have sufficient lipophilic properties to partition into the stratum corneum, but it would also have sufficient hydrophilic properties to partition into the underlying layers of the skin (Kalia & Guy, 2001:159).

Pheroid™ technology was also implemented during this study, in order to establish whether it would enhance penetration of the active ingredients across the skin. The Pheroid™ consists of vesicular structures that contain no phospholipids, nor cholesterol, but consists of the same essential fatty acids that are present in humans (Grobler *et al*., 2008:283).

The aim of this study hence was to investigate the transdermal delivery of vitamin A acetate and zinc acetate, jointly formulated into four topical formulations for acne treatment. Vitamin A acetate (0.5%) and zinc acetate (1.2%) were formulated into a cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. An existing commercial product, containing vitamin A acetate, was used to compare the results of the formulated products with. The transdermal, epidermal and dermal diffusion of the formulations were determined during a 6 h diffusion study, using Franz diffusion cells and tape stripping techniques.

Experimental determination of the diffusion studies proved that vitamin A acetate did not penetrate through the skin. These results applied to both the formulations being developed during this study, as well as to the commercial product.

Tape stripping studies were done to determine the concentration of drug present in the epidermis and dermis. The highest epidermal concentration of vitamin A acetate was obtained with the Pheroid™ emulgel (0.0045 µg/ml), whilst the emulgel formulation provided the highest vitamin A acetate concentration in the dermis (0.0029 µg/ml). Contrary, for the commercial product, the total concentration of vitamin A acetate in the epidermis was noticeably lower than for all the new formulations studied. Vitamin A acetate concentrations of the commercial product in the dermis were within the same concentration range as the newly developed formulations, with the exception of the emulgel that delivered approximately 31% more vitamin A acetate to the dermis, than the commercial product.

Zinc acetate was able to diffuse through full thickness skin, although no flux values were obtained. To eliminate the possibility of endogenous zinc diffusion, placebo formulations (without zinc) were prepared for use as control samples during the skin diffusion investigation. The emulgel and Pheroid™ emulgel formulations were unable to deliver significant zinc acetate concentrations transdermally, although transdermal diffusion was attained from both the cream and Pheroid™ cream. Tape stripping experiments with placebo formulations relative to the formulated products revealed that zinc acetate concentrations in the epidermis and dermis were significantly higher when the placebo formulations were applied. However, the average zinc acetate concentration in the dermis, after application of the cream formulation, was significantly higher, compared to when the placebo cream was applied. It could therefore be concluded that no zinc acetate had diffused into the epidermis and dermis from the new formulations, except from the cream formulation. The zinc acetate concentration being measured in the epidermis

thus rather represented the endogenous zinc acetate. The cream formulation, however, was probably able to deliver detectable zinc acetate concentrations to the epidermis.

Stability of the formulated products was tested under a variety of environmental conditions to determine whether the functional qualities would remain within acceptable limits over a certain period of time. The formulated products were tested for a period of three months under storage conditions of 25°C/60% RH (relative humidity), 30°C/60% RH and 40°C/75% RH. Stability studies included stability indicating assay testing, the determination of rheology, pH, droplet size, zeta-potential, mass loss, morphology of the particles and physical assessment.

The formulations were unstable over the three months stability test period. A change in viscosity, colour and concentration of the active ingredients were observed.

Keywords: Vitamin A, zinc, acne, transdermal diffusion, formulation, Pheroid™.

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UITTREKSEL

Aknee vulgaris is een van die mees algemene siektetoestande, wat weens die kompleksiteit, voorkoms en verskeidenheid van kliniese manifestasies daarvan, groot uitdagings aan dermatoloë bied. Hierdie toestand kom onder ongeveer 85% tienerseuns en 80% tienermeisies voor (Gollnick, 2003:1580). Onbehandelde aknee kan tot ernstige sielkundige nagevolge, soos „n lae selfbeeld, sosiale onttrekking en depressie, aanleiding gee, wat genoeg rede mag wees dat hierdie toestand as „n ernstige afwyking erken behoort te word (Webster, 2001:15). Die patogenese van aknee is veelsydig en sluit faktore, soos blokkering van die follikel, opeenhoping van sebum, „n toename in *Propionibacterium acnes (P. acnes)* en inflammasie van die omliggende weefsel, in (Wyatt *et al*., 2001:1809).

Aknee behandeling fokus primêr op die vermindering van die inflammatoriese en nie-inflammatoriese aknee areas en beperk dus letselvorming (Railan & Alster, 2008:285). Nie-inflammatoriese aknee areas word as oop en en geslote komedoes beskryf, terwyl inflammatoriese areas uit papules, pustules, nodules en siste bestaan (Gollnick, 2003:1581). Aknee behandeling kan topikaal of oraal van aard wees. Topikale aanwending is gewoonlik die aanvanklike keuse vir die behandeling van nie-inflammatoriese komedoes, asook vir matige geïnflammateerde areas. Topikale behandeling het die voordeel dat dit die moontlike sistemiese newe effekte, wat met orale behandelings gepaard gaan, uitskakel (Federman & Kirsner, 2000:80).

Topikale retinoïedes is in die tagtigerjare met groot sukses in aknee behandeling gebruik. Lokale velirritasies in sommige pasiënte het egter die langtermyn effektiwiteit van hierdie middels beperk (Julie & Harper, 2004:S36). Vitamien A asetaat behandelings het egter „n nuwe alternatief tot aknee behandeling gebied, met die voordeel dat dit minder newe-effekte het (Cheng & Depetris, 1998:7).

In hierdie studie is vitamien A asetaat en sink asetaat in semi-soliede, kombinasie formulering ontwikkel, as moontlike alternatiewe tot aknee behandeling. Terwyl vitamien A asetaat ingesluit is om die vorming van mikro-komedoes te beheer, die bestaande komedoes te verminder, sebumproduksie te inhibeer en om matige inflammasie te verminder (Verschoore *et al*., 1993:107), is sink asetaat ingesluit om hormoonwanbalanse en sebumsekresie te normaliser (Hostýnek & Maibach, 2002:35) (Nutritional-supplements-health-guide.com, 2005:2).

Alhoewel topikale toediening van geneesmiddels baie voordele vir die aflewering daarvan inhou, het dit ongelukkig ook verskeie nadele. Die grootste uitdaging tydens transdermale aflewering is om die natuurlike velskans te penetreer. Die transdermale gedrag van „n geneesmiddel kan suksesvol deur sy fisies-chemiese eienskappe voorspel word. „n Ideale

geneesmiddel vir transdermale aflewering behoort "n goeie balans tussen beide lipofiele en hidrofiele eienskappe te kan handhaaf vir goeie partisie tussen beide die stratum korneum en die onderliggende velweefsel (Kalia & Guy, 2001:159).

Die effek van Pheroid™ tegnologie, ten opsigte van die moontlike bevordering van die penetrasie van die akiewe bestandele deur die vel, is ook tydens hierdie studie ondersoek. Die Pheroid™ bestaan uit vesikelvormige strukture wat uit dieselfde essensiële vetsure, soos wat algemeen in die mens voorkom, saamgestel is. Dit bevat egter geen fosfolipiedes of cholesterol nie (Grobler *et al.*, 2008:283).

Hierdie studie het dus ten doel gehad om die transdermale aflewering van vitamien A en sink, wat gesamentlik in vier topikale formulerings vir die behandeling van aknee ingesluit is, te ondersoek. Vitamien A asetaat (0.5%) en sink asetaat (1.2%) is in „n room, „n Pheroid™ room, „n emulgel en „n Pheroid™ emulgel geformuleer. Die eksperimentele resultate van die nuwe formulerings is met "n bestaande kommersiële produk, wat vitamien A asetaat bevat, vergelyk. Die formulerings is in terme hul transdermale, epidermale en dermale diffusie, tydens „n 6 h lange diffusie studie getoets, deur van Franz diffusie selle en „tape stripping" metodes gebruik te maak.

Eksperimentele resultate het getoon dat vitamien A nie deur die vel gediffundeer het nie. Hierdie resultate was op beide die eksperimentele formulerings, asook die kommersiële produk van toepassing.

„Tape stripping" eksperimente is uitgevoer ten einde die konsentrasie van die geneesmiddel, wat in die epidermis en dermis teenwoordig was, te bepaal. Die hoogste epidermale konsentrasie van vitamien A is met die Pheroid™ emulgel (0.0045 µg/ml) verkry, terwyl die emulgel-formulering die hoogste vitamien A asetaatkonsentrasie in die dermis meegebring het (0.0029 µg/ml). In teenstelling met al vier die nuwe formulerings, was die totale vitamien A-konsentrasie in die epidermis aansienlik laer vir die kommersiële produk. Vitamien A-konsentrasies vir die kommersiële produk in die dermis was egter in dieselfde orde as vir drie van die vier nuwe formulerings, met die uitsondering van die emulgel, wat ongeveer 31% meer vitamiene A in die dermis gelewer het as die kommersiële produk.

Alhoewel sink wel deur die voldikte vel gediffundeer het, is geen fluks-waardes opgelewer nie. Ten einde die moontlikheid van intrinsieke sink-diffusie uit te skakel, is placebo formulerings berei (sonder sink) vir gebruik as kontrole-monsters, tydens die vel-diffusie ondersoeke. Die emulgel en Pheroid™ emulgel formulerings was nie in staat om beduidende sink asetaatkonsentrasies transdermaal te lewer nie, alhoewel transdermale diffusie met beide die room en die Pheroid™ room verkry is. „Tape stripping" eksperimente op die placebo formulerings, relatief tot die geformuleerde produkte, het getoon dat die sink

asetaatkonsentrasies in the epidermis en dermis beduidend hoër was met die aanwending van die placebo formulerings. Die gemiddelde sink asetaatkonsentrasie in die dermis, na aanwending van die room formulering, was egter beduidend hoër, in vergelyking met die placebo room. Die afleiding kon dus gemaak word dat geen sink vanuit die nuwe formulerings in die epidermis of dermis gediffundeer het nie, behalwe in die geval van die roomformulering. Die sink asetaatkonsentrasie wat in die epidermis gemeet is, het dus eerder die intrinsieke sink asetaat, wat natuurlik in die vel teenwoordig is, verteenwoordig. Die roomformulering was egter wel moontlik daartoe in staat om meetbare sink asetaatkonsentrasies na die epidermis gelewer.

Die formulerings is onder blootstelling aan �because verskeidenheid van omgewingstoestande aan stabiliteitstoetse onderwerp, ten einde te toets of die funksionele eienskappe van die formulerings oor „n bepaalde tydperk binne aanvaarbare grense sou bly. Die geformuleerde produkte is oor „n tydperk van drie maande getoets, tydens blootstelling aan die volgende stoorkondisies: 25 °C/60% RH (relatiewe humiditeit), 30 °C/60% RH and 40 °C/75% RH. Stabiliteitstoetse het onder andere stabiliteitsaanduidende analitiese toetse, rheologiese bepalings, pH meting, deeltjiegrootte-bepaling, zeta-potensiaal meting, massaverlies, morfologiese inspeksie van die deeltjies en fisiese evaluering ingesluit.

Die eksperimentele formulerings was onstabiel oor die drie maande stabiliteitsevalueringstydperk. Veranderings in die viskositeit, kleur en konsentrasie van die aktiewe bestanddele is waargeneem.

Sleutelwoorde: Vitamien A, sink, aknee, transdermale diffusie, formulering, Pheroid™

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 INTRODUCTION

Transdermal drug delivery has become an area of increasing interest to many large industries. The human skin is the largest organ in the body, which makes it an easily accessible organ that offers multiple sites for administering therapeutic agents for local and systemic action (Mukhtar, 1992:4; Williams, 2003:1). However, percutaneous absorption of drugs is limited, due to the efficient barrier properties of the skin. The biggest challenge in transdermal drug delivery is thus to overcome this barrier. The barrier function can be ascribed to the macroscopical structure of the stratum corneum, which consists of alternating lipoidal and hydrophilic regions (Wiechers, 1989:185). A drug with the ideal physicochemical properties for transdermal drug delivery will therefore have both hydrophilic and lipophilic properties (Kalia & Guy, 2001:160).

Pheroid™ technology offers a new approach to transdermal drug delivery by overcoming the barrier function of the stratum corneum and therefore to enhance penetration of the active pharmaceutical ingredients (APIs) across the skin. It consists of vesicular structures that contain no phospholipids, nor cholesterol, but are compiled of customised essential fatty acids, similar to those present in the human body (Grobler *et al.,* 2008:283).

Acne is an extremely common disease that affects 85% of teenage boys and 80% of girls (Gollnick, 2003:1580). This disease presents a significant challenge to dermatologist, due to its complexity, prevalence and range of clinical expressions. This condition affects the pilosebaceous unit in the dermis. Although superficial and not life threatening, acne can cause serious psychological consequences, such as a low self-esteem, social inhibition and even depression, if left untreated (Webster, 2001:15). Although the precise mechanism is unclear, pathogenesis of acne includes factors that lead to plugging of the follicle (hyperkeratinisation), accumulation of sebum, growth of *Propionibacterium acnes (P. acnes)*, and inflammatory tissue responses (Wyatt *et al*., 2001:1809).

Acne comprises a spectrum of diseases, with the invisible microcomedones (first essential step in acne lesion formation) at the one end and deep scarring inflammatory nodules at the other (Gollnick, 2003:1580). Progressive enlargement of microcomedones, due to blockage of sebum flow, results in clinically visible comedones (non-inflammatory lesions) and inflammatory acne lesions (papules, pustules, nodules and cysts) (Gollnick, 2003:1581). Acne treatment therefore focuses on the reduction of inflammatory and non-inflammatory acne lesions, and thus the

halting of the scarring process (Railan & Alster, 2008:285). Acne treatment may be topical, or oral, based on the severity of the disease. Topical treatment is the most suitable first-line therapy for non-inflammatory comedones, or mildly inflammatory disease states, with the advantage of avoiding the possible systemic effects present in oral medications (Federman & Kirsner, 2000:80).

Topical retinoids reverse the abnormal pattern of keratinisation seen in *acne vulgaris* and have therefore been used with immense success (Habif, 2004:178). The occurrence of local skin irritations has, however, altered their effective use (Julie & Harper, 2004:S36). Vitamin A acetate, an ester form of vitamin A, has offered a new approach in the treatment of acne that presents less side effects, due to its gentle biochemical activity (Cheng & Depetris, 1998:7). Zinc acetate, on the other hand, is also known to decrease irritancy when applied to the skin (Hostýnek & Maibach, 2002:35).

During this study, vitamin A acetate and zinc acetate were formulated into semisolid, topical, combination formulations for the possible treatment of acne. The role of vitamin A was to control the development of microcomedones, reduce existing comedones, diminish follicular plugging and moderately reduce inflammation (Verschoore *et al*., 1993:107). Zinc was included for normalising hormone imbalances (Nutritional-supplements-health-guide.com, 2005:2) and to inhibited sebum secretion (Hostýnek & Maibach, 2002:35). Zinc also plays a significant role in the repairing process of the skin (Hostýnek & Maibach, 2002:34).

1.2 AIM AND OBJECTIVES

The aim of this study was to determine the extent of topical and transdermal delivery of vitamin A acetate and zinc acetate, formulated into four semi-solid, combination products, for the treatment of acne.

The objectives included:

- The formulation of a cream and emulgel, each containing both vitamin A acetate and zinc acetate as APIs, for the treatment of acne.
- The formulation of a Pheroid™ cream and Pheroid™ emulgel, each containing both vitamin A acetate and zinc acetate as APIs, for the treatment of acne.
- Stability determination of the four developed formulations.
- The stabilisation of vitamin A in the four topical formulations.
- The development and validation of a high performance liquid chromatography (HPLC) method for quantitatively determining the concentrations of the vitamin A in the new formulations.

- An investigation into the extent of epidermal, dermal and transdermal delivery of vitamin A acetate (0.5%) and zinc acetate (1.2%), when applied to the skin *via* the four topical formulations.

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CHAPTER 2

FACTORS AFFECTING TRANSDERMAL ACNE TREATMENT

2.2 ACNE

Acne vulgaris is an extremely common skin condition (Leyden, 1997:1). It is a disease of the pilosebaceous follicle and the events leading to the formation thereof include plugging of the follicle, accumulation of sebum, growth of *Propionibacterium acnes* (*P. acnes*) and an inflammatory reaction (Wyatt *et al.,* 2001:1809). The clinical definition of acne could be stated as non-inflammatory, open and closed comedones and inflammatory papules, pustules and nodules (Leyden, 1997:2). This condition can be found in 80% of adolescents, as well as in young adults to some extent (Gollnick *et al*, 2003:S2). Acne is, however, not restricted to a specific age group, and it occurs in 3% male and 12% female adults (Tan *et al.,* 2001:439). The most severe forms usually occur in males, but tend to be more persistent in females, because of the periodic flare-ups, before menstrual periods (Habif, 2004:162).

Acne usually appears in regions of the skin having the most dense population of sebaceous follicles, for example the face, chest, and back (Harper & Fulton, 2008:1). It can also occur on the neck, shoulders, and upper arms (AAD, 2008:1).

This disease is not a life threatening condition, and although limited to the skin, the psychological impact could have devastating consequences and is often underestimated (Webster, 2001:15). Studies have shown that people with acne tend to have low self-esteems and self-confidence, which can lead to social inhibition and in some cases, even depression. Acne should therefore be recognised as a serious disorder (Gollnick, 2003:1580) that could be controlled, but not cured (Habif, 2004:170).

2.2.1 PATHOGENESIS OF *ACNE VULGARIS*

The pathogenesis of *Acne vulgaris* is multifactorial. Four key pathogenic factors that influence the development of acne include:

I. Follicular epidermal hyper-proliferation, with subsequent plugging of the follicle.

II. Excess sebum production.

III. The presence and activity of *P. acnes.*

IV. The increased severity of inflammation (Harper & Fulton, 2008:1).

These factors are graphically illustrated in Figure 2.1.

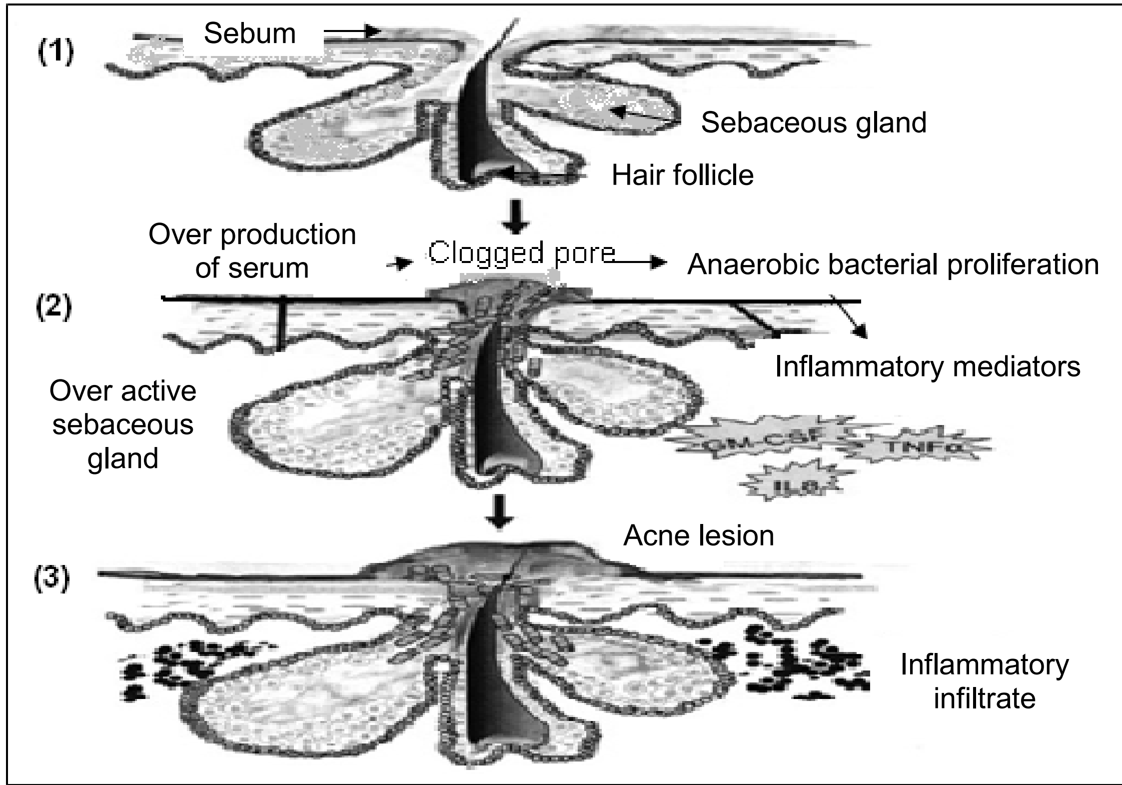


Figure 2.1: Pathophysiology of acne. (1) The normal pilosebaceous unit. (2) Hyper-proliferation and excess sebum provokes clogging of the pore. (3) *P. acnes* recruit inflammatory cells, which release inflammatory signals (Muizzuddin *et al.,* 2008:185)**.**

According to Leyden (1997:2), the most common trigger of acne is puberty, as it is associated with the production of androgen hormones. It is caused by the maturation of the adrenal glands, which secrete more androgens, resulting in an increased sebum production. Most patients with acne do not necessarily have an overproduction of androgens, but rather sebaceous glands that are hyper-responsive to androgen hormones

Other triggers include hormonal changes during the menstrual cycle and pregnancy, occlusive cosmetics and cleansing products, humidity and sweating. No relationship is found between acne exacerbation and diet (Beers, 2006:942).

2.2.1.1 Epidermal hyper-proliferation and plugging of the follicle

Keratinocytes in a normal follicle are shed as single cells to the lumen, with subsequent excretion. In patients with acne, the normal shedding of the hyper-proliferative keratinocytes does not occur.

Abnormal desquamated corneocytes gather in the sebaceous follicle, which cause comedogenesis (Gollnick *et al.,* 2003:S3).

A microcomedone is the earliest microscopic lesion observed and is also a precursor of other acne lesions (Webster, 1996:241). Follicles are gradually filled with lipid droplets, bacteria and monofilaments (Gollnick *et al*, 2003:S3). Eventually the epithelial lining of the sebaceous follicle becomes distended and plugs, called comedones, develop (Habif, 2004:169).

The progressive enlargement of microcomedones, as well as the blockage of sebum flow, result in the clinically visible comedones and inflammatory acne lesions (Gollnick, 2003:1581). Comedones are termed "open" or "closed"; depending on whether they are dilated, or closed at the surface of the skin (Beers, 2006:942).

Non-inflammatory acne lesions, i.e. comedones, include blackheads (open comedones) that are filled with desquamated keratinous cells and sebum. The lesions range between 1 – 5 mm in diameter and appear blackish on the skin surface, due to the refraction of light. Whiteheads (closed comedones) form a white bump just beneath the skin surface, with no open pores, which appear as lesions between 1 – 2 mm in diameter (Gollnick, 2003:1581).

If sebum accumulation continues, the closed comedone becomes expanded and the follicular sac can rupture into the tissue, which then leads to the production of inflammatory acne lesions. Inflammatory acne comprises of papules, pustules, nodules and cysts. The inflammatory lesions are bigger in size than the non-inflammatory lesions and are filled with pus (Gollnick, 2003:1581).

Since the real reason for the hyper-proliferation is unknown, three hypotheses have been proposed to state the hyper-proliferative effect in individuals with acne:

I. Androgen hormones have been implicated as the primary trigger in the development of comedones and are present in the area of the follicles where the comedones form. A defect in the hormone contributes to proliferation *via* the 5-α-reductase enzyme in the infundibulum.

II. Diminished concentrations of linoleic acid are present in individuals with acne and are thought to be the initial step in comedone formation.

III. Inflammation is implicated in comedone formation *via* the interleukin-1α (IL-1α), which is a pro-inflammatory cytokine. Although inflammation is not yet visible in the early lesions of acne, it may still play a crucial role in the development and progression of *Acne vulgaris* and the formation of comedones (Harper & Fulton, 2008:1).

2.2.1.2 Excess sebum production

The excessive production and excretion of sebum is another significant factor in the development of *Acne vulgaris*. Androgen hormones, mainly testosterone, stimulate the production and release of sebum (Gollnick, 2003:1581). The positive correlation between increased sebum production and acne is well established (Wertz & Michniak, 2000:32). That explains why acne first coincides with the onset of puberty.

2.2.1.3 Propionibacterium acnes

An additional factor that tends to be important in the pathogenesis of acne is *P. acnes*, which is a gram-positive, anaerobic organism that organises sebaceous follicles. The prevalence of *P. acnes* is the highest in regions of the body with a high density of sebaceous glands. The large amounts of sebum that is provided by the sebaceous follicles ensure an anaerobic, lipid-rich environment in which *P. acnes* thrives (Gollnick, 2003:1585). *P. acnes* has shown to produce a lipase that metabolises sebum triglycerides into fatty acids and glycerol that may contribute to comedone formation and subsequent inflammation (McInturff & Kim, 2005:73).

Although all individuals have a large number of *P. acnes* present in the skin and also a variable degree of follicular plugging, not all present with active acne. This may be due to the differences in immune response to the organism (Webster, 2001:16).

2.2.1.4 Inflammation

Acne is not primarily a hyper-proliferative disorder of the sebaceous follicle. According to Holland and Jeremy (2005:79), the emphasis has moved to that of an inflammatory skin disorder, generated by *P. acnes.* The highly inflammatory effect of *P. acnes* could be attributed to the release of neutrophils, lymphocytes, and macrophages. These chemostactic factors may cause damage, or rupture to the follicular wall (Gollnick *et al.*, 2003:S4).

2.2.2 TREATMENT OF ACNE

Acne can be effectively treated, although the response to treatment may sometimes be slow (DermNet NZ, 2007:1). Therefore, patients need to understand that it may take between 3 – 6 weeks, before any signs of improvement may be observed (Webster, 2001:17). Successful acne therapy should be based on the treatment of both the pathogenic causes and the clinical symptoms.

The therapeutic goals of acne treatment are to decrease sebum production, reduce *P. acnes* colonisation and inflammation, normalise follicular keratinisation, reverse hyper-proliferation and decrease existing inflammation. To achieve these goals, both topical and systemic acne

therapies are available (Wyatt *et al.,* 2001:1809). Figure 2.2 summarises the various drugs currently being used in the treatment of acne.

Selection of acne treatment is based on the severity, extent and duration of the disease, as well as the type of lesions and the psychological effects (Leyden, 1997:6). Therapeutic treatment options are summarised in Table 2.1.

Table 2.1: Selection of acne treatment (Leyden, 1997:6-7)

Severity	Illustration	Treatment options
Non-inflammatory comedonal acne (blackheads and whiteheads)		Topical tretinoin
Papular inflammatory acne		Topical antibiotics, benzoyl peroxide, or both
Moderate acne		Systemic antibiotics in combination with a topical retinoid
Severe nodular and cystic acne		Systemic isotretinoin, and topical or systemic antibiotics

Drug classes currently being used for the treatment of acne and their effects are summarised in the illustration below.

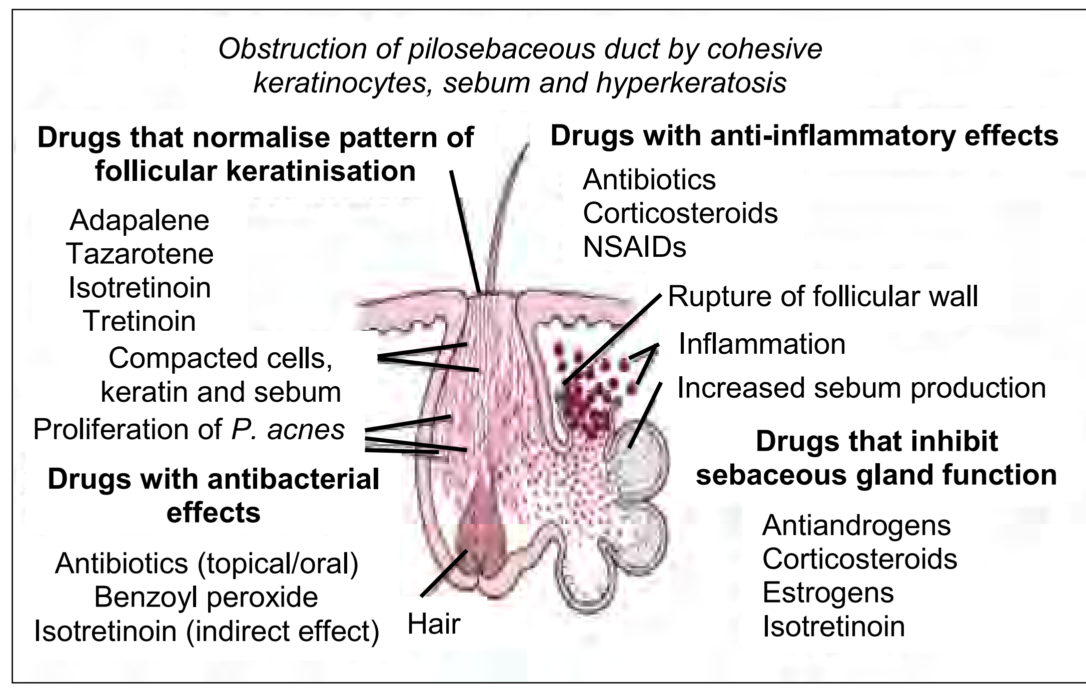


Figure 2.2: Mechanism of action of various drugs in the treatment of acne (Adapted from Beers, 2006:943)**.**

2.2.2.1 Topical agents used in the treatment of acne

Topical treatment is the most suitable first-line therapy for non-inflammatory comedones, or mildly inflammatory disease states. The advantage of topical therapy is the avoidance of the possible systemic effects of oral medications (Federman & Kirsner, 2000:80). Topical treatment of acne involves the use of retinoid analogues, topical antibiotics, benzoyl peroxide and azelaic acid.

2.1.2.2.1 Topical retinoids

Topical retinoids serve as an excellent mono-therapy for the treatment of inflammatory acne, but can also be used for more severe acne. Retinoids are furthermore the preferred agents in maintenance therapy (Gollnick *et al.,* 2003:S6). Topical retinoids have multiple functions in the treatment of acne. They control the development of microcomedones, reduce existing comedones, diminish the formation of new acne lesions, down regulate sebum production and promote normal desquamation of follicular epithelium. Some of the topical retinoids reduce inflammation by modulating the immune response, inflammatory mediators and the migration of inflammatory cells. Retinoids could also enhance the penetration of other compounds, i.e. benzoyl peroxide or topical antibiotics, through altering the follicular microclimate (Gollnick *et al.*, 2003:S6).

The most commonly used topical retinoids include tretinoin, adapalene and tazarotene, each with a different chemical structure. These agents target the microcomedone and are comedone-suppresive, as well as anti-inflammatory in variable strengths (Gollnick *et al*., 2003:S6).

Tretinoin was the first topical retinoid to be used in acne treatment (Wyatt *et al.,* 2001:1809). It is the standard anti-comedonal agent (Webster, 2001:18). Tretinoin inhibits comedone formation by delaying the desquamation process and reducing inflammatory lesions (Kligman *et al.,* 1969:469). A significant side effect of topical tretinoids is local skin irritation, which occurs after a few weeks of therapy (Webster, 1996:256). Another important disadvantage is its photosensitive effect, requiring the use of effective sunscreens (Federman & Kirsner, 2000:82).

Adapalene is a derivative of naphthoic acid and is a synthetic retinoid-like compound, with retinoid effects (Webster, 2001:18). It does not bind to cellular retinoic acid-binding proteins, but appears to produce similar changes in the keratinisation of follicles (Webster, 1996:256). Adapalene is successfully used to treat comedonal acne and also has anti-inflammatory activity (Webster, 2001:18). The efficacy of adapalene and tretinoin seem to be comparable, although adapalene is somewhat less irritating and more stable in sunlight (Webster, 1996:256).

Tazarotene is an acetylenic retinoid pro-drug, used in the treatment of mild to moderate acne. Direct delivery of tazarotene into the skin is provided with topical application. It is then rapidly hydrolysed to the active metabolite, tazarotenic acid (Gollnick, 2003:1591). This metabolite regulates differentiation and proliferation of epithelial tissue. Tazarotene may also demonstrate anti-inflammatory and immuno-modulatory properties (Harper & Fulton, 2008:8).

2.1.2.2.2 Topical antibiotics

Topical antibiotics are indicated for use in patients with mild to moderate inflammatory acne, but have no importance in the treatment of comedonal acne. *P. acnes* plays a significant role in the pathogenesis of acne. Topical antibiotics reduce *P. acnes* populations, therefore lessening the stimulus for inflammation, as well as the percentage of free fatty acids in surface lipids (Webster, 1996:258). The most popular topical antibiotics used to treat acne include erythromycin and clindamycin. However, with the continuous use of these products, resistance may develop against strains of *P. acnes*. To lessen the development of resistance, topical antibiotics could be used in combination with benzoyl peroxide (Harper & Fulton, 2008:5). Efficacy may be increased by using antibiotics in conjunction with topical tretinoin, whilst comedolytic activity may also be added (Webster, 1996:258).

2.1.2.1.3 Benzoyl peroxide

According to White (1999:311), benzoyl peroxide remains one of the most outstanding topical antimicrobial agents in the treatment of acne. It has potent antibacterial and weak comedolytic activity (Webster, 1996:257). Topical application of benzoyl peroxide is used in the treatment of non-inflammatory lesions by reducing the *P. acnes* counts. Benzoyl peroxide tends to have no intrinsic anti-inflammatory properties (Leyden, 1997:5). Adverse effects of benzoyl peroxide include concentration-dependent cutaneous irritation, dryness, and mild dermatitis, developing within the first few days of treatment (Eady *et al*., 1990:215). Benzoyl peroxide does not influence the production, nor composition of sebum and does not have an effect on hyper-keratinisation (Federman & Kirsner, 2000:82).

2.1.2.1.4 Azelaic acid

Azelaic acid is a dicarboxylic acid with both antibacterial and comedolytic effects. It inhibits tyrosinase in proliferative melanocytes and therefore has hypo-pigmentary activity (Webster, 1996:257). The topical application of this drug causes the least irritation of all the preparations (Webster, 2001:18). Azelaic acid is a bacteriostatic drug at low concentrations, whereas it is bactericidal when in higher concentrations (Webster, 1996:257).

2.1.2.3 Systemic agents used in treatment of acne

Systemic agents are the preferred choice to treat inflammatory lesions. Systemic treatment of acne involves the use of retinoids, antibacterial agents, and hormone therapy (Wyatt *et al.,* 2001:1810).

2.1.2.3.1 Systemic retinoids

The oral natural metabolite of vitamin A, isotretinoin, is indicated as a first-line treatment for severe, unmanageable, nodular acne, as well as for moderate or severe acne, unresponsive to topical therapy (Wyatt *et al.,* 2001:1810). Isotretinoin is the only agent that exhibits activity against all four abnormalities found in acne. It decreases the size and secretion of sebaceous glands, normalises epidermal differentiation and prevents the formation of new comedones. *P. acnes* populations are decreased, and therefore isotretinoin exerts an anti-inflammatory effect (Harper & Fulton, 2008:5).

Systemic isotretinoin therapy is associated with several adverse effects, of which the most important are teratogenicity and potential adverse psychiatric events (Wyatt *et al.,* 2001:1810). Mucocutaneous side effects include dryness of the eyes and skin, epistaxes and hair loss (Webster, 1996:261).

Due to the immense adverse effects of isotretinoin, it is important to brief the patient before starting such treatment.

2.1.2.3.2 Systemic antibiotics

Oral antibiotics have two main functions in acne treatment:

- Suppressing the growth of *P. acnes*.
- Acting as an anti-inflammatory agent by inhibiting neutrophil chemotaxis and chemotactic factors (Federman & Kirsner, 2000:83).

Systemic treatment with antibiotics is mainly indicated in the management of more severe, or extensive acne. It is also indicated in acne that is resistant to topical treatment (Federman & Kirsner, 2000:80). The effect of systemic antibiotics depends on their ability to reach the lipid-rich environment of the pilosebaceous follicles where *P. acnes* proliferates (Katsambas & Papadonstantinou, 2004:412).

The most commonly used oral antibiotics are erythromycin and the tetracyclines (Webster, 1997:259). Although they are anti-microbial agents, they have the advantage of additional anti-inflammatory properties (Webster, 2001:18). A common adverse drug reaction attributed to tetracycline and erythromycin is gastrointestinal tract discomfort. Tetracyclines should be avoided during pregnancy and in children younger than eight years, due to skeletal growth inhibition in the foetus and the risk of permanent tooth discolouration (Beers, 2006:1439). Other tetracycline derivatives include minocycline and doxycycline. They are more lipophilic than tetracycline and generally display increased penetration of the pilosebaceous follicle (Harper & Fulton, 2008:5). These two derivatives are normally used if there is no adequate response to tetracycline. The lipophilicity of these drugs causes central nervous system adverse effects, such as headaches, vertigo, and ataxia (Federman & Kirsner, 2000:83). However, photosensitivity is the main, drug related, side effect of doxycycline (Wyatt *et al.,* 2001:1811).

2.1.2.3.3 Hormonal therapy

Androgens, estrogens, growth hormone, and insulin-like growth factor, may all play a significant role in the development of acne (George *et al.,* 2008:188). Sebaceous glands are androgen dependent, therefore the aim of hormonal therapy is to counter the androgen effects on the sebaceous gland through the use of specific hormonal treatment strategies (Gollnick *et al.*, 2003:S22). Estrogens are normally used as oral contraceptives and if given in sufficient amounts, they will suppress sebum production, thus improving acne symptoms (Gollnick *et al.*, 2003:S22).

According to recent studies, estrogens may decrease sebum secretion by one of the following mechanisms:

- Direct opposition of androgens within sebaceous glands.
- Suppression of the ovarian production of androgens through negative feedback on gonadotrophin release.
- Regulating the genes involved in the growth of sebaceous glands, or lipid production (George *et al.,* 2008:189).

Treatment of acne with contraceptives may take up to 2 – 4 months before any improvement is depicted, whilst relapse may occur when medication is discontinued (Leyden, 1997:3).

Gonadotrophin releasing agonists inhibit androgen production by the ovary. Ovulation is suppressed by the disruption of the cyclic release of the follicle stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary gland (George *et al.,* 2008:192). These drugs also suppress estrogen production, and therefore present side effects, such as headaches and bone loss (George *et al.,* 2008:192).

Androgen receptor blockers include cyproterone acetate, spirinolactone and flutamide. Cyproterone acetate reduces sebum production and comedogenesis (Gollnick *et al*., 2003:S22). It also decreases testosterone and gonadotrophin (George *et al.,* 2008:192). Cyproterone acetate has been shown to have similar efficacy in the treatment of acne, than isotretinoin. It should, however, only be used by women, due to the probability of feminisation in men (Gollnick *et al*., 2003:S22).

Spirinolactone is thought to work as an androgen receptor blocker and as an inhibitor of 5α-reductase. It should be reserved for the treatment of acne that is resistant to conventional therapies (Gollnick *et al*., 2003:S22).

Flutamide is a non-steroidal androgen receptor blocker and is effective in the treatment of hirsutism and acne in women. Flutamide is used very rarely, due to its many side effects (Gollnick *et al*., 2003:S23).

2.2 TREATMENT OF ACNE WITH THE VITAMIN A AND ZINC COMBINATION

This section describes vitamin A and zinc and/or their derivatives as active pharmaceutical ingredients (APIs). These actives were employed during this study, due to their significant positive effects in the treatment of acne.

2.2.1 VITAMIN A

2.2.1.1 History

Vitamin A was the first vitamin to be discovered in 1913 by a biochemist, Elmer McCollum and his colleague, Marguerite Davis. The first synthesis of vitamin A was done in 1947 by two Dutch chemists (Semba, 1999:784).

2.2.1.2 Pharmacology and classification

Vitamin A refers to a group of fat-soluble substances that are structurally related to, and possess the same biological properties, as the parent substance of the group, namely all-*trans* retinol (ROL, or retinol). Retinol is also known as the alcohol form of vitamin A and is normally used for transporting vitamin A from the liver to the tissues (Fernandes, 2006:1). ROL is delivered to cells bound to retinol binding protein, and then converted within the cells to various retinoids, depending on the type and the need of the cell (Kang *et al*., 1995:549). Other forms of vitamin A are the aldehyde (retinal), the acid (retinoic acid) and the ester (retinyl ester), collectively known as retinoids. Therefore, if vitamin A is mentioned, it includes all naturally occurring active forms of vitamin A, for example retinol, retinyl esters and the carotenoids.

Retinoids are required in epithelial differentiation, in which the mechanism involves the binding of vitamin A to nuclear retinoid receptors. These receptors can be divided into two classes, the retinoic acid receptor (RARs) and the retinoid-X receptor (RXRs). These receptors function as ligand activated transcription factors that modulate gene transcription (Shapiro & Saliou, 2001:839). Natural cell growth and differentiation is interrupted if there is insufficient vitamin A to bind to these receptors (Drugbank, 2008:8).

Since the alcohol form is unstable, vitamin A is commonly used in the stable ester-forms, such as acetate, propionate and palmitate (British Pharmacopoeia, 2009a:1). Retinyl palmitate is usually found in the skin and is a forerunner of retinol. These ester forms of vitamin A tend to be less irritating to the skin (Fernandes, 2006:2).

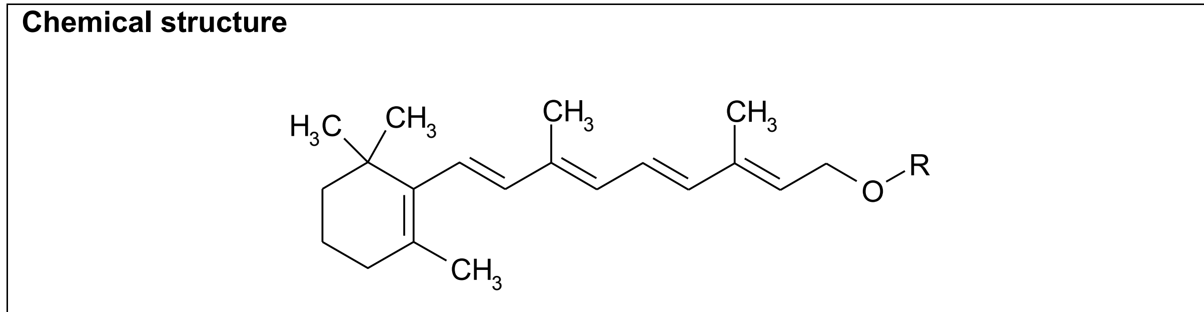
Vitamin A was first used for the treatment of skin disorders, i.e. acne and psoriasis, in the 1940‟s. Unfortunately, the doses were too high and toxic, so scientists developed a derivative of vitamin A, namely retinoic acid (Michaëlsson *et al.,* 1977:31). Today it is used with much clinical success, and is sold as oral or topical prescription medications, for the treatment of acne and keratinisation disorders (Shapiro & Saliou, 2001:839).

2.2.1.3 *Physicochemical properties*

The suitable form of vitamin A selected for use in this study was retinyl acetate, the ester form of vitamin A. Table 2.2 describes the physicochemical properties of vitamin A.

Table 2.2: Physicochemical properties of vitamin A (Sigma-Aldrich, 2010)

Chemical structure



Substance	R	Molecular formula	*M_r (g/mol)*
❖ *all-(E)-retinyl* ❖ *all-(E)-retinyl aceta*	❖ H ❖ *$COCH_3$*	❖ *$C_{20}H_{30}O$* ❖ *$C_{22}H_{32}O_2$*	❖ *286.5* ❖ *328.49*

Synonyms:	Vitamin A acetate, retinol acetate.
Appearance:	Yellow, crystalline, solid, or super-cooled liquid.
Melting point:	57 – 58°C.
Solubility:	Insoluble in water, soluble or partly soluble in anhydrous ethanol, and miscible with organic solvents.
Storage:	Store in airtight containers. Protect from light, oxidising agents, acids and heat. Recommended storage temperature is -20°C.

2.2.1.4 *Absorption, metabolism and excretion*

Ingested retinol acetate is dispersed and emulsified in the stomach. It is then hydrolysed in the lumen of the small intestine by the pancreatic, non-specific, lipase enzyme. The solubilised retinol acetate in the micelles is absorbed by the mucosal cells of the small intestine (Schaeffer & Brooks, 1992:2). In the mucosal cells it is converted into retinol palmitate, which is then transported, coupled to chylomicrons, to the liver for storage. In the liver, the ester is bound to a protein, namely the retinol binding protein (RBP). From the hepatic stores, the ester in combination with the RBP is distributed *via* blood circulation to the target cells, for example the skin. It is proved that RBP is present in the stratum corneum and in the viable layers of the epidermis in human skin (Fu *et al*., 2007:229). In the target cells, the ester and RBP combination is oxidised to retinoic acid, which then enters the nucleus of the cells and binds to the nuclear retinoic acid receptors, resulting in the expression of particular vitamin A dependant proteins. There are many uses of vitamin A, of which the most common is the differentiation of the skin (Wolf, 1997:871).

Vitamin A metabolites are commonly excreted in the urine and are biologically inactive. In the liver, some of the metabolised retinol products are conjugated with glucuronic acid or taurine, for excretion in bile. As the vitamin A exceeds a critical concentration in the liver, the portion of excreted vitamin A metabolites in the bile increases. This is a protective mechanism to minimise the risk of excessive storage of vitamin A (Hicks *et al.,* 1984:1328). The half-life of vitamin A and its derivatives is approximately 1.9 hours (Drugbank 2008:8).

The recommended dietary allowance (RDA) for vitamin A is 3,000 IU for men and 2,300 IU for women. One IU equals 0.3 µg of vitamin A in the form of retinol (Pressman & Buff, 2007:37-38). Sources of vitamin A can be divided into two groups, i.e. one from animal sources and the other from vegetable sources. There are a variety of foods, rich in vitamin A (retinoids) that are categorised as being of the animal source, such as fish, liver, milk and egg-yolk. The vegetable sources contain precursors of vitamin A, known as carotenoids (beta-carotene), and are richly represented in carrots, dark green leafy vegetables and yellow fruits (Kramer, 2005:443).

2.2.1.5 Uses of vitamin A

Vitamin A is one of the most versatile vitamins, with diverse functions such as:

- Enhancement of the immune system.
- Growth and differentiation of epithelial tissue.
- Prevention of cancer and certain malignancies (normal cell development).
- Maintaining good eye condition and vision.
- Enhancement of bone growth (Kramer, 2005:442).

Vitamin A is the dominant vitamin of the skin, because of its fundamental role in the control of the skin cells" normal activities, by protecting the skin and tissues inside and outside of the body. Vitamin A works by genetically promoting cell differentiation, which allows each type of cell to mature so that it is able to fulfil a particular function to help prevent infections (Vitamins & health supplements guide, 2006:2).

It has been used at pharmacological levels for the treatment of several dermatological diseases, which include:

- <u>Psoriase</u>: Vitamin A (tazarotene) normalises keratinocyte differentiation and proliferation. It decreases inflammation by decreasing the expression of inflammatory markers on keratinocytes (Keller & Fenske, 1998:614).

- <u>Photoaging</u>: Vitamin A has a restorative mechanism towards photo-damaged skin, and therefore repairs and limits the progression of existing damage. The effects are believed

to be mediated through the binding of vitamin A to the RARs and RXRs. Thus, it provokes type I and type III, pro-collagen, gene expression in human skin, which results in an increase in the deposition of collagen fibrils in the dermis. The ultraviolet (UV) induction of the matrix metalloproteinases, a family of enzymes responsible for the breakdown of collagen, is blocked (mainly by retinoic acid) (Manela-Azulay & Bagatin, 2009:471). This increase in dermal collagen is associated with wrinkle effacement and a dermis that is more resistant to trauma. Enhanced keratinocyte proliferation leads to the shedding of mature keratinocytes, resulting in a smoother skin surface texture. Vitamin A also reduces pigmentation, due to UV-rays, which includes sun induced, age spots (Shapiro & Saliou, 2001:840).

- Acne: According to literature, topical retinoids have been used with great success in the treatment of various forms of acne, due to their ability to down regulate sebum production, modify cellular differentiation, and their potent, anti-inflammatory properties (Verschoore *et al*., 1993:107).

- Striae: According to Verschoore *et al*. (1993:112), vitamin A can be used to improve striae, due to increased epidermal proliferation and new collagen formation.

- Cellulite: Vitamin A, especially tretinoin (retinoic acid), promotes the synthesis of glycosaminoglycans (GAGs) in normal skin. GAGs are a ground substance, occupying the space between collagen bundles and are highly hygroscopic, binding large volumes of water. As previously described, vitamin A increases the deposition of collagen in the dermis. According to Kligman *et al*. (1999:120), all of this would theoretically add firmness to the dermis.

- Wound healing: Vitamin A can promote and enhance various aspects of wound healing, by reducing an early inflammatory response and by increasing collagen synthesis, epithelialisation, fibroplasias and the stimulation of angiogenesis (Fu *et al.,* 2007:245).

To summarise, vitamin A and its derivatives are among the most important agents in the cosmetic industry for treatment of the skin. They have been used orally and topically in the treatment of psoriasis and acne, topically in photo-damaged skin and are being tested in the topical treatment of striae, cellulite and wound healing (Shapiro & Saliou, 2001:840).

2.2.1.6 Adverse reactions

In spite of the advantages of vitamin A, various side effects occur with the use of excessive amounts. Chronic toxicity is characterised by a condition, called hyper-vitaminosis A, which presents symptoms like fatigue, moderate to extreme weight loss, vomiting, hepatotoxicity, skin

changes (redness, burning and exfoliation of the skin), dry hair, cracking and bleeding lips, anaemia, headaches and pains in bones and joints. Symptoms of chronic toxicity may also include severe visual disturbances and raised intracranial pressure. These effects usually improve and disappear over a period of a week or a month, after withdrawal of vitamin A (Olson, 2001:32).

Acute intoxication of vitamin A occurs when more than 200 mg of vitamin A is ingested by an adult, and more than 100 mg by children daily. It is characterised by sedation, nausea, vomiting, headaches (due to increased intracranial pressure), diarrhoea, blurred vision, vertigo, sore mouth and bleeding gums (Olson, 2001:32).

When vitamin A is used topically, these above effects are absent, due to the lower systemic exposure *via* this route (Verschoore *et al*., 1993:107).

An excess of vitamin A taken during pregnancy can cause birth defects in the foetus. Teratogenicity appears in the first trimester of pregnancy and is mainly associated with 13-*cis*-retinoic acid. Birth defects, such as craniofacial malformations and abnormalities of the central nervous system, thymus and heart have been documented (Martínez-Frías & Salvador, 1990:121). To be safe, topical applications of vitamin A should be avoided, although beta-carotene supplements may be considered (Pressman & Buff, 2007:39).

Excessive alcohol intake should be avoided during the use of vitamin A therapy, because of the depletion of vitamin A stores in the liver, which may then lead to an increased prevalence of vitamin A side effects (Leo & Lieber, 1985:5228).

2.2.4 ZINC

2.2.2.1 Pharmacology and classification

Zinc is an essential trace mineral that is required by every cell in the body. This mineral is concentrated in skeletal muscle (57%), bone (29%), skin (6%), teeth, hair and in the male prostate gland (Hostýnek & Maibach, 2002:34; Pressman & Buff, 2007:232). Zinc is an extremely versatile mineral and is part of many bodily processes, including cell growth and reproduction, wound healing and immunity (Kramer, 2005:466).

The biological functions of zinc can be divided into three categories: catalytic, co-catalytic (regulatory) and structural. A few of the major zinc bio-molecules are highlighted and classified by their general structural type in Table 2.3 (modified from Schwartz *et al.,* 2005:838).

Table 2.3: Summary of major zinc bio-molecules, classified by their general structural type

FUNCTIONAL CLASS	ENZYME	PHYSIOLOGIC FUNCTION OF BIOMOLECULE
Catalytic	Alcohol dehydrogenase	Liver metabolism
	Carboxypeptidase	Protein digestion for nutrition
	Thermolysin	
	Matrix metalloproteinase Collagenase (MMP-1) Elastase (MMP-12) Gelatinase (MMP-2)	Formation of extracellular matrix Hydrolysis of collagen Hydrolysis of elastin Hydrolysis of gelatin
	β-lactamase	
	Carbonic anhydrase	Physiology of CO_2 transport and physiologic buffering
Co-catalytic	Nuclease P1	
	Superoxide dismutase	Scavenges damaging superoxide
	Phosphotriesterases	
	Alkaline phosphatase	
	Leucine aminopeptidase	
	Phospholipase C	
Structural	**Metallothionein**	Storage of zinc
	Nitric oxide synthase	
	Protein kinase	
	Zinc finger class DNA polymerases RNA polymerases	Nucleic acid metabolism Replication of DNA Transcription of RNA
	A-Amylase	
	Aspartate transcarbamoylase	

A few zinc enzymes, with particular importance and relevance to the skin, are discussed in the following paragraphs.

Matrix metalloproteinases (MMP) is an enzyme that specifically hydrolyses matrix proteins (collagen, elastin and gelatin) that cause degradation of the extracellular matrix. As zinc plays an important role in wound repair and healing, this degradation is a driving force behind tissue remodelling and facilitates the movement of new cells throughout the healing wound (Schwartz *et al.,* 2005:841). MMP influences various other physiological and pathological processes, for example, tissue morphogenesis, wound repair, inflammatory diseases, cancer and aspects of

embryonic development,some of which play an important role in acne and the treatment thereof (Sternlicht & Werb, 2001:464).

The second zinc enzyme of importance to healthy skin is superoxide dismutase (SOD). This enzyme catalyses the dismutation of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). It thus eliminates reactive oxygen species (ROS) (Bergendi *et al.*, 1999:1866-1867). This enzyme is therefore known for its activity as an antioxidant, which is of great importance in the skin (Formigari *et al.,* 2007:447).

The third relevant enzyme is metallothionein (MT). Although the primary biochemical role of MT is to store and transport zinc, it also possesses antioxidant activity (Schwartz *et al.,* 2005:839). In previous studies, it was reported that induction of MT in keratinocytes reduced cell damage. Zinc ions are a safe inducer of MT (Masaki *et al*., 2007:73). MT may also have therapeutic relevance in conditions, which include inflammation (Formigari *et al.,* 2007:449).

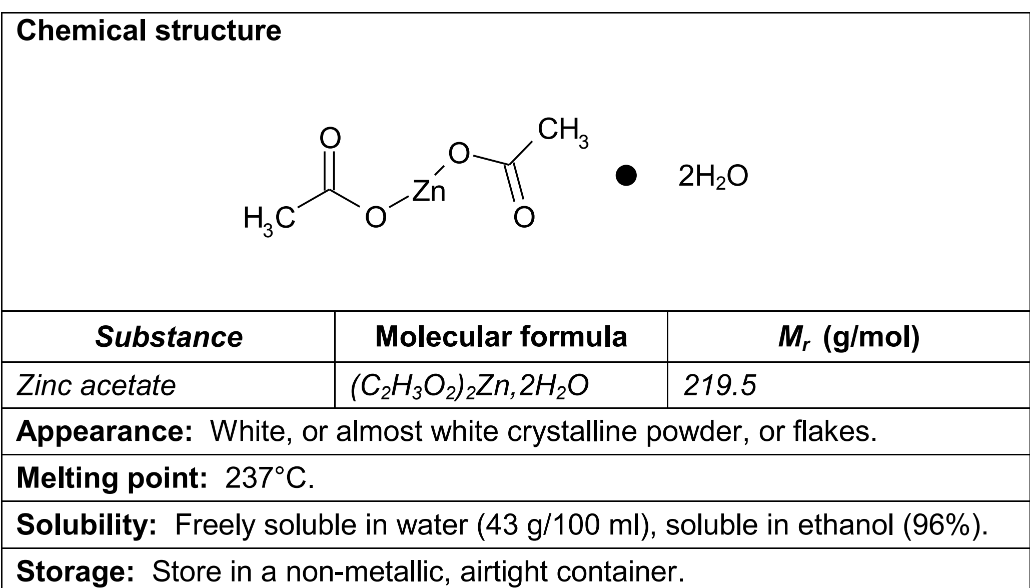
Another important enzyme that suppresses the inflammatory process is alkaline phosphatase (AP) (Schwartz *et al.,* 2005:839). AP is released from the surface of epithelial cells and dephosphorylates adenosine monophosphate (AMP) to generate adenosine. Adenosine participates in the reduction of the inflammatory phase of wound healing and has potent anti-inflammatory activity (Schwartz *et al.,* 2005:841).

The zinc finger proteins are part of the structural function of zinc and are involved in the regulation of gene expression. Examples are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Slater *et al.,* 1971:42). Their function is to catalyse DNA replication and transcription, which is extremely important in the highly proliferative environment of the epidermis (Schwartz *et al.,* 2005:839).

2.2.2.2 Physicochemical properties

The suitable form of zinc to be used in this study is zinc acetate, due to its reduced irritant effect. In Table 2.4 the physicochemical properties of zinc acetate are summarised.

Table 2.4: Physicochemical properties of zinc acetate (British Pharmacopoeia, 2009b)



Chemical structure		
Substance	**Molecular formula**	**M_r (g/mol)**
Zinc acetate	*$(C_2H_3O_2)_2Zn,2H_2O$*	*219.5*
Appearance: White, or almost white crystalline powder, or flakes.		
Melting point: 237°C.		
Solubility: Freely soluble in water (43 g/100 ml), soluble in ethanol (96%).		
Storage: Store in a non-metallic, airtight container.		

2.2.2.3 Absorption, metabolism and excretion

The absorption of dietary zinc takes place in the small intestine. This process comprises both active and passive transport activities. The efficiency of active transport in the intestine lumen increases during low intake of the mineral. Passive transport, on the other hand, is constant during low intake periods. Its efficiency depends on the concentration of zinc present in the lumen (Salgueiro *et al.,* 2000:737). After absorption in the small intestine, zinc may either form an intestinal pool, bound to the intestinal metallothionein, or it may be transported to the liver *via* albumin in the plasma. From there it may form a hepatic pool, bound to the hepatic metallothionein, or it may be distributed to the rest of the body (Salgueiro *et al.,* 2000:744). Figure 2.3 illustrates the overall biological cycle of zinc.

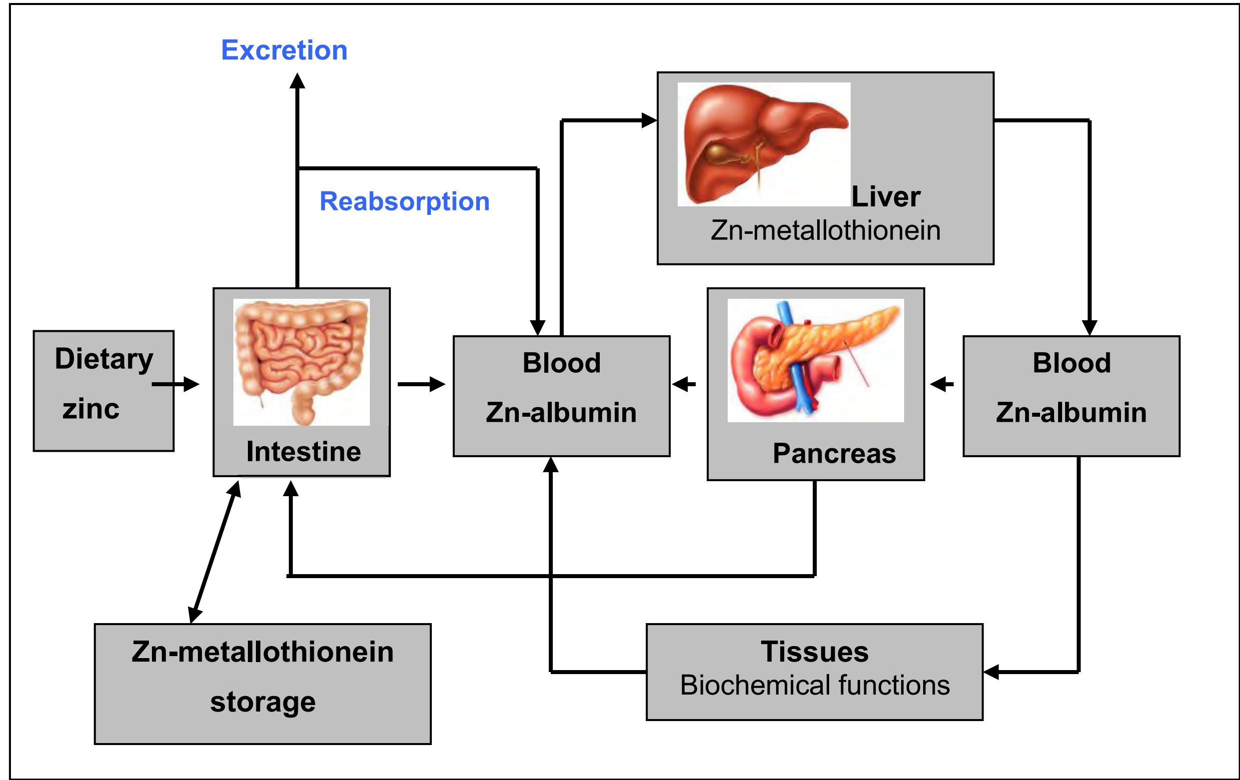


Figure 2.3: Zinc metabolism (Modified from Salgueiro *et al.,* 2000:744)**.**

Apart from dietary zinc, there is also an endogenous zinc secretion from pancreatic, biliar, intestinal, as well as from desquamated mucosal cells into the intestinal lumen (Berger & Schneeman, 1986:265). Dietary and endogenous zinc are both under the same homeostatic regulation and are being transported in the blood *via* plasma albumin (Institute of Medicine, 2001:445).

Many factors and conditions in the body may interfere with the absorption of zinc, and may be considered as activators or inhibitors. The absorption activators include:

- Picolinic acid (a pancreatic secretion).
- Vitamin B_6 (elevates the secretion of picolinic acid).
- Citrate.
- Glycine, histidine, lysine, cystein and methionine (collectively known as amino acids) (Evans & Johnson, 1981:68; Turnlund *et al*., 1991:1059).

The group of absorption inhibitors include:

- Phytic and oxalic acids.
- Tannins.
- Fibre.
- Selenium.
- Iron.
- Calcium (Turnlund *et al.*, 1991:1059).

Phytic acid forms insoluble complexes with zinc and therefore prohibits its absorption. Iron and calcium only interfere with zinc absorption at higher concentrations, than those found at nutritional levels (Sandstead & Smith, 1996:2412S-2413S).

Due to the zinc absorption inhibitors, the body only absorbs 30 – 40% of the ingested zinc (Nutritional-supplements-health-guide.com, 2005:1). The zinc that is not absorbed is excreted with the faeces, or may be reabsorbed at distal segments of the intestine (Salgueiro *et al.,* 2000:744). Under extreme atmospheric conditions and stress, zinc could be excreted through sweat, with higher losses than elimination *via* urine (Hostýnek & Maibach, 2002:35).

There is a wide range of dietary sources rich in zinc. Zinc is abundant in beef, pork, liver, poultry, eggs and seafood (especially oysters). Other foods that are good sources of zinc are beans, cheese, nuts, seeds and wheat germ, although the zinc in these foods are less easily absorbed than the zinc in meat, because of the phytates in the fibre that combine with the zinc (Pressman & Buff, 2007:234).

The RDA for zinc is 15 mg for males and 12 mg for females (Marcus & Coulston, 2001:1747).

A zinc deficiency can occur, because of a limited dietary intake of zinc, or secondary to diseases that decrease intestinal absorption and/or increase intestinal loss of zinc. These include diseases, like cystic fibrosis, Crohn"s disease, liver cirrhosis, alcoholism and stress (Wolfgang & Sandstead, 2006:6). Stunted growth and retarded sexual development in children are of the first physical signs of zinc deficiency (Maret & Sandstead, 2006:6). Other consequences and signs include a suppressed immune system, dermatitis (acne, psoriase, eczema) that appears as the severity of zinc deficiency increases (Wolfgang & Sandstead, 2006:6), poor wound healing, weight loss, alopecia, ataxia, disorientation, loss of smell and taste, and diarrhoea (Salgueiro *et al.,* 2000:741). When these conditions are treated with zinc, a dramatic improvement will occur within a few days (Hostýnek & Maibach, 2002:34).

2.2.2.4 Uses of zinc

The essentiality of zinc in human health is immeasurable. The various uses of zinc include:

- Relief of cold symptoms.
- Boosts the immune system.
- It is needed for cell division.
- Growth and maintenance of muscles.
- Normal growth and sexual development of children.
- Wound healing and anti-inflammatory effects.
- Boosts male fertility by producing healthy sperm and semen.
- Prevents prostate problems (benign prostatic hypertrophy) (Pressman & Buff, 2007:237).

One of the most common uses of zinc in personal care, overall, is in the form of zinc oxide in sunscreens (Hostýnek & Maibach, 2002:34). Zinc is also recommended for health problems, for example Type 2 diabetes, age-related macular degeneration, attention deficit hyper-activity disorder (ADHD) in children and memory loss (Pressman & Buff, 2007:239-240).

Inflammatory conditions are also being treated effectively with oral zinc. These conditions include acne, alopecia, rheumatoid arthritis, colitis, Crohn‟s disease and psoriatic arthritis (Michaëlsson *et al.,* 1977:36; Schwartz *et al.,* 2005:841). The effect of zinc in inflammation is cell type specific on the different cells involved in the process, such as mast cells, platelets, macrophages, neutrophilis, natural killer cells and lymphocytes. The cytokine messengers that assist with communication, are also influenced by zinc. Zinc could have different effects on the cells, when in different amounts present in the body, i.e. the inhibitory effect increases with elevated levels of zinc. In other cases, though, a scarcity in zinc could also have an inhibitory effect. Another important effect of zinc is its ability to inhibit mast cell degranulation, by decreasing histamine secretion, an important mediator of the inflammatory response (Schwartz *et al.,* 2005:842).

Zinc plays a significant role in the treatment of skin conditions. The Egyptians were first to identify the value of topical zinc. Pathological conditions can be treated just as effectively with topical preparations, than with oral zinc supplementation (Hostýnek & Maibach, 2002:34). As zinc is very versatile, the variety of its uses literally covers from head to toe, as shown in Figure 2.4.

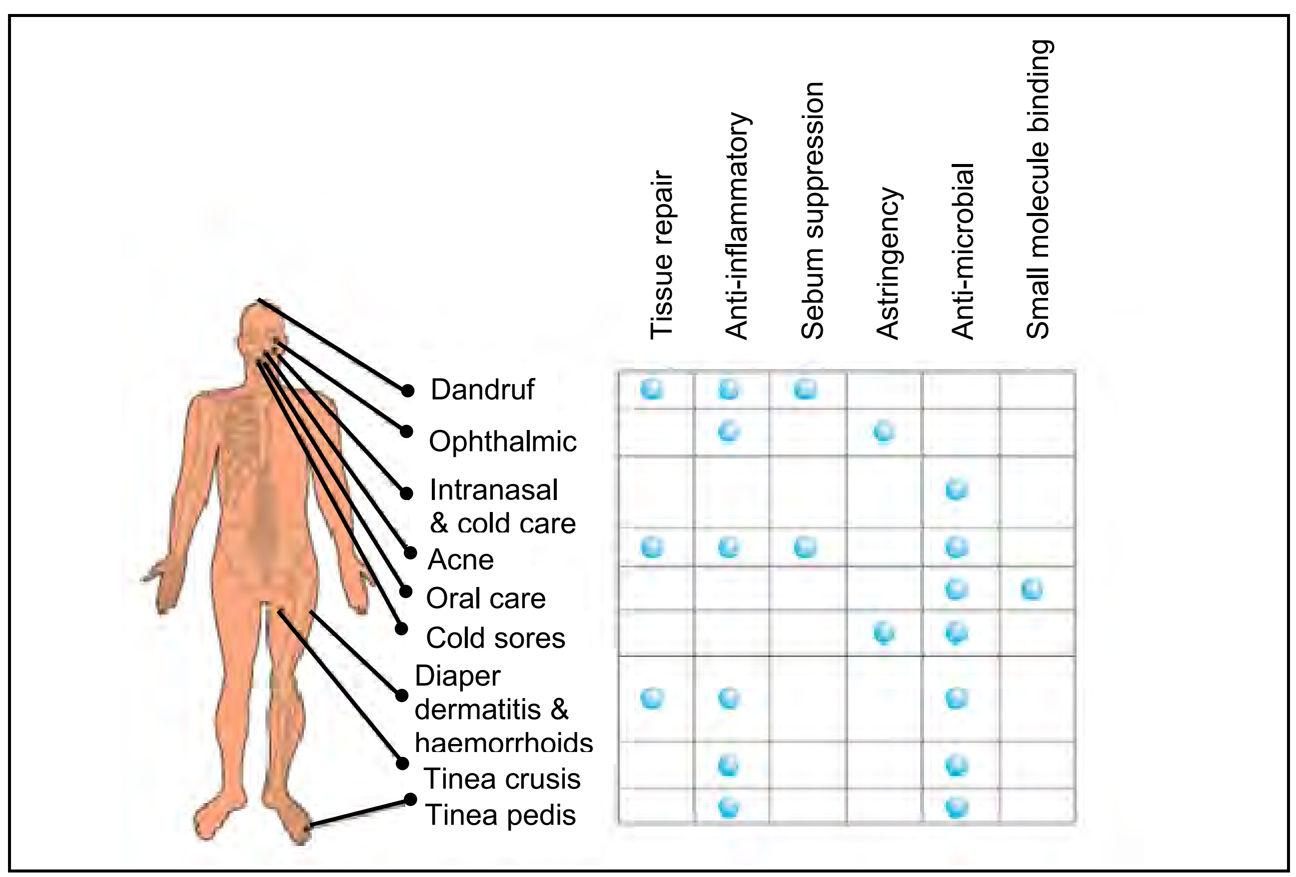


Figure 2.4: Graphic representation of uses of zinc from head to toe (Schwartz *et al.*, 2005:843)**.**

Acne is one of the most common diseases of our century. As previously discussed, acne is characterised by the presence or involvement of a microbe, inflammation, hyper-proliferation, and a high sebum production. For this reason zinc has become popular in the treatment of acne.

The purpose of zinc in the treatment of acne is to:

- Facilitate and regulate the activity of the androgenic hormones, to prevent potential hormone imbalances that may lead to outbreaks (Nutritional-supplements-health-guide.com, 2005:2).
- Decrease and normalise the sebum secretion in the skin by zinc"s inhibiting effect on the bacterial lipase activity (Hostýnek & Maibach, 2002:35).

Although zinc may be extremely effective in the treatment of acne, it must be taken at the correct concentration and in the correct combination to ensure optimal absorption in the body, and thus, optimal efficiency in acne treatment (Holistic Acne Treatments.com, 2007:2). Zinc is best used with tolerable levels of copper, calcium, phosphorous, selenium, vitamin A, vitamin B_6 and vitamin E (Sallamander Concepts (Pty) Ltd, 2009:2).

Zinc is a very important element in the maintenance process of the skin in keeping it healthy, due to its involvement in protein synthesis and collagen formation in the body (Nutritional-supplements-health-guide.com, 2005:2). Other important uses of zinc in skin care applications include its role in wound healing and inflammatory processes. According to previous studies, zinc and protein containing zinc are involved in almost every stage of the cutaneous wound repairing process. The significant role of zinc in the wound healing process includes the modification of extracellular matrix migration of cells, protein synthesis and the reduction of inflammation (Schwartz *et al.,* 2005:841).

2.2.2.5 Adverse effects

Side effects from the intake of natural zinc are rare, but occur with the intake of elevated levels. Chronic intake of more than 100 mg of zinc a day causes adverse effects, i.e. reduction in high-density lipoprotein (HDL) cholesterol, inhibition of the immunological response and a reduced copper status that may lead to anaemia. With the intake of large amounts of zinc, acute adverse effects occur. These include nausea, vomiting, abdominal cramps, loss of appetite, diarrhoea, headaches, dehydration, dizziness and epigastric pain (Institute of Medicine, 2001:482).

2.2.5 SUMMARY

The aim of this study was to combine vitamin A and zinc in topical formulations, like creams and gels, for the possible topical treatment of acne. The function of vitamin A would be to provide a down regulation of sebum production, modify anti-inflammatory properties and cellular differentiation. Zinc would help to normalise the hormone imbalances and sebum secretion, and to maintain the health of the skin (Nutritional-supplements-health-guide.com, 2005:2; Hostýnek & Maibach, 2002:35).

2.3 TRANSDERMAL DRUG DELIVERY

2.3.1 INTRODUCTION

The human skin is the largest organ in the body and accounts for approximately 10% of the total body mass, whilst covering an average area of 1.7 m^2 (Williams, 2003:1). It is an easily accessible organ that offers multiple sites to administer therapeutic agents for local and systemic action (Mukhtar, 1992:4; Williams, 2003:1). The skin forms an interesting and unique barrier between the external and internal environments of our bodies, therefore, its major function is to protect the body against unwanted influences from the outside environment and the loss of endogenous substances, for example water (Williams, 2003:1; Hadgraft, 2004:291). Due to the variety of lipophilic and hydrophilic domains, the barrier is very heterogeneous. An

ideal drug will therefore have to consist of both hydrophilic and lipophilic properties (Kalia & Guy, 2001:160; Hadgraft, 2004:292). However, the biggest challenge in transdermal drug delivery is to overcome this natural barrier.

2.3.2 STRUCTURE AND FUNCTIONS OF HUMAN SKIN

Microscopically, the skin is a multi-layered organ, which is generally divided into three main layers, namely the subcutaneous fat layer, the dermis and epidermis (Abraham *et al.*, 1995:8). A simplified diagram of the skin structure with its three layers is demonstrated in Figure 2.5.

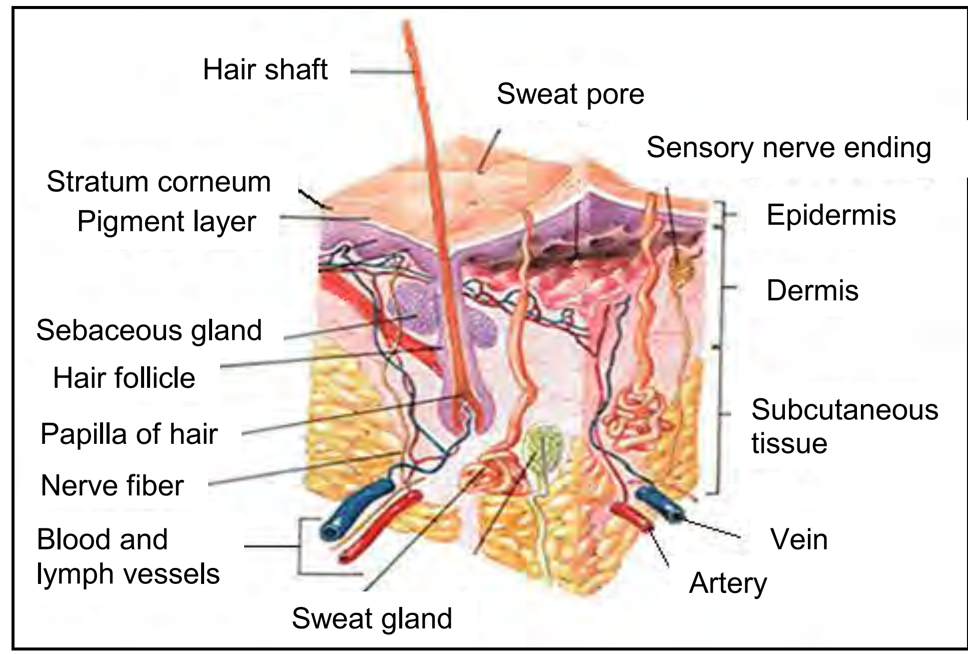


Figure 2.5: Graphic representation of the skin structure with its three main layers (Adapted from Van de Graaf, 2002)**.**

2.3.2.1 Subcutaneous tissue

The subcutaneous tissue (hypodermis) is positioned between the overlying dermis and the underlying body constituents. This is the innermost layer of the skin and is relatively thick in most areas of the body, but is absent in the eyelids and in the male genital region. The thickness of the hypodermis varies with age, sex, endocrine, and nutritional status of the individual. The hypodermis functions as mechanical protection against physical shock, as well as an energy reserve to insulate the body, whilst the principal blood vessels and nerves are carried to the skin in this layer (Williams, 2003:2).

2.3.2.2 Dermis

The dermis (1 – 5 mm in thickness) is situated between the epidermis and a region of the subcutaneous fat layer. The dermis essentially consists of a matrix of connective tissue, mainly collagen fibrils that support the skin and elastic tissue for flexibility (Barry, 2002:502). In terms of transdermal drug delivery, the dermis is not usually a barrier to the permeation of most polar drugs, but the barrier function may be outstanding for highly lipophilic molecules. The dermis is penetrated by blood and lymphatic vessels, nerves and skin appendages. Capillaries provide efficient blood flow in order to dilute and absorb most chemicals *in vivo,* which then penetrate past the stratum corneum and viable epidermis (Barry, 1983:7; Williams, 2003:2).

Hair follicles, sebaceous glands and sweat glands originate in the dermis and are collectively known as the skin appendages (Foldvari, 2000:418). Hair follicles are distributed over the entire skin surface, with the exception of the palms of the hands, soles of the feet and the lips. Every hair follicle holds one or more sebaceous gland(s) that forms an oily substance, called sebum (Williams, 2003:4). Sebum protects the skin from bacterial and fungal infections and controls water loss from the skin.

2.3.2.3 Epidermis

The epidermis is situated above the dermis and is divided into two parts, namely the inner layer, called the viable epidermis (150 μm) and the stratum corneum (10 – 15 μm), which forms the outermost part (Abraham *et al*., 1995:8). The viable epidermis consists of three distinct layers, identifiable from bottom to top are (1) the stratum germinativum (basal layer), (2) stratum spinosum (spinous layer) and the (3) stratum granulosum (granular layer). The stratum lucidum is sometimes described as a fourth layer, but it is usually considered to be the lower layer of the stratum corneum (Flynn, 2002:191).

Predominantly, the epidermis consists of cells called keratinocytes. However, it also contains melanocytes, Merkel cells and Langerhans cells (Wickett & Visscher, 2006:98). The main function of the epidermis is to form the stratum corneum through proliferation and differentiation of the epidermal cells. Therefore, the stratum corneum could provide the rate limiting barrier to transdermal and topical drug delivery, due to its highly hydrophobic nature (Williams, 2003:1). This layer mostly comprises of dead anucleate, keratinised cells (corneocytes), surrounded by lipids. This arrangement is usually simplified to a bricks (corneocytes) and mortar (lipid bilayers) domain (Barry, 2001:102; Williams, 2003:9). It is constantly being regenerated, where new cells replace shedded cells every two weeks in normal individuals (Flynn, 2002:189).

The primary functions of the stratum corneum are to protect the body against chemical and mechanical attacks, as well as from dehydration (Wickett & Visscher, 2006:98). It exhibits

selective permeability and is only able to diffuse lipophilic compounds and drugs with a low molecular weight (Naik *et al.,* 2000:319).

2.3.3 DRUG TRANSPORT THROUGH THE SKIN

According to Ranade and Hollinger (2004:208), transdermal permeation is the movement of the drug from the outside of the skin, through its various layers, into the bloodstream. For the drug to permeate through the skin layers, passive diffusion is involved. The main barrier affecting permeation of some drugs may exist in the aqueous, viable epidermal membrane, but for other molecules the rate limiting barrier to delivery is the stratum corneum (Begoña Delgado-Charro & Guy, 2001:213). Permeation may be described through a multi-step process, which starts at the application of the formulation to the skin surface and ends in the systemic circulation. This process involves:

1. Dissolution within and release of the drug from the topical applicaton to the skin surface.
2. Partitioning into the stratum corneum.
3. Diffusion through the stratum corneum *via* lipidic intercellular pathways (rate limiting step).
4. Partitioning from the stratum corneum into the viable epidermis.
5. Diffusion through the cellular mass of the viable epidermis into the upper dermis.
6. Capillary uptake of the therapeutic agent in order to reach the systemic circulation (Kalia & Guy, 2001:160).

2.3.3.1 Penetration pathways across the skin

Permeation may occur by diffusion *via* three potential entry routes, i.e. the transcellular, intercellular and the transappendageal penetration routes.

2.3.3.1.1 Transcellular penetration route (the shortest route)

The transcellular route transports the solute *via* diffusion directly through the keratinocytes of the stratum corneum, as illustrated in Figure 2.6. Diffusion of hydrophilic molecules is rapid, due to the aqueous environment of this component. The rate limiting barrier for permeation *via* this route is the multiple, bilayered lipids, located between the keratinocytes that must be crossed by the molecules. The diffusional path length is related to the thickness of the stratum corneum, which is approximately 20 μm (Williams, 2003:32-34).

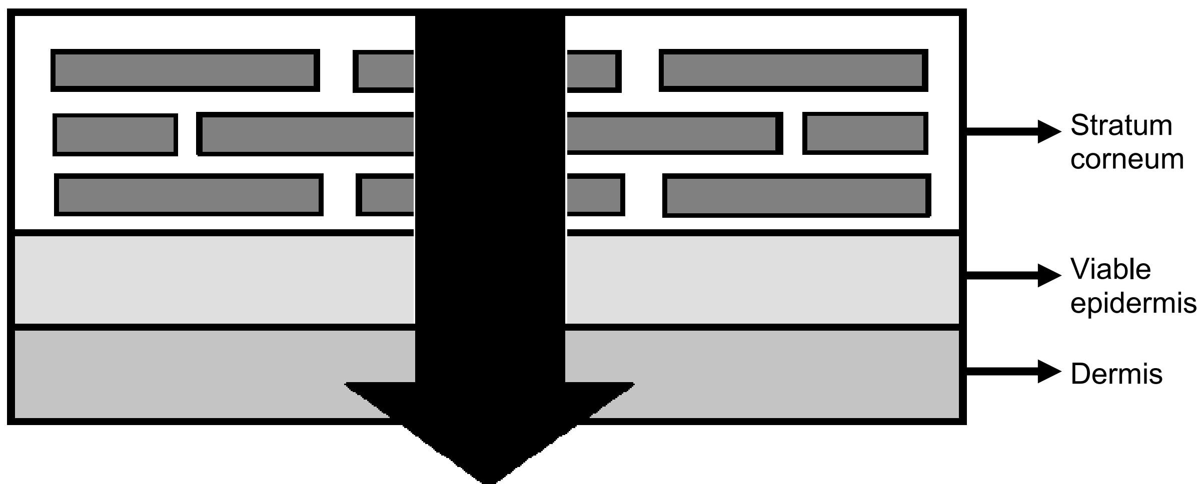


Figure 2.6: Schematic representation of the transcellular penetration route.

2.3.4.2.2 Intercellular penetration route

The intercellular penetration route provides the main pathway for the permeation of small, uncharged molecules through the stratum corneum (Williams, 2003:34). Drugs are transported *via* diffusion through the channels between the cells, as shown in Figure 2.7. The diffusing molecule has to cross a selection of lipophilic and hydrophilic domains, before the junction between the stratum corneum and the viable epidermis is reached. Therefore, the estimated path length is approximately 500 µm, thus, longer than that of the transcellular route (Hadgraft, 2004:292).

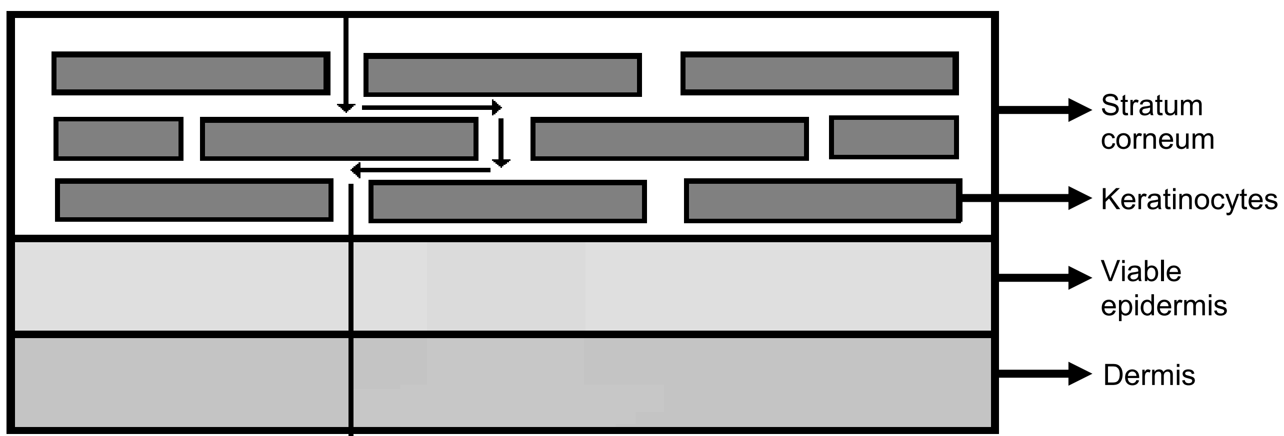


Figure 2.7: Schematic representation of the intracellular penetration route.

2.3.3.1.3 Transappendageal penetration route (shunt routes)

This penetration route provides a parallel pathway, by which solutes can be absorbed *via* the sebaceous pathway of the pilosebaceous apparatus, and the aqueous pathway of the eccrine sweat glands, as demonstrated in Figure 2.8. Absorption takes place without the hindrance of

the stratum corneum (Abraham *et al.*, 1995:9). The area available for transport covers only 0.1 % of the total skin surface. This route may be of great value for large polar molecules, with three or more polar groups that experience high resistance in the stratum corneum (Barry, 2001:101).

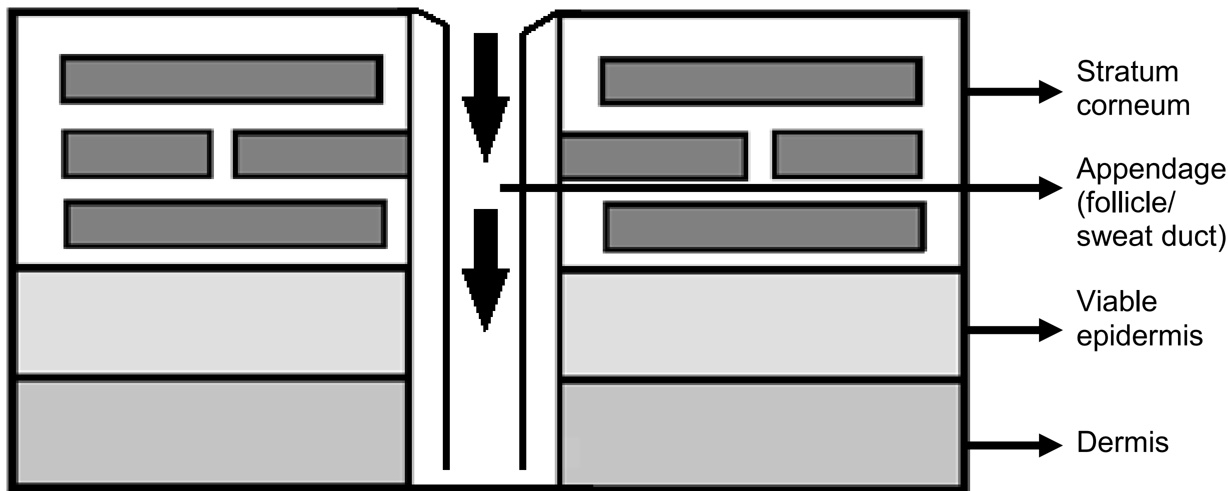


Figure 2.8: Schematic representation of the transappendageal penetration route.

2.3.5 FACTORS AFFECTING PERCUTANEOUS PENETRATION

In the following section, the physiological and physiochemical factors that influence the transdermal delivery of drugs, are discussed.

2.3.5.1 Physiological factors affecting transdermal drug delivery

Besides genetic aspects that cause variation between individuals with regards to the absorption and metabolism of drugs, physiological factors, like skin age, hydration of the skin, region of application, metabolism of the skin and skin disease, may also affect the rate of percutaneous penetration of topical drugs (Farahmand & Maibach, 2009:11).

2.3.4.1.1 Skin age

Skin condition and structure varies with age. The effect of aging alters the barrier functions of the skin and therefore the drug permeation through the skin (Farahmand & Maibach, 2009:11). In the elderly, skin permeation can be affected through the reduction in blood flow that decreases transdermal drug flux, together with reduction in skin elasticity (Williams, 2003:14). In children, the potential for toxic effects of drugs and chemicals are markedly higher, because of the greater surface area per unit body weight, and the variation with age in blood flow and thickness of the skin (American Academy of Paediatrics, 1997:143; Barry, 2002:510).

2.3.4.1.2 Skin hydration

Hydration of the skin is a major factor affecting transdermal absorption (Behl *et al.,* 1993:238; Riviere, 1993:117). The stratum corneum is directly affected and could increase transdermal delivery by increasing the level of hydration, which is why hydration of the skin is considered to be a beneficial penetration enhancer (Barry, 2007:576).

Although the precise mechanism of skin hydration remains unclear, Idson (1975:908) speculates that the opening of the pores contributes to better skin permeation. Water gathers on the outer surface of the protein filaments during hydration, allowing polar and non-polar molecules to penetrate the skin (Idson, 1975:905).

2.3.4.1.3 Region of application

Variations in skin permeability, regarding anatomical site on the body, depends on the nature and thickness of the stratum corneum (Barry, 2007:575). Other properties that include sebum production and the density of hair follicles and glands, may also play a role in the permeation process (Amsden & Goosen, 1995:1975).

Due to regional variation in the structure of the skin, the stratum corneum is thicker on the soles and palms, than on the facial regions (Williams, 2003:16). The face is thus more permeable than other body sites (Barry, 2007:576).

The concentration of a drug that reaches the systemic circulation, is influenced by the region of application and should therefore be taken into account when applying the permeant (Schalla & Schaefer, 1982:57).

2.3.4.1.4 Skin metabolism

The skin is capable of metabolising a large number (approximately 5%) of topical drugs (Amsden & Goosen, 1995:1975). Metabolism occurs mainly in the viable epidermis, which is the most metabolically active layer of the skin (Begoña Delgado-Charro & Guy, 2001:212).

According to Kao *et al.* (1984:289), lipophilic compounds are converted into more water soluble forms to promote elimination from the skin, as hydrophilic drugs penetrate the skin more rapidly when the metabolic rate is higher (Bando *et al.,* 1997:759).

2.3.4.1.5 Damage and diseases of the skin

Since the skin is the first part of the body that is exposed to the outside world, it also is the first to sustain damage. An intact skin limits absorption, as it has barrier properties.

Therefore, a diseased or damaged skin reduces the barrier action and enhances percutaneous absorption. Diseased skin includes acne, psoriasis, atopic dermatitis, cracks, scratches and cuts (Jackson, 1993:179).

2.3.5.2 Physicochemical factors affecting transdermal drug delivery

When considering the general overview of the permeation process, an ideal drug candidate would have sufficient lipophilic properties to partition into the stratum corneum, but also sufficient hydrophilic properties to partition into the viable epidermis and finally the systemic circulation (Kalia & Guy, 2001:160).

2.3.4.2.4 Solubility and melting point

Lipid solubility of a drug is considered to be an essential feature in transdermal absorption, because of the corneocytes in the stratum corneum that are relatively hydrophobic. As the skin is a multiple layered tissue that consists of both hydrophilic and lipophilic domains, it is also important for the molecule to exhibit measurable aqueous solubility, in order for it to penetrate the aqueous boundary layer (viable epidermis). In order to avoid potential bioavailability problems, the aqueous solubility of the substance needs to be more than 1 mg/ml (Naik *et al.*, 2000:319).

Although the aqueous solubility of vitamin A is unknown, it was found to be insoluble in water, but soluble, or partly soluble in anhydrous ethanol and miscible with organic solvents. Zinc is freely soluble in water (430 mg/ml) and soluble in ethanol (96%). Therefore, zinc is an ideal candidate for penetrating the skin.

Some authors (Hadgraft & Wolff, 1993:163; Hadgraft & Finnin, 2006:365) state that the lower the melting point of a material, the greater the ability to penetrate the skin, due to the relationship between the melting point and solubility, *via* the latent heat of fusion. A crystal with a strong lattice will express a low melting point and low heat of fusion (Wells & Aulton, 2007:349). This limits solubility and also the mass transfer across the skin, due to the fact that the drug with the highest melting point will not easily give up molecules to dissolve (Buckton, 2007:112).

It is therefore clear that compounds with a low melting point (< 200°C) (Naik *et al.*, 2000:319) will have a direct effect on the solubility and will have a comparatively higher transdermal permeation ability (Barry, 2001:102).

2.3.4.2.5 Partition coefficient

The partition coefficient (P) is the ratio of the distribution of molecules between two phases (oil and aqueous phase) (Ansel & Popovich, 1990:36).

Due to the occurrence of experimental difficulties in determining the appropriate skin/water partition coefficient in transdermal delivery studies, the octanol/water partition coefficient (log P) is the parameter most widely used for predicting the partitioning of the drug between the lipophilic stratum corneum and the underlying hydrophilic viable epidermis (Farahmand & Maibach, 2009:2).

According to Williams (2003:36), a compound with a log P between 1 - 3 will permeate the skin comparatively fast, and it is also an indication of the ability of the drug to dissolve in both oil and water. Both low and high log P values may be associated with poor skin permeability. Drugs with a low log P value have minute partitioning in the skin lipids, and therefore, show low permeability, whereas drugs with a high log P value subside in the stratum corneum and also have low permeability (Thomas & Finnin, 2004:699).

The experimental log P value for zinc is 1.22, which indicates that zinc is able to permeate the skin (Drugbank, 2008). No log P value for vitamin A was found in the literature, but due to its high lipophilicity, skin penetration is likely to be poor.

2.3.4.2.6 Molecular size

The molecular size of a drug substance influences various physical and chemical properties, including the penetration rate of the drug and mainly, the absorption of the drug (Ansel & Popovich, 1990:100). Considering that the stratum corneum is a compact membrane and that diffusing molecules follow a tortuous path through it, it might seem obvious that the penetration rate of a drug decreases exponentially with an increase in molecular size. The same conclusion can be made in terms of the absorption of a drug and its molecular size (Naik *et al.,* 2000:319).

It is well documented that large molecules (more than 500 Dalton) (Naik *et al*., 2000:319) may have difficulty diffusing through the skin, whereas small molecules will diffuse relatively easier, and thus permeate the skin more efficiently (Hadgraft & Finnin, 2006:365).

The molecular weight of zinc and vitamin A is 219.51 Da and 328.49 Da, respectively (Sigma-Aldrich, 2010).

2.3.4.2.4 Hydrogen bonding

As described in the literature, the number of hydrogen bonds within a molecule is important in

determining its permeability. If there are too many hydrogen bonds present within a molecule, the absorption could be influenced negatively. For a molecule to be well absorbed, there should be no more than 5 hydrogen bond donors and no more than 10 hydrogen bond acceptors present. The reason why some drugs, e.g. peptides, are poorly absorbed, is because of the large hydrogen bond capacity present (Ashford, 2002:244). Pugh (1999:189) further states that the stratum corneum is predominantly a hydrogen bond donor barrier.

Lipid solubility of a drug molecule can be increased by substitution, esterification or alkylation, through which the tendency of hydrogen bonding of a drug molecule is minimised. Although it may decrease the aqueous solubility, an increase can be observed in the partitioning of the drug into lipidic membranes (Hillery, 2001b:78).

2.3.4.2.5 State of ionisation

An ionisable drug means that there are both charged and uncharged species present in the penetrant (Smith, 1990:27). When considering the non-polar nature of the horny layer, it is widely believed that charged (ionised) drugs are poor transdermal penetrants (Williams, 2003:38). The pH partition hypothesis states that the unionised form of a drug can permeate the skin better, due to its higher lipid solubility (Barry, 2007:576), hence to summarise:

- Unionised form of a drug = lipophilic → membrane transport.
- Ionised form of the drug = hydrophilic → minimal membrane transport (Hillery, 2001a:24).

As was previously mentioned, permeation through the skin can occur *via* several pathways. Although ionisable drugs are poor transdermal candidates, they can cross the membrane by shunt routes. The quantity of drugs permeating *via* these routes may be somewhat less than drugs traversing the stratum corneum membrane *via* the lipoidal intercellular route in the unionised form (Williams, 2003:38). To overcome this limitation, the ionised drug is combined with an oppositely charged ion, forming an ion pair of which the overall charge is neutral. The neutral drug complex enhances the transdermal flux (Shargel *et al.,* 2005:382).

Most drugs are either weak acids, or weak bases (Billany, 2007:364). To achieve optimum bioavailability and to maintain solubility, knowledge of the drug"s pKa and the pH of its surrounding environment is required (Wells & Aulton, 2007:338). The stratum corneum is extremely resistant to variations in pH, tolerating a range of 3 – 9 (Barry, 2007:576). A weak acid is at its maximum solubility at a high pH in its fully ionised form, and *vice versa* under low pH conditions (Aulton, 2007:37).

2.3.4.3.6 Diffusion coefficient

The diffusion coefficient of the permeant is a term concurrently used with diffusivity. Diffusivity is defined as a property of the permeant in the membrane and is a measure of how easily a molecule will traverse through the tissue (Williams, 2003:27). The diffusion coefficient can therefore be defined as the amount of a drug that diffuses across a membrane (in this case the stratum corneum), or within the various strata of a given area per time unit (cm^2/s or cm^2/h) (Williams, 2003:223). The diffusion constant is influenced by the properties of the drug, the medium through which it diffuses, and the degree of interaction between these (Smith & Surber, 2000:29).

The drug"s affinity for the vehicle (formulation), the temperature of the vehicle and its viscosity are other parameters that influence the diffusion coefficient. The diffusion coefficient increases when a drug with a low affinity for the vehicle is used. Due to the fact that diffusion through the stratum corneum is a passive process, increasing the temperature noticeably increases the permeant diffusion coefficient. The temperature gradient across the human skin is approximately 32 °C at the outer surface. Thus, with elevation of the skin temperature, diffusion through the tissue may increase. Appropriate temperature control is thus vital for good experimental design (Williams, 2003:18). According to the literature, the diffusion coefficient in the stratum corneum is $10^{-9} – 10^{-13}$ (cm^2/s), and in the viable epidermis approximately 10^{-6} (cm^2/s) (Scheuplein & Blank, 1971:762).

2.3.5.3 Mathematics of skin permeation

Passive diffusion is the process by which molecules diffuse spontaneously from a high concentration of the substance (application of formulation on the stratum corneum) to a lower concentration (within the strata of the skin). Permeation through the outermost layer of the skin occurs by passive diffusion, and can often be described mathematically by Fick"s first law of diffusion (Begoña Delgado-Charro & Guy, 2001:213). It states that the rate of transfer of diffusing substances through a unit area of a section is proportional to the concentration gradient measured, normal to the section. Fick"s second law states that the rate of change in concentration with time at a point within a diffusional field is proportional to the rate of change in the concentration gradient at that point (Williams, 2003:41-42).

Fick"s law of diffusion can be written in the following generalised form:

$$J = \frac{K \times D}{h} \Delta C$$ *Eq 2.1*

Where: J = the flux (µg/cm^2.h)

K = the partition coefficient of the drug into the membrane

D = the diffusion coefficient of the drug in the membrane (cm^2/h)

h = the membrane thickness (cm)

ΔC = the concentration gradient (µg/cm^3) (Hillery, 2001a:13)

The total amount of permeant that diffuses through the skin in a predetermined amount of time is termed the flux (Williams, 2003:41). From Equation 2.1 it is evident that there are three important factors that influence the rate at which drugs penetrate the skin. Firstly, the concentration of the drug in the vehicle, secondly the partition coefficient of the drug between the stratum corneum and the vehicle, and lastly the diffusivity of the drug within the stratum corneum (Walters, 1990:86).

Percutaneous absorption could be enhanced through the following:

- An increase in the total delivered dose from a single application, by increasing the quantity of drug in the vehicle (the rate is not necessarily enhanced).
- Minimising the barrier function of the stratum corneum by either disturbing the intercellular lipid domains, and the intracellular keratin networks, or enlarging the appendageal transport *via* the sweat glands or follicles.
- Smoothing the progress of drug partitioning from the vehicle into the skin, i.e. increasing the solubility of the drug in the stratum corneum.
- Supporting partitioning at the interface of the stratum corneum and the viable epidermis (Begoña Delgado-Charro & Guy, 2001:228).

2.3.5 PENETRATION ENHANCERS

The multiple bilayered lipids of the stratum corneum provide the principle pathway for permeation through this membrane. Therefore, the stratum corneum consists of a barrier that is highly efficient and effective (Williams, 2003:83). Some compounds are unable to permeate through the skin and this factor makes it necessary to either optimise the permeation of the chosen drugs through the skin, or to reduce the impermeability of the skin. This could be achieved through the use of permeation enhancers (Barry, 2002:522).

The characteristics of an ideal enhancer are the following:

- It should show no pharmacological action of its own.
- It should be non-allergenic, non-toxic and non-irritating.
- The material should have a rapid onset of action with a suitable and predictable effect.
- The stratum corneum should immediately and fully recover its normal barrier properties, after removal of the material.
- It should be physically and chemically stable and compatible with all drugs.
- It should be cosmetically acceptable, by showing good spreadability.
- It should be readily incorporated into the preparations being used topically.
- It should be colourless, odourless and tasteless (Barry, 2002:522).

A variety of penetration enhancement techniques are available, including the enhancement *via* chemicals, vesicles, such as liposomes, modification of the stratum corneum and electrically assisted enhancement techniques. A complete description of these penetration enhancers and techniques is beyond the scope of this dissertation. Hence, only techniques that are relevant to this study will be discussed briefly.

2.3.5.1 Chemical penetration enhancers

Chemical penetration enhancers provided the starting point for research in the transdermal delivery section. According to Williams (2003:86), the ideal chemical must offer a reversible reduction in the barrier properties of the skin, without causing long-term damage to the underlying skin cells.

Chemical penetration enhancers consist of two major categories, i.e. those that impact diffusion across the stratum corneum and those that adjust partitioning into the stratum corneum. The former class of permeation enhancers assist permeation by disruption of the well organised nature of the skin lipids and by an increase in the fluidity. The latter class of permeation enhancers works by altering the solubility properties of the skin and thereby increasing the solubility of the drug within the stratum corneum (Thomas & Finnin, 2004:700).

Water is the safest and most widely used penetration enhancer, as it opens up the compact structure of the horny layer (Barry, 2002:510). Under normal conditions, the water content of the stratum corneum is approximately 20% (w/w). Hydration of the stratum corneum may result from occlusion of the skin, which allows for increased drug delivery of topically applied drugs (Williams, 2003:84).

Other existing chemical penetration enhancers include alcohols, polyalcohols, pyrrolidones, amines, amides, fatty acids, sulphoxides, esters, terpenes, alkanes, surfactants and phospholipids (Barry, 2002:523).

An important factor to keep in mind is that absorption into circulation might not necessarily be improved when the diffusion through the stratum corneum is maximised (Hadgraft & Wolff, 1993:164).

2.3.5.2 Physical penetration enhancers

Physical penetration enhancers make use of an energy source to overcome the barrier properties of the skin. A reservoir of drug is provided on the skin surface, from which the needed levels of delivery can be reached (Thomas & Finnin, 2004:699).

Iontophoresis and electroporation are examples of physical penetration enhancers that have been used for the delivery of highly potent, or large molecular weight compounds (Thomas & Finnin, 2004:699).

Low-frequency sonophoresis (ultrasound) is a technique that is primarily used in physiotherapy. It is defined as sound of a frequency higher than 20 kHz. Sonophoresis enhances drug delivery through a combination of thermal, chemical and mechanical adjustments within the skin tissue. Ultrasonic energy induces the formation of small gaseous pockets within cells (cavitation), increases pore size and causes modifications in the lipid structure of the stratum corneum, which enhance drug penetration into the tissue (Naik *et al*., 2000:324).

An alternative approach to bypass the stratum corneum involves the use of micro needles. This device consists of 400 micro needles that are inserted into the skin, through which the drug is transported across the stratum corneum. This method of administration is not painful (Barry, 2001:107).

2.4 DRUG DELIVERY VEHICLES

2.4.1 PHEROID™ TECHNOLOGY

Pheroid™ technology, the patented delivery system, is based on what was earlier known as Emzaloid™ technology. It is reported to enhance the absorption of various categories of drugs into the viable epidermis, or into the underlying dermis (Grobler *et al.,* 2008:284).

The Pheroid™ drug delivery system is a submicron, emulsion type formulation that consists of essential and plant fatty acids (Grobler, 2004:4). These fatty acids are compatible with the fatty acids present in the human body, since they are oriented in the *cis*-formation. They enhance

penetration and delivery of drugs, due to the affinity that exists between the fatty acids and the human cell membrane (Grobler, 2004:4). The Pheroid™ drug delivery system is highly efficient in comparison with other delivery systems, due to its effective entrapment of the drug, the significant rate of transportation of the drug and the delivery of the drug to specific target sites in the body (Grobler, 2004:4; Grobler *et al.,* 2008:305).

Penetration of the skin, keratinised tissue, fungi, bacteria and parasites occurs readily with the use of Pheroids™, due to the morphological manipulation and alteration in the size and shape of the vesicles. The uptake mechanism of Pheroids™ by cells is still unclear, but it is thought to be actively facilitated by the process of protein mediated transfer (Grobler, 2004:4).

Pheroid™ technology, known as a skin friendly carrier, consists of many advantages, including the following:

- Increases the therapeutic value.
- Increases the delivery of active compounds.
- Reduces the cytotoxicity.
- Decreases time to onset of action.
- Reduces minimal effective concentration.
- Penetrates the most known barriers in the body and cells.
- Targets treatment areas.
- Deficient immunological response.
- Reduces resistance of the drug.
- Transfers genes to the cell nuclei (Grobler, 2004:3).

Various successes as reported in using Pheroid™ technology, for example, the entrapment of viruses, bacteria and anti-malarials (Grobler, 2004:14). It was decided to use Pheroids™ in this study was, due to its reported effectiveness and versatility as a delivery system in pharmaceutical applications (Grobler *et al.,* 2008:293).

Pheroids™ exist in different types, each with a particular composition. The different types include:

- Lipid bilayer vesicles with nano- and micrometer diameters.
- Reservoirs or depots that contain pro-Pheroid™.
- Micro-sponges (Grobler, 2004:5).

The Pheroid™ delivery system is a colloidal system, containing micron- and submicron sized spherical structures that are lipid-based and evenly dispersed in the formulation (Grobler, et al., 2008:284). One of the most impressive characteristics of the Pheroid™ is its particle size that ranges between 200 nm - 2 µm, whereas normal colloidal systems only contain particles that are between 1 – 1000 nm in diameter (Grobler *et al.,* 2008:285).

The formation of Pheroids™ is through a self-assembly process, similar to that of low energy emulsions and micro-emulsions. In comparison with its peer, the liposome, it generally contains a lipid bilayer, but without the presence of phospholipids or cholesterol. As with emulsions, the Pheroids™ are also dispersed in a dispersed medium with two liquid phases, as well as an extra dispersed gas phase. The gas phase is related to the dispersed fatty acid phase (Grobler *et al.,* 2008:288).

The main component of Pheroids™ is the *cis*-formatted ethylated and pegylated polyunsaturated fatty acids, with the exclusion of arachidonic acid. These essential fatty acids in Pheroids™ cannot be manufactured by human cells. As it is important for various cell functions, it has to be ingested. Some of the significant advantages of these fatty acids are:

- Energy homeostasis.
- Maintenance of the cell"s membrane integrity.
- Modulation of the immune system through the prostaglandine/leukotrin cascade (Grobler, 2004:5).

Apart from fatty acids, Pheroids™ also contain a unique nitrous oxide (N_2O) component. This dispersed gas is distributed in union with the dispersed phase throughout the continuous phase. Its association contributes to the miscibility of the oil and water phases of fatty acids, the self-assembly process and the stability of the Pheroid™ (Grobler *et al.,* 2008:289). Interaction between the nitrous oxide and the fatty acids results in a stable vesicular structure of the Pheroid™. It therefore provides a matrix that is able to transport hydrophobic and hydrophilic drugs effectively through the skin layers (Grobler *et al.,* 2008:290).

The presence of fatty acids in Pheroids™ inhibits water loss by the epidermis and supports the intercellular lipid layers that maintain the barrier properties of the skin. That is why the Pheroid™ is an ideal drug delivery system to use in transdermal delivery of drugs (Grobler *et al.,* 2006:25).

Clearly, the Pheroid™ drug delivery system is very different from other conventional macromolecular carriers, such as liposomal delivery systems. Due to the various advantages associated with Pheroids™, it was decided to use this technique in the transdermal delivery of

retinyl acetate and zinc acetate. Table 2.5 summarises the similarities and differences between other lipid-based delivery systems and the Pheroid™ drug delivery system, as well as the essential advantages of both systems (Grobler, 2004:6-8).

Table 2.5: Similarities and differences between Pheroid™ and other lipid-based delivery systems (Grobler, 2004:6).

Pheroid™	Other delivery systems
Mainly consists of fatty acids that are natural to the body.	Delivery systems usually contain substances foreign to the body.
No immune responses in man are obtained, according to cytokine studies.	Some liposomal formulations cause immune responses in man.
A wide range of Pheroid™ is formulated, depending on the method of manufacture and the composition.	Various liposomes have been described, namely, single lamellar vesicles, multi-lamellar vesicles, multivesicular vesicles and nanosomes.
Manipulation in terms of size, charge, lipid composition and membrane packing can be obtained. The desired type(s) can repeatedly be obtained.	The degree of repeatability of liposomal systems, liposomal types and sizes has been described as problematic.
The presence of fatty acids in the formulation causes affinity between the Pheroid™ and cell membranes.	Specific binding and uptake mechanisms have not been described for other delivery systems in mammalian.
The polyphilic nature of the Pheroid™ can entrap drugs that have different solubility values, as well as insoluble drugs.	Most delivery systems are either lipophilic or hydrophilic.
Sterically stabilised without the disadvantages of increased size, or decreased elasticity.	Delivery systems generally need to be sterically stabilised.
Enhances the bioavailability of oral, topical and buccal administration of active compounds in most products tested to date, as well as increased absorption.	Some delivery systems tend to enhance absorption, whilst others decrease absorption.
Entrapment efficiency in all tested compounds is between 85 - 100%.	Entrapment efficiencies may be problematic, due to charge and steric limitations.
For combination therapies, micro-sponges can be used, as one drug can be entrapped in the interior volume and the other in the sponge spaces.	Combination therapies are problematic for most delivery systems.

2.5 CONCLUSION

Acne vulgaris is a common pilosebaceous disease, which occurs in the dermis. The pathogenesis includes excess sebum production, *P. Acnes* activity, hyperproliferation of the sebaceous follicle and inflammatory tissue response. Acne may have an enormous psychological impact, and should therefore be treated accordingly. There is a wide range of topical and systemic treatments available for acne. The advantage of topical treatment is its reduced adverse effects.

This study focused on the transdermal delivery of vitamin A and zinc, formulated into topical, combination formulations, for use in the possible treatment of acne. Vitamin A was used to diminish sebum production, reduce inflammation and modify cellular differentiation, whereas zinc was included to normalise hormone imbalances and the secretion of sebum. Zinc is known as being overall very advantageous in the maintenance of skin health.

The unique structure of the skin allows it to perform specialised functions, of which the most important function is its excellent barrier properties, due to the outermost layer, the stratum corneum. Penetration of environmental chemicals into the skin is hence limited. The target of acne treatment is located in the dermis. To overcome these barrier properties of the stratum corneum and enhance the penetration of vitamin A and zinc across the skin, it was decided to investigate the use of Pheroid™ technology during this study. Pheroid™ technology was incorporated into two of the four cosmeceutical formulations developed during this study, i.e. in a cream and in a gel, in order to investigate any positive correlation between the use of Pheroid™ technology and the transdermal delivery of the drugs.

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CHAPTER 3

ARTICLE FOR PUBLISHING IN SKIN PHARMACOLOGY AND PHYSIOLOGY

Chapter 3 is written in an article format for the purpose of publication in Skin Pharmacology and Physiology. The complete author‟s guide of this journal is given in Appendix E.

Formulation, *in vitro* release and transdermal diffusion of vitamin A and zinc for the treatment of acne

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Keywords: Vitamin A, zinc, acne, transdermal diffusion, formulation, Pheroid™

Abstract

Background/Aim: Acne is a disease of the pilosebaceous unit in the dermis. Topically applied vitamin A (retinoic acid), has mainly been used in the treatment of acne, but has the disadvantage of causing skin irritation. New strategies for acne treatment are needed in which the side effects can be eliminated. The acetate derivatives of vitamin A and zinc, in combination, are a new approach to acne treatment. The aim of this study was to conduct transdermal diffusion studies on four topical formulations, containing vitamin A and zinc. Of these products, two were formulated with Pheroid™ technology. **Methods:** Diffusion studies and tape stripping methods were used to determine the transdermal, epidermal and dermal diffusion of the actives. **Results/Conclusions:** Vitamin A was not delivered transdermally, but to its intended target site for the treatment of acne (dermis). Although zinc showed transdermal diffusion with the cream and Pheroid™ cream, no zinc was delivered to the dermis, when compared to the placebo formulation. Only the cream formulation may have possibly delivered zinc to the epidermis.

1 Introduction

Acne vulgaris is the single, most common disease, affecting the pilosebaceous unit in the dermis [1]. This condition affects approximately 80% of adolescents and to some extent young adults [2]. The common trigger for the development of acne is puberty, associated with the androgen hormone. Factors that contribute to the pathogenesis of acne include plugging of the follicle, accumulation of sebum, growth of *Propionibacterium acnes* (*P. acnes*) and inflammatory tissue response [3].

Although acne is not life threatening, it can have serious psychological consequences (low self-esteem, social inhibition, depression), if left untreated, and should therefore be recognised as a serious disorder [1]. Acne could clinically be expressed as non-inflammatory, open and closed comedones, and inflammatory papules, pustules and nodules [4]. The aim of acne treatment therefore focuses on the reduction of the inflammatory and non-inflammatory acne lesions, and thus on halting the scarring process [5]. To fulfil this aim, different topical and systemic acne therapies are available. Topical treatment for acne is the most suitable first-line therapy for non-inflammatory comedones, or mildly inflammatory disease states, with the advantage of avoiding the possible systemic effects of oral medications [6].

Topical treatment of acne involves the use of retinoid analogues, for example tretinoin, isotretinoin, adapalene and tazarotene. Although they have been used with great success, their effectiveness in long term therapy has been reduced, due to local skin irritations that occur [7]. Vitamin A acetate does not have such harsh side effects and is therefore regarded as a new approach in the treatment of acne [8]. The acetate derivative of zinc is also known for its lower irritancy when applied to the skin [9]. During this study, the vitamin A acetate and zinc acetate combination was formulated into four semisolid formulations, i.e. two creams and two emulgels, in an attempt to find effective acne treatment therapies. Vitamin A was included with the aim of controlling the development of microcomedones, to reduce existing comedones, diminish sebum production and moderately reduce inflammation [10], whereas zinc was used for its ability to normalise hormone imbalances [11], as well as the secretion of sebum [9].

Transdermal delivery of drugs has become increasingly popular, due to its numerous advantages over other traditional delivery methods. Although it offers an attractive route, there

are unfortunately many factors limiting diffusion, of which the biggest challenge is to overcome the natural skin barrier [12]. The physicochemical properties of a drug provide a good indication(s) of its transdermal behaviour properties. For a drug to be effectively used in transdermal delivery, it should consist of sufficient lipophilic properties to partition into the stratum corneum, but also sufficient hydrophilic properties to partition into the underlying layers of the skin [13].

Pheroid™ technology was implemented in one of the two cream and emulgel formulations each, in order to establish whether the Pheroid™ would enhance penetration of the active ingredients across the skin. This patented technology offers a new approach to transdermal drug delivery by overcoming the barrier function of the stratum corneum and therefore to enhance penetration of the active pharmaceutical ingredients across the skin. It consists of vesicular structures that contain no phospholipids, nor cholesterol, but are compiled of customised essential fatty acids, similar to those present in the human body [14]. Other than the fatty acids, Pheroid™ also contains nitrous oxide. This dispersed gas assists in the miscibility of fatty acids in the dispersal medium. It also contributes to the stability of the Pheroid™. The interactions between the nitrous oxide and the fatty acids present a steadfast vesicular Pheroid™ structure that is able to effectively transport hydrophilic and hydrophobic drugs [14].

The four formulations (cream, Pheroid™ cream, emulgel and Pheroid™ emulgel), were used as donor phases in determining the transdermal, epidermal and dermal diffusion concentrations of vitamin A and zinc after application. A commercial product that contains vitamin A acetate was used to compare the results of the newly formulated products with. A diffusion study of 6 hours was conducted, and Franz diffusion cells and tape stripping techniques were used.

2 Materials and Methods

2.1 Materials

The active ingredients vitamin A acetate (retinyl acetate) and zinc acetate, were obtained from Sigma-Aldrich (Kempton park, South-Africa) and BASF chemical company (Midrand, South-Africa), respectively. Ingredients used in the formulation of semisolid products were liquid paraffin, cetyl alcohol, tween-80, propyl paraben, methyl paraben and butylated hydroxyanisole (BHA), and were obtained from Merck Chemicals (Wadeville, South-Africa). Span-60 was

supplied by Brunel manufacturers, and butylated hydroxytoluene (BHT) by Sigma-Aldrich (Kempton park, South-Africa). The xanthan gum was obtained from Warren Chem Specialities and tocopherol was received from Chempure (Pretoria, South-Africa). Water used during all the experiments and formulations was purified with a Milli-Q® water purification system (Millipore, Milford, USA). The phosphate buffered solution (PBS) was prepared from potassium orthophosphate crystals and sodium hydroxide pearls, both received from Merck Laboratory Supplies (Midrand, South-Africa). High performance liquid chromatograph (HPLC) analytical grade ethanol was used as solvent, and methanol acted as HPLC mobile phase (Merck Laboratory Supplies, Midrand, South-Africa). Nitric acid 65% were used for the analysis of zinc (Merck Laboratory Supplies, Midrand, South-Africa).

2.2 Sample analysis

2.2.1 HPLC analysis of vitamin A for diffusion studies

The HPLC method for the analysis of vitamin A was developed and validated at the North-West University, Potchefstroom campus, South Africa. Samples collected from the diffusion studies as well as from the tape stripping experiments were analysed with HPLC. In order to accurately determine the concentration of vitamin A in the samples, an Agilent® 1200 series HPLC system was used. It was equipped with an Agilent® 1200 quaternary pump, ultraviolet (UV) detector, autosampler, degasser and thermostat, with the temperature set at 5°C. Data analysis was performed by Chemstation Rev. A.10.02 analysis software. A Verusil XBP C_{18} (2) (4.6 mm x 150 mm) silica column with 5 µm particle size was used during the analysis.

The mobile phase consisted of 100% analytical HPLC grade methanol. The flow rate was set at 1 mL/min, with an injection volume of 25 µl and a runtime of 10 min. Vitamin A was detected at 349 nm and it eluted at 4.7 min. All analyses were performed under controlled environmental conditions (25 ± 2°C).

2.2.2 Analysis of zinc for diffusion studies

Zinc in the samples was tested with a Varian SpectrAA - 250 Plus Atomic Absorption Spectrometer (AA). An air and acetylene flame was used to detect zinc. Varian hollow cathode lamps were employed at a wavelength of 213.9 nm. Analysis was done by Eco-Analytica from

the North-West University, Potchefstroom Campus, using a standard, validated and accredited method.

For sample preparation, 1 mL was withdrawn from the Franz diffusion cell as well as from the politop with tape strips or politop containing dermis samples. It was transferred into a 10 mL volumetric flask. Nitric acid (65%) was used as solvent, and diluted with HPLC graded water to attain a concentration of 1.6%. 9 mL of the nitric acid (1.6%) was added to the 1 mL in the volumetric flask, to prepare a 10 mL solution.

2.3 Preparation of vitamin A and zinc containing semisolid formulations

A cream, Pheroid™ cream, emulgel and Pheroid™ emulgel were formulated, with vitamin A and zinc as active pharmaceutical ingredients.

2.3.1 Ingredients

Ingredients used in the semisolid formulations and the function of each were as follows: vitamin A acetate (active pharmaceutical ingredient), zinc acetate (active pharmaceutical ingredient), liquid paraffin (emollient/solvent), cetyl alcohol (thickener), Tween-80 (emulsifier), Span-60 (emulsifier), methyl paraben and propyl paraben (preservatives), BHA and BHT (anti-oxidants) and xanthan gum (gelling agent).

2.3.2 Preparation of a cream and Pheroid™ cream

The water phase (Phase B) consisted of 72.86 g distilled water and 1.2% zinc. The distilled water was weighed and heated to 40 ± 2°C, where after the zinc were added and heated to 80 ± 2°C. The oil phase (Phase A) of the cream consisted of 12% liquid paraffin, 10% cetyl alcohol, 1.5% Tween-80, 1.5% Span-60, 0.2% methyl paraben, 0.04% propyl paraben and 0.1% of both BHA and BHT. These ingredients were weighed, add together and heated to 80 ± 2°C.

Phase A was added to Phase B whilst being homogenised at a speed of 13 500 rpm until the formulation cooled down and reached a temperature of exactly 40 ± 2°C. The formulation was then stirred with a glass rod until room temperature (25 ± 2°C) was reached. Exactly the same procedure, ingredients and quantities were used to prepare the Pheroid™ cream formulation, except for the addition of the Pheroid™ ingredients, which were added to the oil-phase in the aforementioned procedure.

2.3.3 Preparation of an emulgel and Pheroid™ emulgel

The water phase (Phase B) consisted of 71.12 g distilled water, 1.2% zinc and 1.5% xanthan gum. The distilled water was weighed and heated to 40 ± 2°C. Xanthan gum was slowly added to the distilled water, while homogenising at 777 rpm. The zinc were added to the distilled water and xanthan gum and heated to 80 ± 2°C. The oil phase (Phase A) of the emulgel consisted of 20% liquid paraffin, 4.5% Tween-80, 0.5% Span-60, 0.4% methyl paraben, 0.08% propyl paraben and 0.1% of both BHA and BHT. These ingredients were weighed, add together and heated to 80 ± 2°C. Phase A was added to Phase B whilst being homogenised at a speed of 13 500 rpm until the formulation cooled down and reached a temperature of exactly 40 ± 2°C. The formulation was then stirred with a glass rod until room temperature (25 ± 2°C) was reached.

Exactly the same procedure, ingredients and quantities were used to prepare the Pheroid™ emulgel formulation, except for the addition of the Pheroid™ ingredients, which were added to the oil-phase in the aforementioned procedure.

2.4 Stabilisation of vitamin A

An instability problem was detected during a 24 h stability test through the HPLC, where the quantity of the vitamin A was reduced with 10.90% during the first 12 h. In an attempt to stabilise the vitamin A, different anti-oxidants were added, for example sodium-bisulphite, ethylene-diamine-tetra-acetic acid (EDTA) and ascorbic acid. Different quantities of the anti-oxidants were weighed in a 100 mL volumetric flask and filled to volume with ethanol:PBS (50:50) after adding 5 mL of a 12.5 μg/mL vitamin A solution. The different mixtures were transferred to HPLC vials and injected into the HPLC.

2.5 Franz cell diffusion experiments

2.5.1 Skin preparation for diffusion studies

Abdominal skin of Caucasian female patients, who had undergone abdominoplastic surgery, was obtained from various medical institutions. The Research Ethics Committee of the North-West University granted ethical approval for acquiring and utilising the donated skin under reference number 04D08. Informed consent had been obtained from all donors before surgery and their identities kept anonymous.

The skins had been frozen at -20°C within 24 h after surgery. Full thickness skin was used during this study, consisting of the stratum corneum, epidermis and dermis. Subcutaneous fat

and connective tissue were removed with a blunt scalpel, taking care not to damage the skin. The skin was placed on a flat surface for inspection for any defects, such as stretch marks, scars, or blemishes. Circles, having a diameter of approximately 15 mm, were punched from the skins, placed on Whatman® filter paper and covered with aluminium foil. These samples were then frozen at -20°C until use. According to [15], the skin circles would be stable under such conditions for a period of three to six months. Before diffusion studies were performed, the frozen skin circles were thawed at room temperature and mounted onto the diffusion apparatus.

2.5.2 Donor phase preparation for diffusion studies

A cream, Pheroid™ cream, emulgel and Pheroid™ emulgel, containing vitamin A and zinc, and a commercial product were used as donor phase during diffusion studies (see Section 2.3). The commercial product used for diffusion studies, contained vitamin A acetate with a concentration that was unknown. This product was used to compare the transdermal diffusion with the formulated products, containing vitamin A, in this study. According to [9], zinc is the most abundant element in the human body, of which 6% is present in the skin. To eliminate the possibility of endogenous zinc diffusion, placebo formulations (without zinc) were prepared for use as control samples during the skin diffusion investigation. A placebo formulation was formulated for the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. A Franz cell for each of the four formulations was used and the extraction of the receptor phase was conducted after 6 h.

2.5.3 Receptor phase preparation for diffusion studies

A mixture of ethanol (50%) and PBS (50%) was used as receptor phase for both the membrane and skin diffusion studies. The solution of the receptor phase must be able to dissolve the ingredients that require analysis, therefore an organic solvent, ethanol, was added to dissolve the lipophilic vitamin A and other oily ingredients. PBS was prepared by dissolving potassium orthophosphate crystals (13.62 g) in 500 mL of HPLC grade water. Subsequently, sodium hydroxide pearls (3.174 g) were dissolved in 786.8 mL HPLC grade water. The two solutions were mixed and the pH was adjusted to 7.4 with 10% phosphoric acid. A 50:50 (v/v)

ethanol:PBS solution and a fresh receptor phase were prepared before every diffusion study, in order to prevent the formation of crystals.

2.5.4 Procedure of Franz cell diffusion experiments

Diffusion studies were conducted in amber Franz diffusion cells, in order to prevent photo-isomerisation of vitamin A. The Franz diffusion cells consist of a donor compartment and a receptor compartment with a receptor capacity of approximately 2 mL and a diffusion area of 1.075 cm^2. Five diffusion studies were performed; one for each formulation (cream, Pheroid™ cream, emulgel, Pheroid™ emulgel and commercial product). Fifteen Franz diffusion cells were used per diffusion study, of which ten cells were used for skin diffusion investigation, and five cells as controls (placebo).

In order to compare the results, skin of the same donor was used for each individual diffusion study [15]. Full thickness skin circles were mounted between the receptor and donor compartments, with the stratum corneum facing upwards and in contact with the donor phase. The unit was sealed with Dow Corning® vacuum grease and fastened with a metal horseshoe clamp to secure the donor and receptor compartments. The donor compartments of the ten experimental cells were each filled with approximately 1 mL of the formulated product, i.e., cream, Pheroid™ cream, emulgel, Pheroid™ emulgel or the commercial product. Samples were submerged in a water bath set at 32°C, the same as human skin temperature. 1 mL of the placebo formulations (without vitamin A or zinc) were transferred into four of the control cells as well as into another cell with a 50:50 (v/v) ethanol:PBS solution (pH 7.4). In order to prevent evaporation of volatile components, the donor compartment was covered with Parafilm® and a cap. The receptor phase was magnetically stirred in order to maintain homogeneity throughout the experiment. The receptor compartment was filled with 2 mL of the 50:50 (v/v) ethanol:PBS (pH 7.4) solution at a temperature of 37°C. Care was taken to prevent air bubbles beneath the skin samples. The fifteen cells were each securely clamped and placed on a Variomag® magnetic stirrer plate in a Grant water bath, with the receptor compartment covered under water. After placing the Franz cells in the water bath (37°C), it was confirmed that the mounted skin samples were at a temperature of 32°C. The content of the receptor compartments were

withdrawn at specific time intervals, and immediately replaced with 2 mL of the preheated ethanol:PBS (50:50) solution, to maintain sample conditions.

1 mL each of the withdrawn receptor phase samples was transferred into a 10 mL volumetric flask and made up to volume for the analysis of zinc on the AA spectrometer. The remaining solutions was each transferred into amber HPLC vials and immediately analysed on the HPLC in order to determine the concentration of vitamin A within the receptor compartment, for each withdrawal time.

2.5.4.1 Procedure of Franz cell membrane diffusion experiments

The aim of the membrane diffusion study was to determine whether vitamin A and zinc were released from the formulated products. For this reason, the extraction times differed from those of the skin diffusion study. The exact same method, as described in Section 2.5.4, was used for the membrane diffusion studies, except for the use of polytetrafluoroethylene (PTFE) membranes, with a thickness of 0.4 μm, instead of skin. The samples from the receptor compartments of the Franz cells were withdrawn after 0.5, 1.0, 2.0, 4.0, and 6.0 h. The receptor compartment was immediately refilled with fresh (50:50) ethanol:PBS solution (37°C). Analyses of vitamin A and zinc were performed as described in Sections 2.2.1 and 2.2.2.

2.5.4.2 Procedure of Franz cell skin diffusion experiments

The method, as described above (Section 2.5.4), was used for the skin diffusion studies. Originally, the contents of the receptor phase was withdrawn after 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 h. The generated results were not measurable, because of the degradation and inherent instability of vitamin A. Subsequently, only one extraction for both vitamin A and zinc was done after 6 h, after which the receptor compartment was filled with fresh (50:50) ethanol:PBS solution (37°C).

2.5.5 Tape stripping procedure

Tape stripping is a basic method to remove the outermost layer of the skin. This technique was used to investigate the penetration and reservoir behaviour of topically applied formulations [16]. After the 6 h diffusion study, the skin samples were carefully removed from the Franz diffusion cells. The diffusion area of each skin circle was dabbed dry with clean paper towel. 3M Scotch® Magic™ tape was cut into strips, big enough to cover the diffusional area. Sixteen

tape strips were needed per skin circle, of which the first strip was discarded, due to possible residue from the formulated product on the skin‟s surface [16]. The remaining 15 strips were used to remove the stratum corneum-epidermis and placed in a politop filled with 5 mL of ethanol:PBS (50:50) solution. Complete removal of the stratum corneum-epidermis was indicated by the glistening of the viable epidermis. The non-diffusion area of each skin sample was cut off and discarded, whereas the remaining area of diffusion of the skin was cut into pieces, thus enlarging the surface area. These were placed into a politop filled with 5 mL of ethanol:PBS (50:50) solution, in order to determine the concentration of actives being diffused into the epidermis-dermis. Politops, containing the tape strips, as well as the epidermis-dermis samples, were sonicated for approximately 30 min directly after sampling. All the samples were centrifuged for 10 min with an Eppendorf centrifuge 5804R and analysed on the HPLC to determine the permeant contents.

2.5.6 Statistical analysis

All data was statistically analysed, using quantitative statistical methods. Analyses were performed utilising the SPSS and R statistical software. Descriptive statistics in the form of median values were reported and data (with the data points superimposed) was graphically represented. Inferential statistics were performed, involving formal hypothesis testing. These tests included a two-way design, where any relationship between the application of Pheroid™ in the topical formulations (i.e. the cream and emulgel) was investigated on epidermis (stratum corneum-epidermis), dermis (epidermis-dermis) and the diffusion concentrations of vitamin A and zinc. Should a significant variation in the data be observed, it would be more accurate to use the median value (centre of data set) [17]. Hence, based on the small sample sizes and skew distributions of the data, the non-parametric test, as proposed by [18], henceforth referred to as the BDM test, was used for this purpose.

The non-parametric Mann-Whitney test was performed to investigate the main effects of Pheroid™ and the formulations on the concentrations of vitamin A and zinc. All statistical inferences were performed at a 5% (0.05) level of significance. A p-value of less than 0.05 indicated a statistically significant difference among the groups of data, whereas a p-value of

more than 0.05 was statistically insignificant, meaning no noticeable difference between the data.

It should be noted that the standard two-way analyses of variance (ANOVA), followed by T-tests, were also performed, but were not reported, due to model assumption violation, since more data points were required.

The tape stripping experiment conducted on the commercial product, delivered very consistent (no variation) results in the stratum corneum-epidermis and epidermis-dermis. These values were therefore only used as reference values in the graphical representations and discussions.

3 Results and discussion

3.1 Stabilisation of vitamin A

Table 1: Anti-oxidants, their quantities and results on the stability of vitamin A

The reader is referred to Table 1, which contains the different anti-oxidants used in this study as well as their quantities and the percentage decreased after 12 h. According to these results, the minimum degradation was visible in the sodium-bisulphite and EDTA combination with degradation of 6.04%. Normally, a sample is allowed a 5% reduction in concentration during a standard stability test [19]. These results were unacceptable according to the acceptance criteria. A Pheroid™ cream was formulated, as a final attempt, to establish whether the characteristics and components of the cream would be able to stabilise vitamin A. Ingredients like tocopherol, BHA, BHT methyl paraben and propyl paraben were present in the formulation. A positive result was depicted, with a percentage degradation of 0.9% over a 12 h period. A contributing factor in the stabilisation process of vitamin A, is the role of the fatty acids present in the Pheroid™ formulation. Loss of vitamin A during a 12 h period was reduced from 10.90%, without any stabilising agents, to 0.9%, when formulated into a cream with stabilising agents. These results proved acceptable.

3.2 Franz cell diffusion experiments

3.2 1 Membrane diffusion studies

The average, cumulative concentration of vitamin A being released after 6 h from the Pheroid™ cream (49.518 μg/cm^2) was the highest, followed by the cream (18.314 μg/cm^2), emulgel (0.720

µg/cm^2) and Pheroid™ emulgel (0.554 µg/cm^2). The Pheroid™ cream formulation released more than two and a half times the amount of vitamin A, than the cream formulation, with an average percentage being released of 0.555% after 6 h. More vitamin A diffused through the membrane from the cream formulations, than from the emulgel formulations.

The average cumulative concentration of zinc being released from the Pheroid™ emulgel (14.313 µg/cm^2) was the highest after 6 h, followed by the emulgel (8.760 µg/cm^2), cream (8.277 µg/cm^2), and lastly the Pheroid™ cream formulation, with a concentration of 6.125 µg/cm^2 . The Pheroid ™ emulgel formulation released almost twice the amount of zinc, compared to that of the emulgel, cream and Pheroid™ cream formulations.

Vitamin A and zinc were released from all the formulations, but only in small percentages. Since low concentrations were expected to diffuse during the diffusion studies, it was decided that only one extraction for the skin diffusion studies would be done at 6 h.

3.2.2 Skin diffusion studies

3.2.2.1 Skin diffusion of the commercial product and formulations containing vitamin A

No vitamin A was detected in the receptor compartment during the diffusion study of the commercial product, therefore indicative that it did not penetrate through the skin. The high lipophilicity of vitamin A may have prevented diffusion through the skin into the Franz cell receptor. Vitamin A might therefore remain in the epidermis-dermis [20], which was an acceptable result, when considering the intended target area (dermis) of treatment.

According to the literature, vitamin A generally penetrates the skin very poorly [21], as was confirmed by the results from this study (formulations), since no vitamin A was detected in the receptor compartment. Vitamin A did not penetrate through the full thickness skin, possibly due to its high lipophilicity that may have formed a reservoir in the stratum corneum [22] that prevented diffusion from the skin into the Franz cell receptor. Since the target area for the treatment of acne is the dermis, these results were acceptable [23].

When the diffusion concentrations of vitamin A of the formulations were compared to those of the commercial product, it was observed that no vitamin A had diffused through the skin during the 6 h diffusion studies.

3.2.2.2 Determination of intrinsic zinc that diffused by using placebo formulations

Figure 1: Diffusion concentrations of zinc in the formulations compared to the placebos

According to the literature, zinc does not normally diffuse through the skin [24]; [25]. However, [9] found that the skin inherently contains approximately 6% of zinc. To eliminate uncertainty, transdermal diffusion experiments were conducted on placebo (without zinc) formulations. These placebo formulations were also a placebo cream, placebo Pheroid™ cream, placebo emulgel and placebo Pheroid™ emulgel. Zinc was detected in the receptor phase, thus confirming the natural presence of zinc in the skin, whenever a placebo formulation was applied. The highest average zinc concentration resulted from the Pheroid™ cream (0.205 $\mu g/cm^2$), followed by the emulgel (0.096 $\mu g/cm^2$), cream (0.067 $\mu g/cm^2$) and Pheroid™ emulgel (0.059 $\mu g/cm^2$) formulations (see Figure 1).

3.2.2.3 Skin diffusion of the formulations containing zinc

Zinc diffused through the skin, after the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel formulations containing zinc was used as donor phases. However, since no flux was observed, a sample was extracted only once, after 6 h. The highest average zinc concentration that permeated through the skin, was from the Pheroid™ cream (9.231 $\mu g/cm^2$), followed by cream (8.710 $\mu g/cm^2$), Pheroid™ emulgel (0.123 $\mu g/cm^2$) and emulgel (0.050 $\mu g/cm^2$) formulations as could be seen in Figure 1. After comparing the results from the cream formulations with those of the emulgel formulations, it was observed that the cream formulations showed higher diffusion concentrations than the emulgel formulations. The cream formulations were more lipophilic than the emulgel formulations, due to the higher contents of oil ingredients that may have diffused through the lipid domain of the stratum corneum [28]. This in turn may have resulted in the higher diffusion concentrations through the skin.

When comparing the test outcomes of the Pheroid™ formulations with the non-Pheroid™ formulations, it was established that the incorporation of Pheroid™ into the topical formulations positively affected the diffusion potential of zinc. This was probably due to the presence of the fatty acids associated with Pheroid™, which caused the formulations to be more lipophilic. The hydrophilic zinc was thus possibly entrapped into the lipophilic Pheroid™ vesicle, rendering it more permeable through the skin [14]. Different salt forms show different behaviour in the

transdermal diffusion of metals. The hierarchy in the behaviour between the different salt forms of zinc are as follows: acetate > nitrate > sulphate > chloride [26]. Therefore, the chosen zinc acetate may have contributed to the good diffusion results of zinc through the skin.

Figure 1 illustrates the diffused zinc concentrations from the different formulations and placebos. A comparison of the diffusion concentrations of the zinc from the formulations to those from the placebos showed that the emulgel and Pheroid™ emulgel formulations, relative to the placebos, delivered insignificant concentrations of zinc transdermally. Contrary, the cream and Pheroid™ cream formulations transferred significant amounts of zinc transdermally.

3.3 Concentrations in the stratum corneum-epidermis and epidermis-dermis

3.3.1 The commercial product and formulations containing vitamin A

Detectable concentrations of vitamin A acetate from the commercial product (cream) were able to penetrate the stratum corneum-epidermis as well as the epidermis-dermis, although these concentrations were significantly low.

The total concentration of vitamin A acetate in the epidermis-dermis (0.0020 µg/mL) was noticeably higher than the concentration in the stratum corneum-epidermis (0.0012 µg/mL). These results were favourable, due to the target area of acne treatment, which is in the dermis.

Figure 2: Graphical representation of vitamin A concentrations (µg/mL) in the **a)** stratum-corneum-epidermis and **b)** the epidermis-dermis for the different formulations. (In the box-plot, the dotted and solid lines represent the average and median values, respectively; whilst the dotted line over the whole graph represents the concentration of the commercial product).

The Pheroid™ emulgel (0.0045 µg/mL) formulation obtained the highest average concentration in the stratum corneum-epidermis as could be seen in Figure 2a. The emulgel, cream and Pheroid™ cream formulation followed, with concentrations of 0.0042 µg/mL, 0.0018 µg/mL and 0.0017 µg/mL, respectively. A possible explanation for this phenomenon may be the hydration effect of the emulgel formulation on the stratum corneum, which increased epidermal delivery of vitamin A [27]. However, no significant differences were observed from a comparison of the Pheroid™ formulations with the non-Pheroid™ formulations.

From Figure 2a it was evident that the highest median vitamin A concentration in the stratum corneum-epidermis was obtained from the Pheroid™ emulgel (0.0040 µg/mL), followed by the emulgel (0.0038 µg/mL), Pheroid™ cream (0.0018 µg/mL) and lastly, the cream (0.0017 µg/mL) formulation. No significant differences between the average and median concentration values of the cream and Pheroid™ cream were observed. Therefore, both the median and average values could be used to determine the vitamin A concentration released from the cream formulations. However, there was a minor difference between the median and average concentrations of the emulgel and Pheroid™ emulgel. It was determined that the median value would be a more accurate representation of the true concentration, since it considered all the data points and would not be affected by outliers in the data [17].

From Figure 2b it was clear that the highest average vitamin A concentration in the epidermis-dermis was obtained from the emulgel (0.0029 µg/mL) formulation, followed by the Pheroid™ emulgel (0.0017 µg/mL) formulation. The lowest vitamin A concentrations in the epidermis-dermis were obtained when vitamin A was delivered from both the cream and Pheroid™ cream formulations, both with average concentrations of 0.0005 µg/mL. Due to the hydrophilic nature of the dermis [28], permeation into the dermis of the hydrophilic emulgel was expected to be higher than for the cream formulation, which had a more lipophilic nature. The vitamin A concentration being detected in the epidermis-dermis with the Pheroid™ emulgel, was slightly lower than that of the emulgel. This may have been attributed to the more lipophilic nature of Pheroid™ ingredients, which may have increased the lipophilicity of the emulgel formulation and subsequently decreased the delivery of vitamin A into the hydrophilic epidermis-dermis. The concentration in the epidermis-dermis showed that the Pheroid™ vesicles did not significantly contribute to the penetration of vitamin A through the skin.

Considering the median concentration values, it was clear from Figure 2b that the highest median vitamin A concentration in the epidermis-dermis was obtained from the emulgel (0.0020 µg/mL) formulation, followed by Pheroid™ emulgel (0.0017 µg/mL), cream (0.0004 µg/mL) and Pheroid™ cream (0.0004 µg/mL). Since insignificant differences between the average and median concentrations of the cream, Pheroid™ cream and Pheroid™ emulgel were observed, both the median and average concentrations could be used to determine the vitamin A being

released from the cream formulations. Since a significant difference was observed between the median and average concentrations of the emulgel formulation, the median concentration was a more accurate value to use, as it recognised all the data points and would not be affected by outliers in the data [17].

Degradation products of vitamin A were detected in both the stratum corneum-epidermis and epidermis-dermis concentrations, by means of HPLC, but were not quantifiable. The degradation in the stratum corneum-epidermis concentrations were only depicted in the emulgel and Pheroid™ emulgel formulations with 8.89 and 17.24% degradation, relative to the amount of vitamin A that had diffused into the stratum corneum-epidermis. The degradation in the epidermis-dermis concentrations were 8.89 and 17.65% degradation, relative to the amount of vitamin A that had diffused into the epidermis-dermis.

As a result, the total concentration of vitamin A in the stratum corneum-epidermis was noticeably higher than in the epidermis-dermis, when the four topical formulations were applied. This may have been due to the hydrophobic nature of vitamin A, causing it not to partition effectively from the lipid rich stratum corneum to the underlying hydrophilic skin layers [29]. Due to acne that develops in the sebaceous follicles in the dermis [23], it was evident from the test outcomes, that these four formulations reached their intended target site. The reason for the small concentrations may have been due to the small amounts of vitamin A being released by the formulations.

Results obtained from the vitamin A concentrations being released into the stratum corneum-epidermis and epidermis-dermis from the commercial product, compared well with the formulated products. The vitamin A concentrations in the stratum corneum-epidermis, released from the commercial product (0.0012 μg/mL) were, however, lower, compared to the vitamin A concentrations, released from the formulated products. Contrary, the vitamin A concentrations being released from the commercial product into the epidermis-dermis (0.0020 μg/mL), was slightly higher than from the formulated Pheroid™ emulgel, cream and Pheroid™ cream. It was not higher than the concentration of vitamin A that was released from the emulgel into the epidermis-dermis (0.0029 μg/mL), though.

3.3.2 Placebo formulations to determine the intrinsic zinc concentration

Figure 3: Concentrations (μg/mL) of zinc in the stratum corneum-epidermis and epidermis-dermis from the four formulations, compared to the placebos

During the tape stripping experiments on the different placebo formulations, traces of zinc were detected in both the stratum corneum-epidermis and epidermis-dermis. These results indicated the natural presence of zinc in human skin, as was also observed by [9]. Tape stripping results revealed that the highest average concentration of zinc in the stratum corneum-epidermis was obtained after placebo Pheroid™ cream (0.086 μg/mL) was applied to full thickness skin, followed by the application of placebo emulgel (0.069 μg/mL), placebo cream (0.045 μg/mL) and placebo Pheroid™ emulgel (0.019 μg/mL) formulations.

The highest average zinc concentration was detected in the epidermis-dermis after the application of placebo Pheroid™ cream (0.062 μg/mL) to full thickness skin, followed by the placebo cream (0.039 μg/mL), placebo Pheroid™ emulgel (0.024 μg/mL) and placebo emulgel (0.010 μg/mL) formulations.

3.3.3 Formulations containing zinc

Zinc was detected in the subcutaneous layer of the skin, using different formulations. However, the diffused concentrations of zinc in the stratum corneum-epidermis and epidermis-dermis were significantly low.

The following rank order for the concentration of zinc being released from the formulations into the stratum corneum-epidermis was established, and illustrated in Figure 3: the cream (0.1336 μg/mL) formulation delivered the highest zinc concentration, followed by the Pheroid™ cream (0.0314 μg/mL), Pheroid™ emulgel (0.0009 μg/mL) and emulgel (0.000 μg/mL) formulations. No detectable concentration of zinc diffused from the emulgel formulation into the stratum corneum-epidermis, which may have been due to the affinity of the hydrophilic zinc for the aqueous phase of the emulgel [29]. [9] concluded from their studies that zinc should be delivered transdermally, if it is in its ionised form and formulated in a lipophilic vehicle, for example a cream formulation. It could therefore be concluded that the reason for the relatively high zinc concentration being delivered by the cream formulation during this study, was due to the lipophilicity of the cream formulation. This enhanced the penetration of zinc through the lipid

domain of the stratum corneum [28]. Zinc could also have been delivered from the cream formulation into the stratum corneum-epidermis, due to the affinity of the zinc for the hydrophilic surroundings [29].

From a comparison of the zinc concentrations being detected in the epidermis-dermis, the cream formulation (0.0144 μg/mL) depicted the highest concentration. This concentration was more than 14 times higher than the zinc concentration delivered to the epidermis-dermis by the Pheroid™ cream (0.0010 μg/mL) and 48 times higher than that of both the emulgel (0.0003 μg/mL) and Pheroid™ emulgel (0.0003 μg/mL). Zinc concentrations were slightly higher in the epidermis-dermis than in the stratum corneum-epidermis for the emulgel and Pheroid™ emulgel formulations. Contrary, zinc concentrations were higher in the stratum corneum-epidermis than in the epidermis-dermis for the cream and Pheroid™ cream formulations, which may possibly have contributed to the higher concentrations of the cream formulations in the epidermis-dermis.

Figure 3 clearly shows that zinc concentrations in the stratum corneum-epidermis were significantly higher when the placebo Pheroid™ cream, placebo emulgel and placebo Pheroid™ emulgel were applied. It could therefore be assumed that zinc concentrations, detected in the stratum corneum-epidermis after individual application of the Pheroid™ cream, emulgel and Pheroid™ emulgel formulations, was not zinc that had diffused from these formulations into the stratum corneum-epidermis, but rather represented endogenous zinc, normally present in the skin [9]. However, from a comparison of the zinc concentrations in the stratum corneum-epidermis, when the cream or placebo cream was applied, it was found that the possibility existed that zinc did indeed diffuse from the cream formulation into the stratum corneum-epidermis.

Zinc concentrations being measured in the epidermis-dermis, after application of the four formulations and placebo formulations on full thickness skin, are illustrated in Figure 3. It was apparent that zinc concentrations in the epidermis-dermis were significantly higher when the placebo formulations were applied. The zinc concentration, as depicted in the epidermis-dermis after application of the cream formulation, was significantly lower than when the placebo cream was applied. Thus, it could be assumed that the zinc concentrations present in the epidermis-

dermis after individual application of the four formulations containing zinc, was not zinc that had diffused from these formulations into the epidermis-dermis, but rather the intrinsic zinc, normally present in the skin [9].

3.4 Statistical analysis for diffusion studies

3.4.1 Diffusion concentrations of vitamin A and zinc

Vitamin A was not able to diffuse through the skin; therefore no statistical data was available for the transdermal delivery of vitamin A.

The BDM test did not show any significant relationship (p-value = 0.84) between the application of Pheroid™ in the formulations and the zinc concentrations. Similarly, the Mann-Whitney test revealed that the Pheroid™ did not have any statistically significant effect on the delivery of zinc (p-value = 0.99). However, the cream and emulgel formulations depicted a statistically significant effect (p-value = 0) on zinc concentrations.

3.4.2 Vitamin A concentrations in the stratum corneum-epidermis and epidermis-dermis

According to the BDM test, no significant relationship existed between the application of Pheroid™ in the topical formulations and the concentration of vitamin A in the stratum corneum-epidermis (p-value = 0.92), nor epidermis-dermis (p-value = 0.44). Similarly, the Mann-Whitney test further revealed that Pheroid™ did not have any statistically significant effect on the delivery of vitamin A to the stratum corneum-epidermis (p-value = 0.93), nor epidermis-dermis (p-value = 0.56). However, the cream and emulgel formulations showed a statistically significant effect on vitamin A concentrations in the stratum corneum-epidermis and epidermis-dermis, both having a p-value of 0.

3.4.3 Zinc concentrations in the stratum corneum-epidermis and epidermis-dermis

According to the BDM test, there was a significant relationship between the application of Pheroid™ in formulations and the zinc concentration in the stratum corneum-epidermis (p-value = 4.41×10^{-5}), whilst an insignificant relationship existed with regards to zinc in the epidermis-dermis (p-value = 0.78).

Since a positive relationship was observed, the effect of Pheroid™ on the zinc concentration was tested for both the cream and emulgel formulations. The effect of the cream and emulgel formulations on zinc concentrations was also determined for both the Pheroid™ and non-

Pheroid™ formulations. The Mann-Whitney test observed a statistically significant effect of Pheroid™ on zinc concentrations in the cream formulation. A p-value of 0.000 for the cream formulation was observed. For the emulgel formulation, no significant difference of Pheroid™ on zinc concentrations were observed (p-value = 0.087).

The effect of cream and emulgel formulations on zinc concentrations in Pheroid™ and non-Pheroid™ formulations, showed a statistically significant difference in the median concentration on both levels of Pheroid™ application. The p-values was 0.000 and 0.002 for the Pheroid™ and non-Pheroid™, respectively. However, a more significant difference between zinc concentrations was observed when no Pheroid™ was applied.

Considering the epidermis-dermis, the Mann-Whitney test revealed that the application of Pheroid™ did not have any statistically significant effect on the delivery of zinc to the epidermis-dermis (p-value = 0.99). However, the cream and emulgel formulations depicted a statistically significant effect on zinc concentrations, with a p-value of 0.000 in the epidermis-dermis. However, in the latter case, a more focused study would be required to investigate the repeatability of the observed distributions.

4 Conclusion

Membrane release studies were conducted on the formulated topical cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. Vitamin A and zinc were both released from these formulations, although the released concentrations were significantly low.

When conducting a 6 h skin permeation study, vitamin A in the formulated products, as well as in the commercial product, did not penetrate through the skin. However, when diffusion into the different layers of the skin was examined, the Pheroid™ emulgel delivered the highest average concentration of vitamin A to the stratum corneum-epidermis, followed by the emulgel. Contrary to these results, the average vitamin A concentration that diffused from the emulgel into the epidermis-dermis was the highest, followed by that of the Pheroid™ emulgel. A possible explanation for these results was the hydrophilic nature of the underlying layers of the skin, which caused enhanced permeation of the hydrophilic emulgel. The emulgel formulations might also have hydrated the stratum corneum to a higher extent than the creams, causing it to be more permeable [27].

The commercial product, containing vitamin A, produced lower concentrations of the active pharmaceutical ingredients in the stratum corneum-epidermis than the four new formulations. Concentrations measured in the epidermis-dermis, showed that the average vitamin A concentration released from the commercial product was within the concentration range delivered by the formulations. However, the emulgel without Pheroid^{TM} delivered approximately 31% more vitamin A to the epidermis-dermis, than the commercial product.

Franz cell diffusion studies showed that it was possible to deliver zinc transdermally, although no flux values were obtained. It was found that the emulgel and Pheroid^{TM} emulgel formulations were unable to deliver significant zinc concentrations transdermally. However, transdermal diffusion of zinc from both the cream and Pheroid^{TM} cream formulations were attained. The lipophilic nature of these cream formulations may have enhanced diffusion through the lipid domain of the stratum corneum, which resulted in higher diffusion concentrations of zinc through the skin. Incorporation of the Pheroid^{TM} technology might have achieved a positive effect on the penetration of zinc through the skin. Although zinc reached the systemic circulation, it was in sub-therapeutic concentrations that could not produce any effects of significant concern. The lethal dose for zinc salts administered subcutaneously is 330 mg/kg [30].

During tape strip studies, it was observed that no zinc diffused into the stratum corneum-epidermis and epidermis-dermis from the formulated products. It was rather the endogenous zinc that is naturally present in the stratum corneum-epidermis and epidermis-dermis, which was measured. The cream formulation, however, may have delivered zinc to the stratum corneum-epidermis.

Acne develops from the sebaceous follicles in the dermis. It was thus proven that vitamin A that was released from all four formulations, reached its target site of treatment. Zinc, however, did not reach the dermis and therefore its target site [23].

Emulgel formulations would significantly enhance delivery of vitamin A into the dermis and the cream formulation would effectively deliver zinc to the dermis. Based on the results from this study, if transdermal zinc delivery is required, the Pheroid^{TM} cream as delivery system is recommend.

It was concluded that it would, therefore, be very difficult to formulate a product containing both vitamin A and zinc, which would be able to effectively deliver both active ingredients to their specific target area, the dermis.

Conflicts of Interest

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Tables

Table 1: Anti-oxidants, their quantities and results on the stability of vitamin A

Anti-oxidant(s)	Quantity	% Decreased
Sodium-bisulphite	0.5%	7.18%
EDTA	0.1%	7.02%
Ascorbic acid	0.5%	6.14%
Sodium-bisulphite + EDTA	0.5% and 0.1%, respectively	6.04%
Ascorbic acid + EDTA	0.5% and 0.1%, respectively	6.34%
BHA + BHT (preservatives)	0.1% respectively	6.10%
Pheroid™ cream	See section 2.3.2	0.9%

FIGURE LEGENDS

Figure 1: Diffusion concentrations of zinc in the formulations compared to the placebos

Figure 2: Graphical representation of vitamin A concentrations (µg/mL) in the **a)** stratum-corneum-epidermis and **b)** the epidermis-dermis for the different formulations. (In the box-plot, the dotted and solid lines represent the average and median values, respectively; whilst the dotted line over the whole graph represents the concentration of the commercial product).

Figure 3: Concentrations (µg/mL) of zinc in the stratum corneum-epidermis and epidermis-dermis from the four formulations, compared to the placebos

Figures

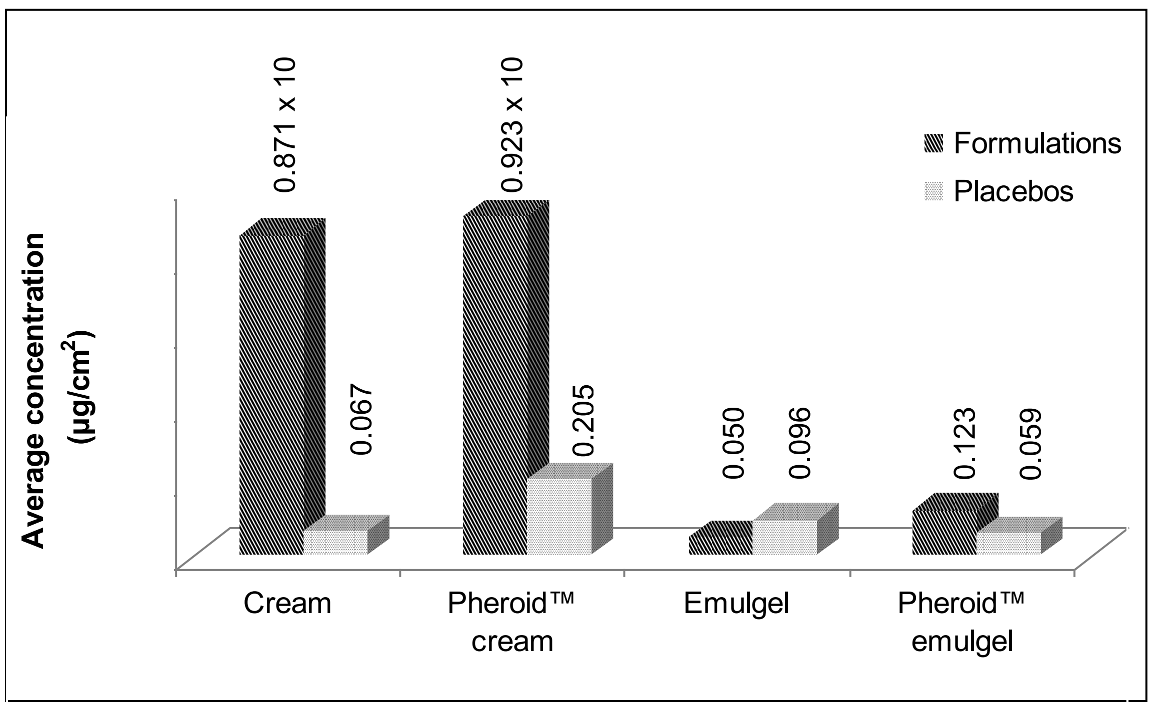
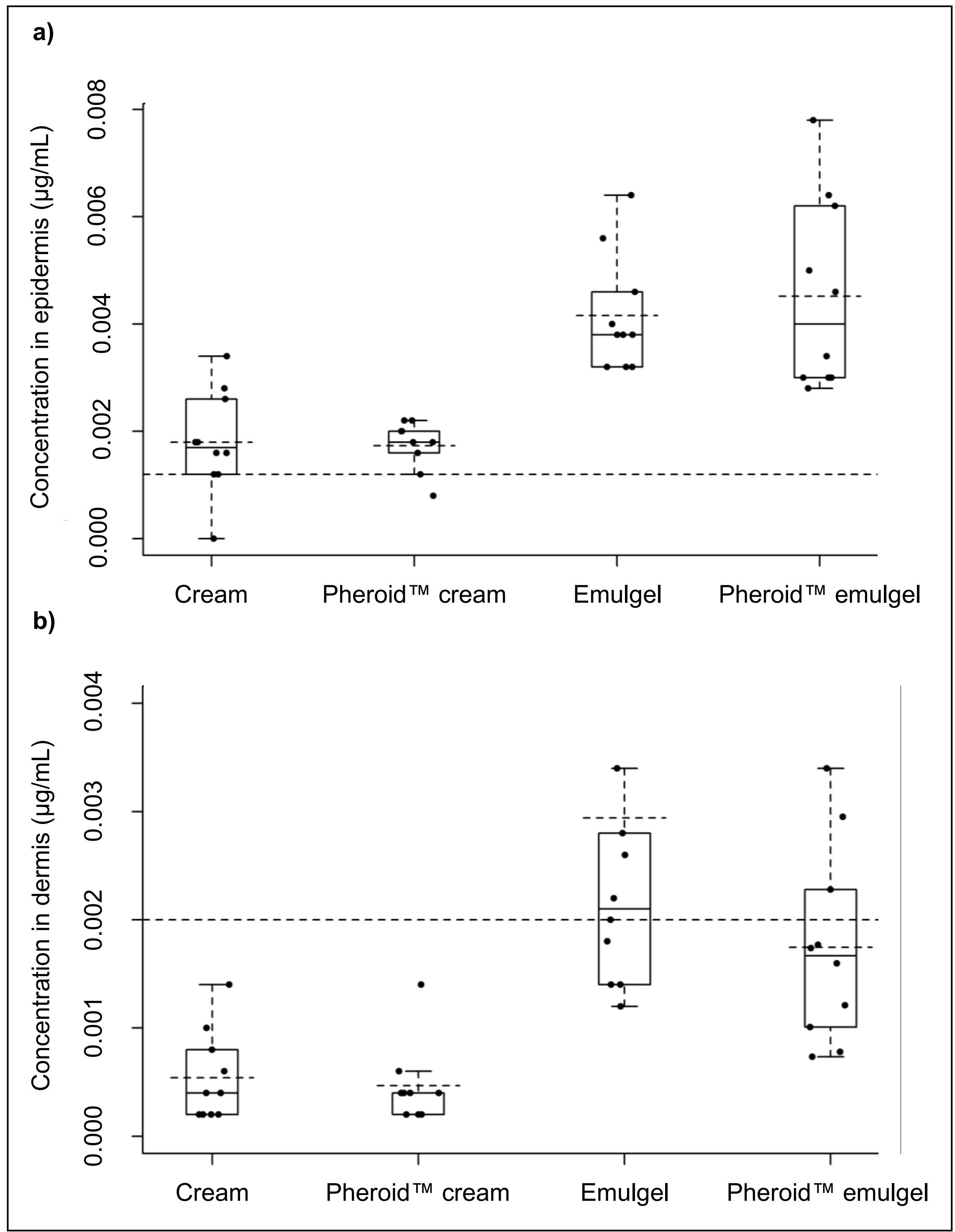


Figure 1: Diffusion concentrations of zinc in the formulations compared to the placebos



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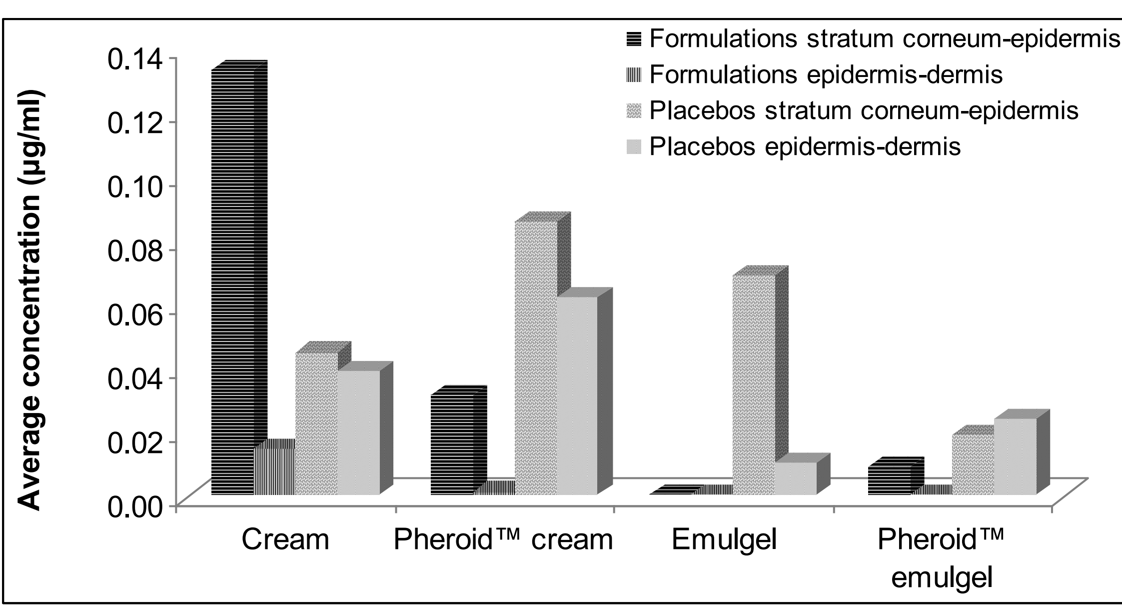

Figure 3: Concentrations (µg/mL) of zinc in the stratum corneum-epidermis and epidermis-dermis from the four formulations, compared to the placebos

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVES

Acne too often is dismissed as being a minor affliction, not worthy of treatment. Uninformed people may be unaware of the permanent scarring that this disease may cause, not only to the skin, but also to the psyche, if left untreated (Habif, 2004:162). Acne is a disease that develops in the pilosebaceous unit of the dermis and causes inflammatory and non-inflammatory lesions. The treatment of acne thus focuses on the reduction of these lesions and hence the inhibition of the scarring process (Railan & Alster, 2008:285). Topical retinoids had for long been viewed as the most successful treatment for acne, but drawbacks, such as their chemical instabilities and the causing of local skin irritations, have resulted in the discontinuation of such treatments (Julie & Harper, 2004:S36). The combination of vitamin A acetate and zinc acetate has been introduced as a new approach in the treatment of acne, with less side effects (Cheng & Depetris, 1998:7).

The aims of this study were to:

- Develop and validate of an HPLC method to quantitatively determine the concentrations of the APIs in the formulations.
- Formulate a cream, Pheroid™ cream, emulgel and Pheroid™ emulgel, containing both vitamin A acetate and zinc acetate as APIs ingredients as possible new acne treatment products.
- Stabilise vitamin A in these formulations.
- Determine the stability of the four formulations when exposed to different temperature - and humidity conditions over a three-month period.
- Determine the transdermal, epidermal and dermal diffusion of the vitamin A (0.5%) and zinc (1.2%) for the four new combination products.

An HPLC method was successfully developed and validated. Hence, all the ingredients in the formulations could be analysed.

Due to the known unstable nature of vitamin A, a 24 h stability test was conducted during the preformulation phase. It was found that the vitamin A concentration had reduced by 10.90% during the first 12 h, confirming its instability. Different anti-oxidants (sodium-bisulphite, ethylene-diamine-tetra-acetic acid (EDTA) and ascorbic acid) were added in an attempt to stabilise vitamin A in formulation, without any success. However, vitamin A depicted a positive

result, showing a percentage degradation of only 0.9% over a 12 h-period, when formulated into a Pheroid™ cream. The Pheroid™ ingredients therefore may have had a stabilising effect on the vitamin A. However, further studies would be required to verify this assumption, or to develop similar formulations in which vitamin A would remain stable.

The stability of the newly formulated products and commercial product, containing vitamin A, were tested over a period of three months under storage conditions of 25°C/60% RH, 30°C/60% RH and 40°C/75% RH. Stability indicating assay testing, the determination of rheology, pH, droplet size, zeta-potential, mass variation, morphology and physical assessment were conducted on the formulations over the stability period. Assay results showed a higher instability of vitamin A when exposed to elevated temperatures. However, for the commercial product being tested, the vitamin A was found to be even more unstable than in the newly formulated products.

Results showed that the zinc concentrations remained relatively stable over the three months of stability testing. BHT and dl-α-tocopherol did not comply with the accepted standards and were thus unsuitable for use as anti-oxidants. The zeta-potential and mass variation remained relatively constant. A drastic decrease in viscosity, and a decrease in pH and droplet size were all indicative of the instability of the new formulations. The cream formulation proved to be the most stable over the duration of the test period.

Membrane release studies were performed on all four formulations in order to determine whether the two active ingredients would be released from the formulations. Results confirmed that both the vitamin A and zinc were released from all the formulations. The highest vitamin A concentration being released after 6 h was from the Pheroid ™ cream (49.518 $\mu g/cm^2$), whereas the Pheroid™ emulgel (14.313 $\mu g/cm^2$) released the highest zinc concentration after 6 h.

Franz-cell diffusion studies were also conducted, using full thickness, Caucasian, female, abdominal skin over a period of 6 h. Vitamin A in the formulated products, as well as in the commercial product, did not penetrate through the skin.

Tape stripping studies were further done to determine the concentration of actives present in the epidermis and dermis. Whilst vitamin A showed the highest epidermal concentration with the application of the Pheroid™ emulgel (0.0045 µg/ml), the emulgel formulation delivered the highest vitamin A concentration in the dermis (0.0029 µg/ml). Contrary, the commercial product showed lower concentrations of vitamin A in the epidermis than all four the new formulations studied. Concentrations, as measured in the dermis, showed that the vitamin A concentration being released from the commercial product, was within the same concentration range as

delivered by the new formulations. However, the emulgel without Pheroid™ delivered approximately 31% more vitamin A to the dermis, than the commercial product.

Due to the ambiguous results obtained from the diffusion and tape stripping studies on the penetration of zinc being released from the formulations, the same tests were conducted using placebo formulations. Zinc was able to diffuse through full thickness skin, although no flux (cumulative concentration over time) values were obtained. To eliminate the possibility of endogenous zinc diffusion, placebo formulations (without zinc) were prepared for use as control samples during the skin diffusion investigation. The emulgel and Pheroid™ emulgel formulations were unable to deliver significant zinc concentrations transdermally. However, significant transdermal diffusion of zinc from both the cream and Pheroid™ cream formulations were attained.

The results obtained from the tape stripping experiments with placebo formulations relative to the formulated products, revealed that zinc concentrations in the epidermis and dermis were significantly higher when the placebo formulations were applied. However, the average zinc concentration in the dermis, after application of the cream formulation, was significantly higher than when the placebo cream was applied.

The conclusion could therefore be drawn that no zinc diffused into the epidermis and dermis from three of the new formulations, except from the cream formulation. Hence, except for the cream formulation that delivered detectable zinc concentrations to the epidermis, it was rather the endogenous zinc, already present in the epidermis and dermis that was being measured for the other three formulations.

Future perspectives for further investigation could include:

- The development of an effective formulation in which vitamin A would successfully be stabilised in topical formulations.
- Thorough investigation of the natural vitamin A and mineral concentrations in the skin that may influence diffusion study results of similar topical formulations than those being investigated during this study.
- Sample bracketing could be used during the analysis of diffusion study data. This would serve as special precautionary measure to compensate for a loss of or degradation of vitamin A, in order to ensure more accurate results.
- The determination of the significant decrease in viscosity of the formulated products during stability testing. Thickening agents may be added in higher concentrations in future formulation processes.

- The incorporation of other delivery systems may be investigated that would be able to optimally deliver vitamin A and zinc to their target site, the dermis.

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APPENDIX A

VALIDATION OF THE ANALYTICAL METHOD

According to the International Organisation for Standardisation, the definition of validation is the confirmation by examination and provision of objective evidence that a method meets the particular requirements for a specified intended use (Araujo, 2009:2224). This could be simplified and stated that validation is the process of demonstrating that the method developed will consistently produce results that meet its pre-determined specifications. In section A.1 the high performance liquid chromatography (HPLC) method of analysis will be discussed for the active ingredient, vitamin A. This method will be used to analyse samples containing vitamin A during transdermal diffusion studies. The HPLC method used during stability studies will be discussed in Section A.2. The last mentioned method made use of the Pheroid™ cream formulation, due the fact that it was the most complex formulation that contained all the ingredients needed to be analysed. Vitamin A acetate and zinc acetate were used as active ingredients, however in this study they will be referred to simply as vitamin A and zinc.

The zinc present in the various formulations could not be analysed by HPLC, due to the fact that it is too polar and does not have a chromophore. Samples were tested with a standard, validated and accredited method by Eco-Analytica from the North-West University, Potchefstroom Campus. A Varian SpectrAA - 250 Plus Atomic Absorption Spectrometer was used during the study. Zinc was detected through the use of an air and acetylene flame. Varian hollow cathode lamps were employed at a wavelength of 213.9 nm. Detected zinc values were presented in µmol/L and then converted to mg/L.

A.1 VALIDATION OF ACTIVE PHARMACEUTICAL INGREDIENTS (APIS)

The HPLC method development was performed in conjunction with Professor Jan du Preez, from the School of Pharmacy, North-West University, Potchefstroom Campus.

This method needs to be sensitive, accurate and consistent, in order to accurately determine the concentration of vitamin A in the formulated products permeating human skin, as well as the concentration released *in vitro.* Analyses were performed in a controlled laboratory environment at a temperature of 25 °C (± 2 °C).

A.1.1 CHROMATOGRAPHIC CONDITIONS

Analytical instrument:	An Agilent® 1200 series HPLC system was used during the analysis. The system was equipped with an Agilent® 1200 quaternary pump, UV detector, autosampler, thermostat, degasser and analysis software (Chemstation Rev. A.10.02) for analysis and data acquisition.
Column:	A Venusil XBP C18 (2) (4.6 mm x 150 mm) silica column, with a 5 µm particle size was employed. (Agela Technologies, Newark, DE).
Mobile phase:	Methanol – analytical HPLC grade (100%).
Flow rate:	1.0 ml/min
Injection volume:	25 µl
Detection:	UV at 349 nm
Run time:	10 min
Retention time:	Vitamin A eluted at 4.7 min.
Solvent:	A 50:50 mixture of ethanol and phosphate buffered solution (PBS) was used during the validation process. Ethanol was used as solvent to ensure the solubility of the lipophilic agents i.e., vitamin A, Pheroid™, and the skin tissue lipids.
Precautions:	Amber glassware was used and all experiments and actions were performed under reduced lighting conditions. The thermostat of the HPLC system was set to 5 °C.

A.1.2 STANDARD PREPARATION

Approximately 5 mg of vitamin A was weighed in a 100 ml volumetric flask and filled to volume with ethanol:PBS (50:50). The solution was sonicated for a few minutes to assure that the vitamin A dissolved completely, and then transferred to an amber HPLC vial to be analysed. This was the 100% standard solution.

A.1.3 VALIDATION PARAMETERS

The following parameters were used to validate the analytical method:

- Linearity
- Accuracy
- Precision (which include interday and intraday)
- Ruggedness (which include stability and repeatability)

A.1.3.1 LINEARITY

Linearity of an analytical method is the ability to achieve results that are directly proportional to the concentration of the ingredient present in the sample. A specific range should be set for the method to be proved linear or not (Howard, 2003:8). Linearity of the method of analysis was determined by plotting the ingredient concentration (µg/ml) versus the peak area ratios. The regression line was fitted on the plot. Linearity data is described by the following equation:

$y = mx + c$ **Equation A.1**

Where:

y: peak area

m: slope

x: concentration

c: y-intercept

The regression coefficient (r^2) is determined according to the linear equation. The acceptance criterion for linear regression is a regression coefficient of ≥ 0.99.

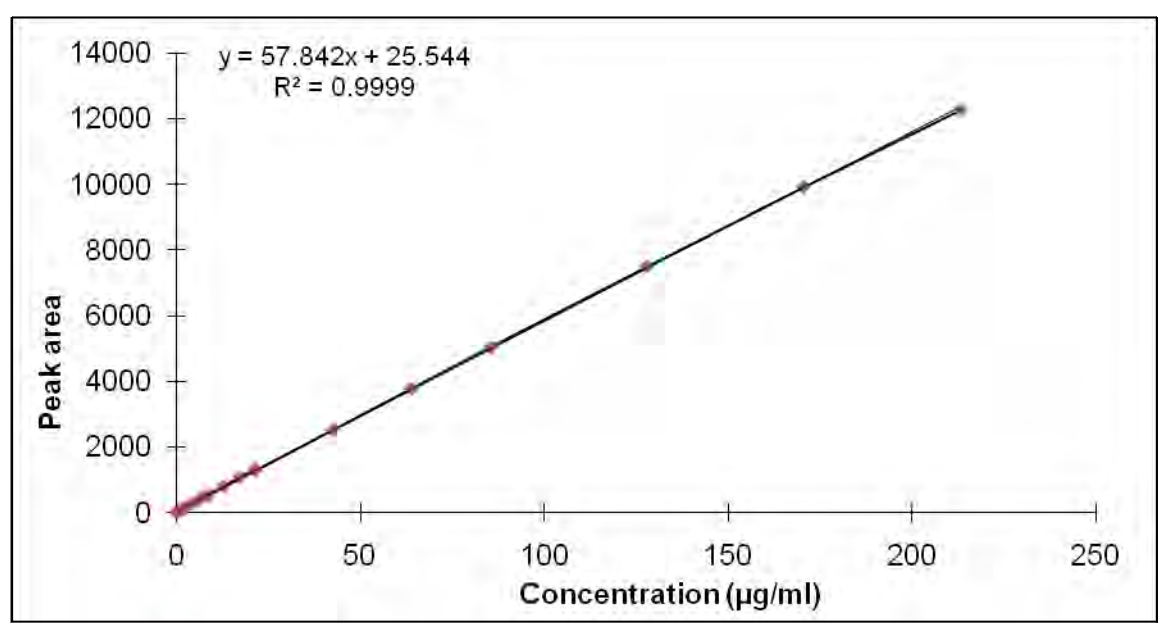

Figure A.1: Linear regression curve of vitamin A.

Three different vitamin A standard solutions (2.13 µg/ml, 21.3 µg/ml and 213 µg/ml) were prepared to investigate linearity. A range of volumes (1.0 µl, 2.5 µl, 5.0 µl, 7.5 µl, 10.0 µl, 15.0 µl, 20.0 µl and 25.0 µl) were analysed on the HPLC from each flask in order to obtain a broader range of concentrations. A wide range was needed, as the concentration vitamin A that will be delivered transdermally is unclear. The data obtained is stipulated in Table A.1 and the regression curve is showed in Figure A.1.

Table A.1: Linearity of vitamin A

Standard solution	Injection volume	Standard concentration (µg/ml)	Peak area ratios
2.13 ug/ml	1.0	0.09	5.17
	2.5	0.21	12.85
	5.0	0.43	25.80
	7.5	0.64	38.65
	10.0	0.85	51.83
	15.0	1.28	77.39
	20.0	1.70	103.51
	25.0	2.13	129.23
21.3 ug/ml	1.0	0.85	53.47
	2.5	2.13	132.39
	5.0	4.26	264.66
	7.5	6.39	396.50
	10.0	8.52	528.65
	15.0	12.78	792.83
	20.0	17.04	1054.09
	25.0	21.30	1315.34
213 ug/ml	1.0	8.52	507.92
	2.5	21.30	1260.60
	5.0	42.60	2509.19
	7.5	63.90	3761.08
	10.0	85.20	5005.36
	15.0	127.80	7483.85
	20.0	170.40	9907.31
	25.0	213.00	12239.20
		r^2	0.9999
		y-intercept	25.5
		slope	57.8

The method showed a high degree of linearity in the range 0.09 µg/ml to 213 µg/ml and therefore, demonstrated the stability of the analytical system.

A.1.3.2 ACCURACY

Accuracy was defined by Araujo (2009:2226) as the degree of agreement between the experimental value obtained and the accepted reference value (actual value).

Standard solutions were prepared by weighing 3 x 5 mg; 3 x 10 mg and 3 x 15 mg vitamin A in

100 ml volumetric flasks. These flasks were filled to volume with ethanol:PBS (50:50). Each of the before mentioned solutions were diluted by extracting 5 ml and filled to 100 ml with ethanol:PBS (50:50). The standard solutions were transferred to amber HPLC vials and injected in duplicate on the HPLC.

The mean percentage recovery value, standard deviation (SD) and percentage relative standard deviation (RSD) are shown in Table A.2, where the percentage recovery is an indication of the accuracy. The acceptance criteria for accuracy should be a recovery between 98 – 102%.

Table A.2: Accuracy of vitamin A

Concentration spiked	Peak area			Recovery	
(µg/ml)	1	2	Mean	(µg/ml)	(%)
71.60	199.62	200.35	199.99	70.04	97.82
71.60	200.49	200.15	200.32	70.15	97.98
71.60	199.80	200.24	200.02	70.05	97.83
115.20	328.88	325.61	327.25	114.60	99.48
115.20	325.38	326.34	325.86	114.12	99.06
115.20	325.97	325.40	325.68	114.05	99.01
158.70	455.31	453.69	454.50	159.17	100.29
158.70	454.00	453.92	453.96	158.98	100.18
158.70	452.36	452.52	452.44	158.45	99.84
				Mean	99.05
				SD	0.93
				RSD	0.94

An average recovery of 99.05% was obtained, which proved the method to be accurate.

A.1.3.3 PRECISION

Precision indicates the proximity of a value obtained from replicate measurements under the prescribed conditions. Precision is divided into intra-day and inter-day variation, under normal operating conditions (Araujo, 2009:2227).

A.1.3.3.1 Intra-day precision

Intra-day indicates the repeatability of the method and expresses the precision evaluated under the same experimental conditions within the same day (Araujo, 2009:2227).

Intra-day was assessed by weighing 3 x 2.5 mg; 3 x 5.0 mg and 3 x 7.5 mg vitamin A in 100 ml volumetric flasks and filled to volume with ethanol:PBS (50:50). Each of the aforementioned solutions was diluted after extracting 5 ml by filling it to 100 ml with ethanol:PBS (50:50). A standard solution was also prepared as described in section A.1.2. The samples, as well as the standard solutions, were sonicated and transferred to amber HPLC vials. All the samples as well as the standard were injected in duplicate into the HPLC and the procedure was repeated on the same day.

Results are shown in Table A.4. The acceptance criterion for intra-day precision is a % RSD of ≤ 2.

Table A.3: Intra-day precision for vitamin A

				Concentration	
Mass (g)	**Peak area 1**	**Peak area 2**	**Mean peak area**	**(µg/ml)**	**%**
2.6	143.1	142.6	142.9	2.7	102.2
2.8	153.4	154.8	154.1	2.9	102.5
2.7	146.6	148.0	147.3	2.8	102.9
5.3	289.5	290.1	289.8	5.4	102.4
5.2	276.4	276.1	276.3	5.2	100.0
5.0	264.0	267.1	265.6	5.0	99.3
7.5	397.5	396.3	396.9	7.5	98.9
7.7	408.2	408.6	408.4	7.7	100.2
7.7	405.9	402.9	404.4	7.6	99.3
				Mean	**100.9**
				SD	**1.5**
				% RSD	**1.5**

The obtained % RSD of 1.5 was within the acceptable range. Therefore, precision of vitamin A was satisfactory.

A.1.3.3.2 Inter-day precision

Inter-day precision was performed by spacing out the measurements over a period of three consecutive days, in order to change factors that could vary between days, for example temperature.

Samples were prepared in triplicate as described in Section A.1.2, on the three different days to determine the between-day variability of the method. The samples were transferred into amber HPLC vials and injected in duplicate into the HPLC. The acceptance criterion according to

pharmaceutical standards for inter-day precision is a % RSD of ≤ 5. Results of the data on the three different days are shown in Table A.4.

Table A.4: Inter-day precision for vitamin A

	Day 1	Day 2	Day 3	Between days
	102.4	99.3	99.4	
	100.0	102.7	96.5	
	99.3	102.9	107.6	
Mean	100.6	101.6	101.2	**101.1**
SD	1.3	1.7	4.7	**0.4**
%RSD	1.3	1.6	4.6	**0.4**

Inter-day precision complied with the pharmaceutical standards with a % RSD of 0.4. The assay will perform well when carried out under standard laboratory conditions.

A.1.3.4 RUGGEDNESS

Ruggedness can be defined as the measure in which test results can be duplicated under normal conditions, whether using different analysts or laboratories.

Ruggedness of a sample solution can be divided into two categories, namely sample stability and system repeatability.

A.1.3.4.1 Stability of sample solutions

A sample was prepared as described in Section A.1.2. The stability and degradation of the sample was determined by analysis on the HPLC. A sample was left in the autosampler tray and analysed at one hour time-intervals over a period of 12 h. Due to the inherent instability of vitamin A, only a 12 h study was performed on the samples. If sample solutions degrade by 2%, it may not be used from that time-interval forward. In Table A.5 the stability values of vitamin A are shown.

Table A.5: Stability values of vitamin A

Time (h)	Peak area	% remaining
0	310.51	100.00
1	304.95	98.21
2	301.84	97.21
3	299.43	96.43
4	295.79	65.26
5	293.48	94.52
6	290.38	93.52
7	287.90	92.72
8	285.77	92.03
9	283.06	91.16
10	280.35	90.29
11	276.65	89.10
12	273.84	88.19
Mean	291.1	91.4
SD	10.8	8.3
% RSD	3.7	9.1

Vitamin A proved instable, with an average degradation of 8.6% during the 12 h-period. This problem was attended to in Appendix B.2.2.1, in an attempt to compensate for the loss. It was found that vitamin A was more stable in the formulation of the Pheroid™ cream, due to the stabilising effect of the anti-oxidants and other protective ingredients (see Table A.6).

A.1.3.4.2 System repeatability

Repeatability was determined by preparing a sample of 10 mg vitamin A in a 100 ml volumetric flask, filled to volume with ethanol:PBS (50:50). It was transferred to an amber HPLC vial and the sample was injected six consecutive times into the HPLC on the same day. The peak area and retention times should have a % RSD of ≤ 2 in order for the system to be repeatable. In table A.6 the peak areas and retention times are shown to determine the repeatability of vitamin A.

Table A.6: System repeatability of vitamin A

Injection	Peak area	Retention time (min)
1	329.94	4.75
2	325.31	4.74
3	323.91	4.76
4	323.61	4.76
5	320.64	4.76
6	320.74	4.76
Mean	324.03	4.76
SD	3.14	0.01
% RSD	0.97	0.16

System repeatability for vitamin A proved to be acceptable with a % RDS value of 0.97 for peak area and 0.16 for retention time, respectively.

A.1.3.5 SPECIFICITY

According to Hong & Shah (2000:363), specificity can be defined as the ability to clearly distinguish between the active ingredient tested and other compounds expected to be present in the sample, for example impurities and degradation products.

A standard solution was prepared by weighing 10 mg vitamin A in a 100 ml volumetric flask. This flask was filled to volume with ethanol:PBS (50:50). Forced degradation was induced by preparing four test tubes with 1 ml of the standard solution in each. The standard solution in the test tubes was diluted 1:1 with 10% hydrogen peroxide; 0.1 M hydrochloric acid; 0.1 M sodium hydroxide; and HPLC grade water, respectively. Due to the fact that vitamin A is more prone to degradation, the samples were transferred into amber HPLC vials and injected in duplicate into the HPLC immediately in order to examine if any other peaks were formed.

No degradation products interfered with the peak of vitamin A. Therefore, the method"s specificity was proved and accepted according to the acceptance criteria. Figures A.2 to A.6 illustrate the different chromatograms of standard solutions that were forced to degrade with the use of different chemical compounds.

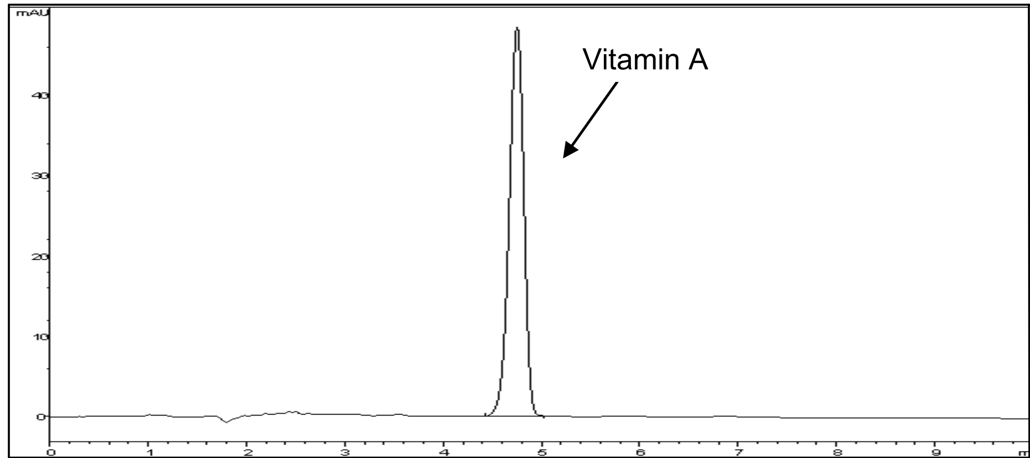

Figure A.2: **Chromatogram of a standard vitamin A solution.**

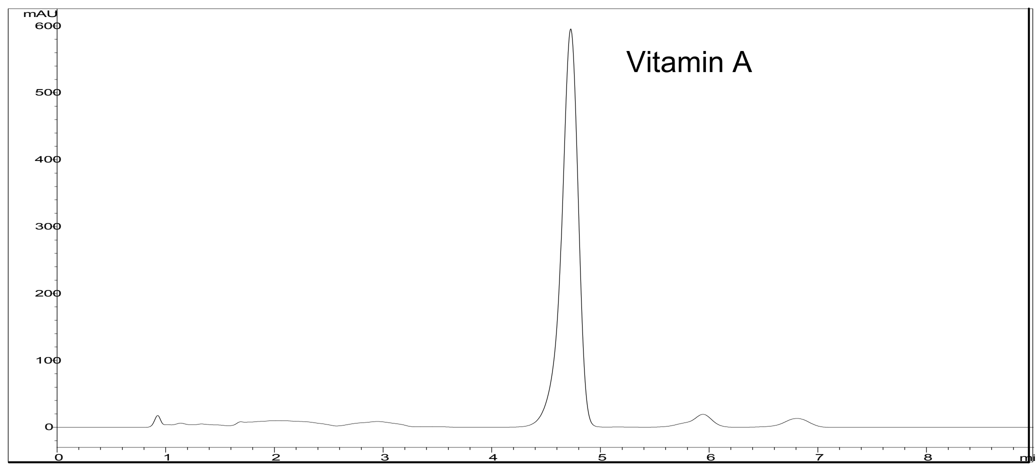

Figure A.3: **Chromatogram of a standard solution stressed in 10% hydrogen peroxide.**

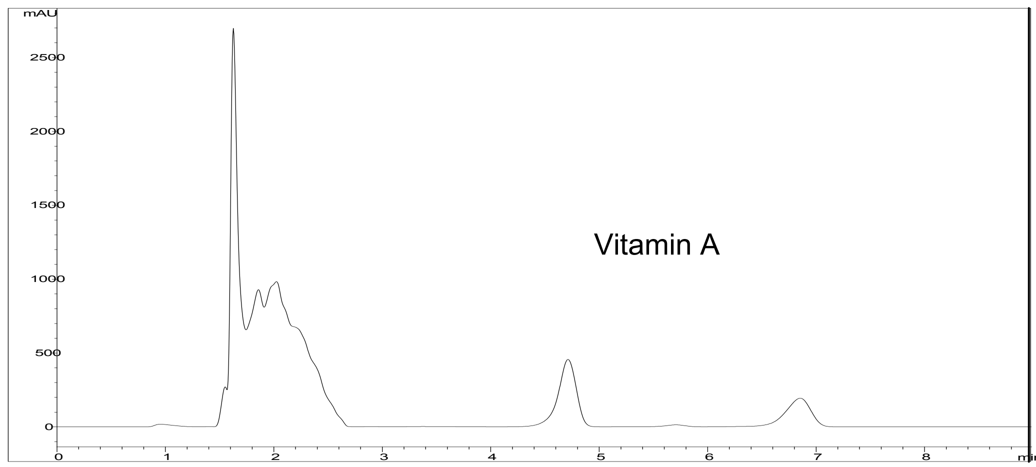

Figure A.4: **Chromatogram of a standard solution stressed in 0.1 M hydrochloric acid.**

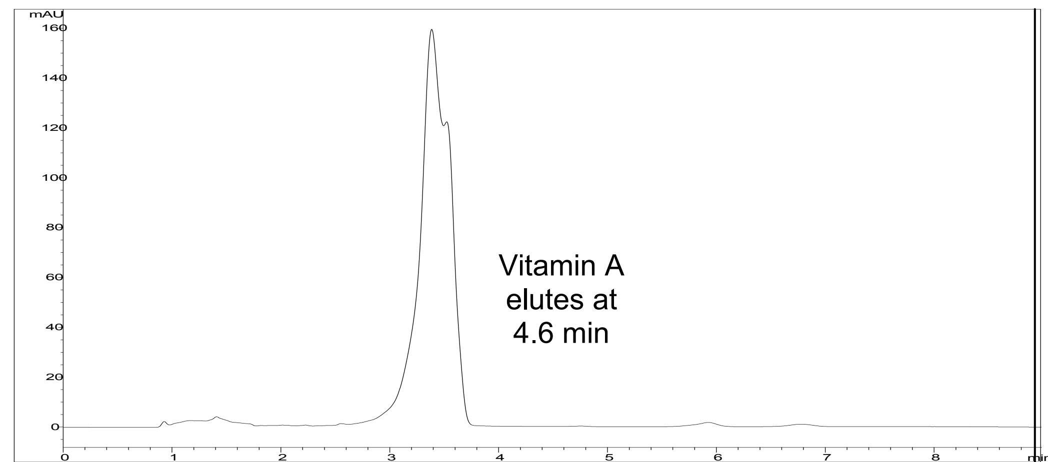

Figure A.5: Chromatogram of a standard solution stressed in 0.1 M sodium hydroxide.

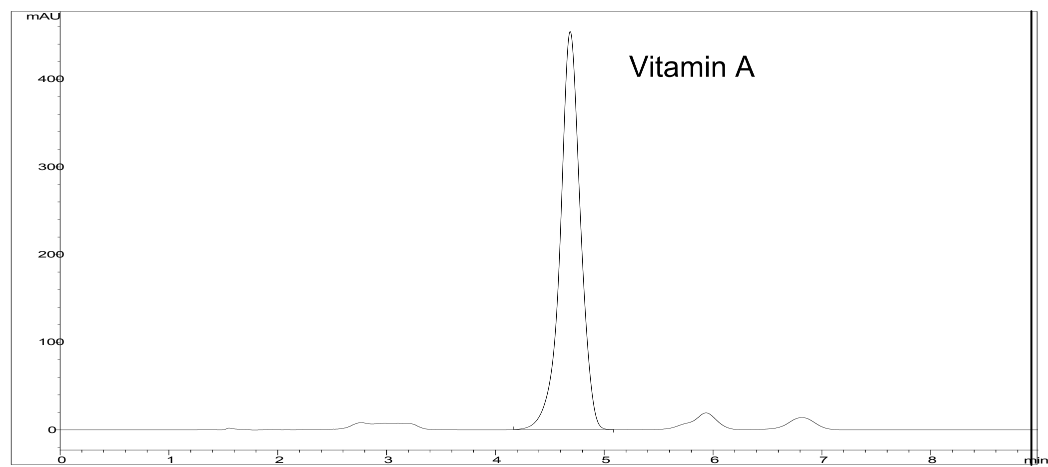

Figure A.6: Chromatogram of a standard solution stressed in 0.1 M HPLC grade water.

Vitamin A is prone to completely degrade in the presence of sodium hydroxide and less prone for degradation in hydrochloric acid. Vitamin A seems to be relatively stable in the presence of water and hydrogen peroxide, respectively.

A.1.3.6 CONCLUSION

The HPLC method developed demonstrated satisfactory results for accuracy, precision and specificity. The stability problem of vitamin A was attended to in an attempt to find possible solutions. Therefore, it could be said that the method for vitamin A proved to be sensitive, accurate and consistent, and could be used for the analysis of vitamin A during the *in vitro* drug release and transdermal drug diffusion studies.

A.2. HPLC METHOD VALIDATION FOR PHEROID™ CREAM

A.2.1 INTRODUCTION

The purpose of this validation was to demonstrate that the HPLC method used, during the six months of stability testing, was sensitive, accurate and consistent in the determination of the six ingredients present in the formulated products. A cream, Pheroid™ cream, gel and Pheroid™ gel were formulated. The method of analysis was done on the Pheroid™ cream, due to the fact that it was the formulation that contained all the ingredients that need to be tested. The ingredients in the formulation included: vitamin A acetate (API); methylparaben and propylparaben (preservatives); butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (anti-oxidants) as well as dl-α-tocopherol. Analyses were performed in a controlled laboratory environment at a temperature of 25 °C (± 2 °C).

A.2.2 CHROMATOGRAPHIC CONDITIONS

The same analytical instrument and column during HPLC analysis was used as described in Section A.1.1 except for the following:

Mobile phase: The mobile phase consisted of two solvents:

A. 0.1% octanesulfonic acid solution was the first solvent used in the mobile phase. The solution was prepared by accurately weighing 1 g octanesulphonic acid and dissolving it in 950 ml HPLC grade water. The pH was buffered with 10% phosphoric acid (H_3PO_4) and 10% ammonium hydroxide (NH_4OH) to 3.5; and the solution was made up to 1000 ml with HPLC grade water. An ultrasonic bath was used to degas the solution.

B. Methanol – analytical HPLC grade (100%).

Table A.7 illustrates the mobile phase composition that consisted of octanesulfonic acid buffer (solvent A) and methanol (solvent B), as well as the timetable used during the gradient elution.

Table A.7: Timetable for the composition of the mobile phase during gradient elution

Time (min)	Solvent A	Solvent B
8.0	0.0	100.0
20.0	0.0	100.0
20.20	60.0	40.0

Flow rate: 1.0 ml/min

Injection volume: 10 µl

Detection: UV at 220 nm

Run time: 25 min

Retention time: Methylparaben eluted first at 5.7 min, followed by propylparaben (7.7 min), BHA (8.6 min), BHT (10.9 min), vitamin A (12.7 min) and dl-α-tocopherol (17.2 min).

A.2.3 STANDARD SOLUTION PREPARATION

Ethanol was used to dissolve vitamin A during the preparation of the standard solutions for the Pheroid™ cream. Table A.8 indicates the amount of each active ingredient weighed.

Table A.8: Standard solution preparation

Substance	Amount weighed (mg)
Methylparaben	2
Propylparaben	5
BHA	1
BHT	1
Vitamin A	5
dl-α-tocopherol	2

A standard solution (as for 100%) was prepared by accurately weighing each of the ingredients and transferring it to a 50 ml volumetric flask. When all the ingredients were added in the 50 ml flask, it was made up to volume with ethanol. The amount of propylparaben was too small to weigh accurately, therefore 5 mg was weighed in a 50 ml volumetric flask and diluted with ethanol. From this solution, 4 ml was extracted and added to the standard solution to ensure the correct concentration. The standard solution was sonicated in an ultrasonic bath for approximately 5 min to ensure that all the ingredients were completely dissolved. Amber HPLC sample vials were filled with the standard solution and analysed on the HPLC, using the method described in Section A.2.2.

In assessing the accuracy of the method, a placebo preparation was needed. The substances used and the amounts weighed are shown in Table A.9.

Table A.9: Placebo preparation for 100 g cream

Substance	Amount weighed (g)
Vitamin F ethyl ester	2.8
Cremophor RH 40	1.0
Cetyl alcohol	10.0
Span 60	1.5
Tween 80	1.5
Liquid paraffin	12.0
Water	71.2

A.2.4 SAMPLE PREPARATION

The cream should be thoroughly stirred with a spatula to ensure homogeneity, before the sample preparation started. The sample was prepared by weighing 1 g Pheroid™ cream in a 50 ml volumetric flask and diluting it by adding ethanol. To ensure that all the cream has dissolved, the sample was sonicated and vigorously shaken repeatedly.

A.2.5 VALIDATION PARAMETERS

A.2.5.1 LINEARITY

As described in Section A.1.3.1, an analytical method is considered linear, if the result delivered is directly proportional to the concentration of the ingredient in the sample within a given range. It is calculated by the linear equation (see Equation A.1) (ICH, 2005:5). The regression coefficient (r^2) is determined according to the linear equation and the acceptance criterion for linear regression is a regression coefficient of ≥ 0.99.

A 130% standard solution was prepared for assessment of the linearity. Various dilutions were made from the 130% standard solution, delivering a wide range of concentrations (See Table A.10 to A.15). The linear regression curves are also plotted and are shown in Figures A.7 to A.12.

Table A.10: The peak area values of vitamin A

Concentration (µg/ml)	Peak area
130.6	918.53
117.54	817.39
104.48	731.95
91.42	636.72
78.36	538.85
65.30	453.59
r^2	0.9995
y-intercept	- 14.7790
slope	7.1222

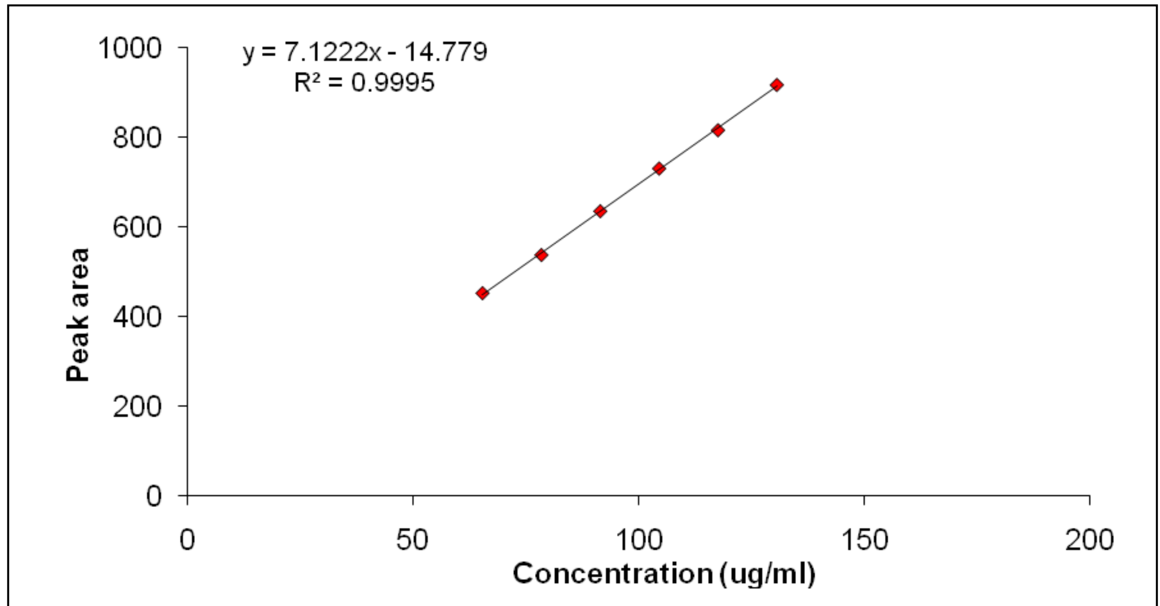

Figure A.7: Linear regression curve of vitamin A.

The method was linear for vitamin A over a concentration range of 65.30-130.6 µg/ml. The attained regression value (r^2) indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.11: The peak area values of methylparaben

Concentration (µg/ml)	Peak area
53.60	908.96
48.25	815.13
42.88	731.55
37.52	631.37
32.16	543.91
26.80	462.85
r^2	0.9993
y-intercept	8.4969
slope	16.7610

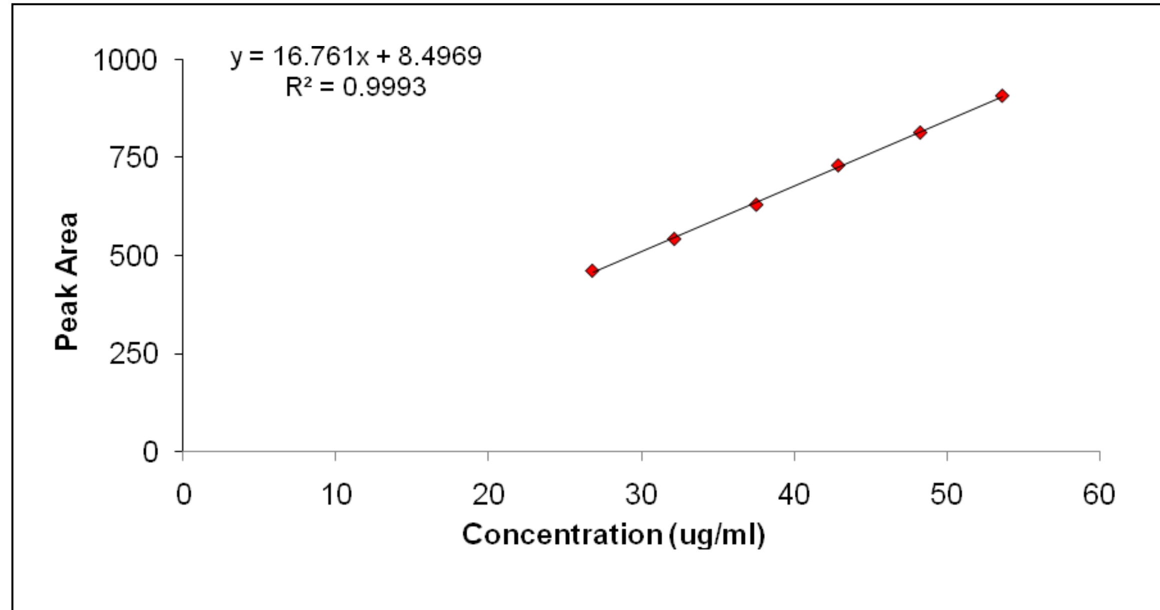

Figure A.8: Linear regression curve of methylparaben.

The method was linear for methylparaben over a concentration range of 26.80-53.60 µg/ml. The attained regression value (r^2) indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.12: The peak area values of propylparaben

Concentration (µg/ml)	Peak area
10.85	157.80
9.76	139.49
8.68	124.21
7.59	108.57
6.51	91.59
5.42	76.68
r^2	0.9993
y-intercept	- 4.6644
slope	14.8790

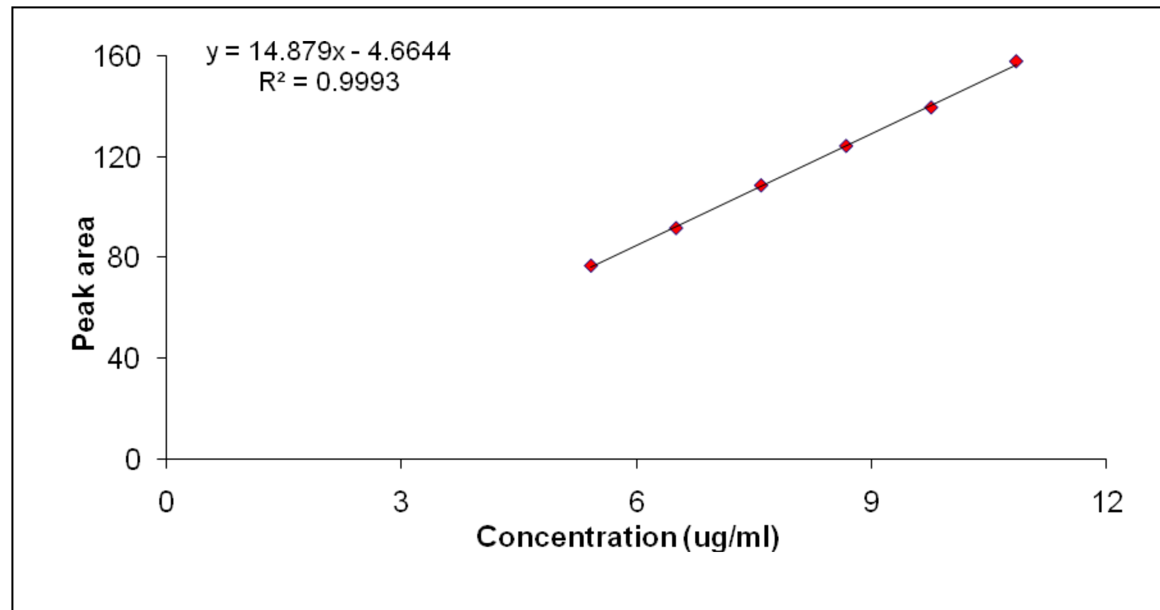

Figure A.9: Linear regression curve of propylparaben.

The method was linear for propylparaben over a concentration range of 5.42-10.85 µg/ml. The attained regression value (r^2) indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.13: The peak area values of BHA

Concentration (μg/ml)	Peak area
30.40	570.10
27.36	502.47
24.32	445.50
21.28	382.77
18.24	327.59
15.20	274.10
r^2	0.9986
y-intercept	- 25.9210
slope	19.4300

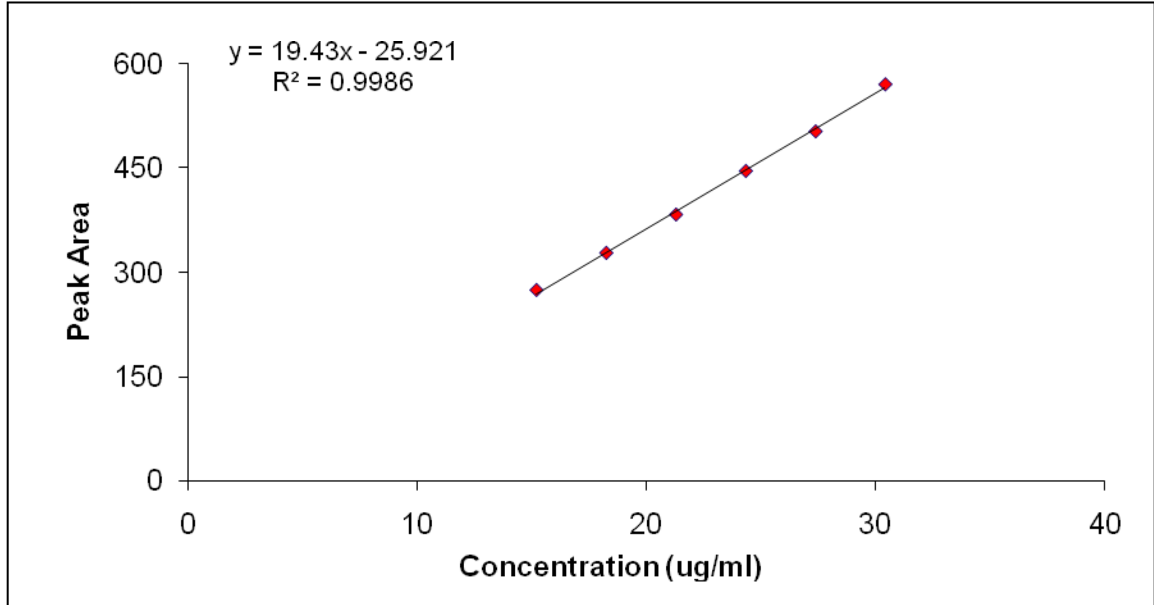

Figure A.10: Linear regression curve of BHA.

The method was linear for BHA over a concentration range of 15.20-30.40 μg/ml. The attained regression value (r^2) indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.14: The peak area values of BHT

Concentration (µg/ml)	Peak area
29.60	665.22
26.64	595.20
23.68	534.18
20.72	463.81
17.76	399.60
14.80	337.73
r^2	0.9996
y-intercept	7.5923
slope	22.1490

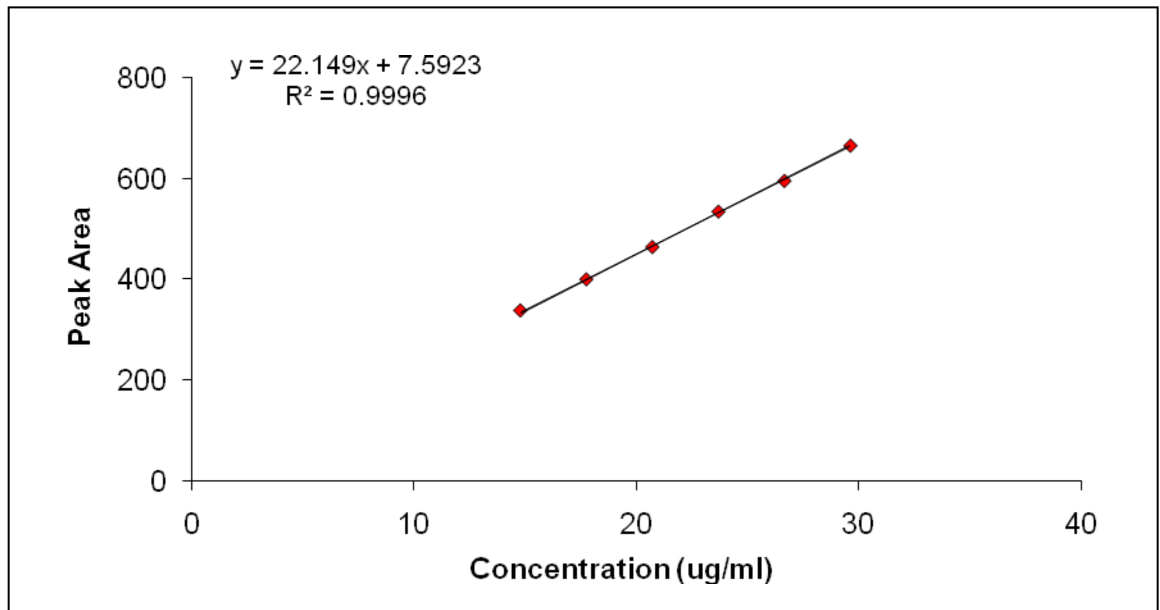

Figure A.11: Linear regression curve of BHT.

The method was linear for BHT over a concentration range of 14.80-29.60 µg/ml. The attained regression value (r^2) indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

Table A.15: The peak area values of dl-α-tocopherol

Concentration (µg/ml)	Peak area
55.60	766.21
50.04	681.08
44.48	615.57
38.92	534.27
33.36	449.58
27.80	379.20
r^2	0.999
y-intercept	- 9.9262
slope	13.931

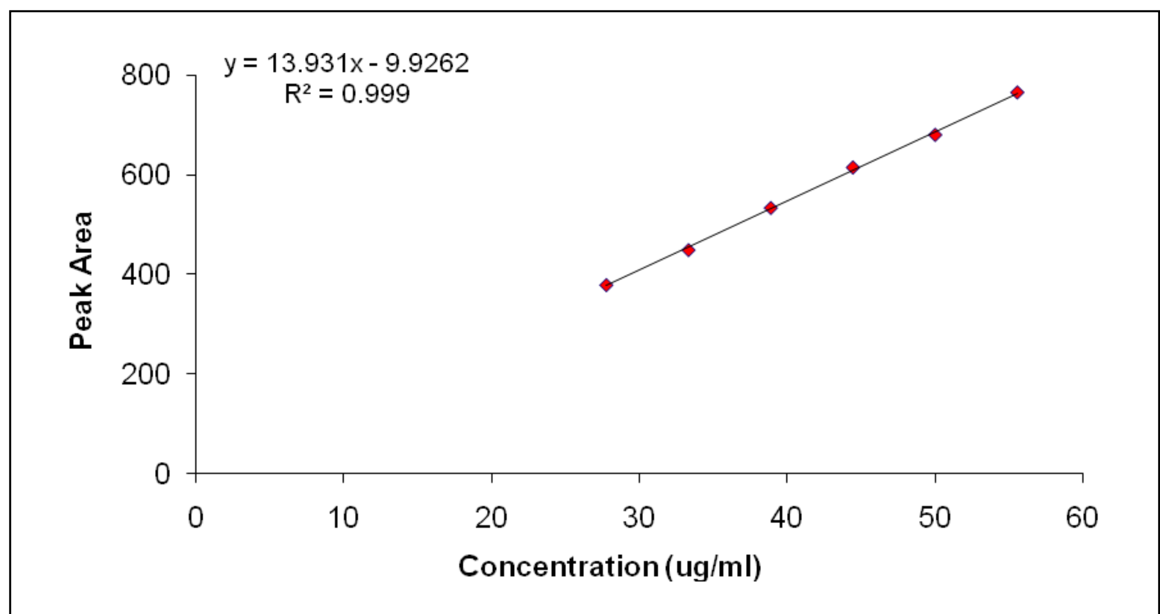

Figure A.12: Linear regression curve of dl-α-tocopherol.

The method was linear for dl-α-tocopherol over a concentration range of 27.80 – 55.60 µg/ml. The attained regression value (r^2) indicated a high degree of linearity and therefore demonstrated good stability of the analysis system.

A.2.5.2 ACCURACY

Accuracy is the most crucial step in any analytical method and is described in Section A.1.3.2. Accuracy was assessed by weighing approximately 3 x 0.8 g (80%); 3 x 1.0 g (100%) and 3 x 1.2 g (120%) placebo cream, as described in Table A.9, into 50 ml volumetric flasks. Normally, a ten times stronger standard solution was prepared, but due to the difficulties with the solubility of vitamin A, only a 130% solution could be prepared. The placebo creams that were weighed, were spiked with known amounts of the 130% standard solution to produce solutions with specific concentrations. The following solutions were prepared:

- 32 ml standard solution added to the 80% placebo cream;
- 40 ml standard solution added to the 100% placebo cream and
- 48 ml standard solution added to the 120% placebo cream.

The samples were filled to volume with ethanol (solvent), sonicated and transferred to amber sample vials. It was analysed in duplicate on the HPLC.

The percentage recovery is an indication of the accuracy and the results are shown in the Tables A.16 to A.21. The acceptance criterion for accuracy is a recovery between 98 – 102%.

Table A.16: Accuracy of vitamin A

Concentration spiked		Peak area			Recovery	
%	µg/ml	1	2	Mean	µg/ml	%
80	83.30	697.62	697.47	697.55	85.61	102.77
80	83.30	698.91	696.85	697.88	85.65	102.82
80	83.30	689.85	686.89	688.37	84.48	101.42
100	104.13	867.17	871.19	869.18	106.67	102.45
100	104.13	867.31	865.14	866.22	106.31	102.10
100	104.13	874.46	871.46	872.96	107.14	102.89
120	124.95	1039.30	1037.10	1038.20	127.42	101.97
120	124.95	1039.10	1039.33	1039.22	127.54	102.07
120	124.95	1038.94	1038.79	1038.87	127.50	102.04
					Mean	102.28
					SD	0.46
					RSD	0.45

The percentage vitamin A recovered ranged between 101.42% and 102.89%, with an average recovery of 102.28%. According to the type of formulation prepared, these values was still

inside the range and acceptable. Therefore, the method could be considered as accurate for vitamin A.

Table A.17: Accuracy of methylparaben

Concentration spiked		Peak area			Recovery	
%	**µg/ml**	**1**	**2**	**Mean**	**µg/ml**	**%**
80	34.02	1090.68	1091.28	1090.98	34.21	100.54
80	34.02	1098.13	1096.70	1097.42	34.41	101.14
80	34.02	1096.93	1098.15	1097.54	34.41	101.15
100	42.53	1358.00	1358.98	1358.49	42.60	100.16
100	42.53	1356.26	1357.53	1356.90	42.55	100.04
100	42.53	1365.24	1366.70	1365.97	42.83	100.71
120	51.03	1613.68	1611.80	1612.74	50.57	99.09
120	51.03	1608.39	1609.47	1608.93	50.45	98.85
120	51.03	1601.22	1603.24	1602.23	50.24	98.44
					Mean	100.01
					SD	0.94
					RSD	0.94

The percentage methylparaben recovered ranged between 98.44% and 101.15%, with an average recovery of 100.01%. These values were acceptable according to the criteria, and therefore the method could be considered as accurate for methylparaben.

Table A.18: Accuracy of propylparaben

Concentration spiked		Peak area			Recovery	
%	µg/ml	1	2	Mean	µg/ml	%
80	6.72	178.96	177.35	178.15	6.69	99.60
80	6.72	177.11	177.22	177.16	6.66	99.05
80	6.72	179.35	179.15	179.25	6.73	100.22
100	8.40	225.84	227.81	226.82	8.52	101.45
100	8.40	224.00	225.78	224.89	8.45	100.59
100	8.40	227.94	227.87	227.91	8.56	101.94
120	10.08	271.98	275.12	273.55	10.28	101.96
120	10.08	270.42	271.69	271.06	10.18	101.03
120	10.08	270.48	270.78	270.63	10.17	100.87
					Mean	100.74
					SD	0.94
					RSD	0.93

The percentage propylparaben recovered ranged between 99.05% and 101.96%, with an average recovery of 100.74%. These values were acceptable according to the criteria, and therefore the method could be considered as accurate for propylparaben.

Table A.19: Accuracy of BHA

Concentration spiked		Peak area			Recovery	
%	**µg/ml**	**1**	**2**	**Mean**	**µg/ml**	**%**
80	17.01	275.00	274.92	274.96	17.47	102.67
80	17.01	275.79	275.09	275.44	17.50	102.85
80	17.01	274.07	273.48	273.77	17.39	102.23
100	21.26	340.29	340.83	340.56	21.63	101.73
100	21.26	339.56	340.77	340.17	21.61	101.61
100	21.26	338.55	337.96	338.26	21.49	101.04
120	25.52	409.68	408.01	408.84	25.97	101.77
120	25.52	408.38	407.99	408.18	25.93	101.61
120	25.52	407.41	409.39	408.40	25.94	101.66
					Mean	101.91
					SD	0.54
					RSD	0.53

The percentage BHA recovered ranged between 101.77% and 102.85%, with an average recovery of 101.77%. These values were acceptable according to the criteria, and therefore the method could be considered as accurate for BHA.

Table A.20: Accuracy of BHT

Concentration spiked		Peak area			Recovery	
%	**µg/ml**	**1**	**2**	**Mean**	**µg/ml**	**%**
80	16.83	911.27	912.65	911.96	17.39	103.31
80	16.83	909.59	909.54	909.57	17.34	103.04
80	16.83	910.73	911.29	911.01	17.37	103.20
100	21.04	1144.17	1145.77	1144.97	21.83	103.77
100	21.04	1140.03	1141.24	1140.64	21.75	103.37
100	21.04	1144.13	1146.09	1145.11	21.84	103.78
120	25.25	1386.32	1388.83	1387.58	26.46	104.79
120	25.25	1384.83	1382.66	1383.75	26.39	104.51
120	25.25	1380.69	1381.27	1380.98	26.33	104.30
					Mean	103.79
					SD	0.59
					RSD	0.56

The percentage BHT recovered ranged between 103.78% and 104.79%, with an average recovery of 103.79%. According to the type of formulation prepared, these values was still inside the range and acceptable. Therefore, the method could be considered as accurate for BHT.

Table A.21: Accuracy of dl-α-tocopherol

Concentration spiked		Peak area			Recovery	
%	µg/ml	1	2	Mean	µg/ml	%
80	33.80	873.23	875.90	874.57	34.08	100.82
80	33.80	877.52	876.65	877.08	34.18	101.12
80	33.80	877.92	876.05	876.98	34.18	101.10
100	42.26	1085.28	1086.82	1085.82	42.31	100.14
100	42.26	1084.50	1084.24	1084.37	42.28	100.00
100	42.26	1082.25	1097.92	1090.09	42.48	100.53
120	50.71	1315.32	1316.97	1316.15	51.29	101.15
120	50.71	1310.89	1307.16	1309.03	51.01	100.60
120	50.71	1309.76	1308.56	1309.16	51.02	100.61
					Mean	100.67
					SD	0.39
					RSD	0.39

The percentage dl-α-tocopherol recovered ranged between 100.82% and 101.15%, with an average recovery of 100.67%. These values were acceptable according to the criteria, and therefore the method could be considered as accurate for dl-α-tocopherol.

A.2.5.3 PRECISION

A simplified definition of precision was given by Hong and Shah (2000:361), as the distribution of test results around their average. Precision can be divided into intra-day precision and inter-day precision.

A.2.5.3.1 Intra-day precision

Intra-day was assessed by weighing 3 x 0.8 g (80%); 3 x 1.0 g (100%) and 3 x 1.2 g (120%) into 50 ml volumetric flasks and filled to volume with ethanol. The solutions were sonicated and transferred to amber HPLC vials. A 100% standard solution was prepared as described in section A.2.3. The samples and the standard were injected in duplicate into the HPLC. The procedure was repeated on the same day.

Results are shown in the Tables A.22 to A.27. The acceptance criterion for intra-day precision is a % RSD of ≤ 2 as previously mentioned in Section A.1.3.3.1.

Table A.22: Intra-day precision for vitamin A

				Concentration	
Mass	**Peak area 1**	**Peak area 2**	**Mean**	**(µg/ml)**	**%**
0.80835	615.6	620.7	618.1	93.47	115.63
0.83340	647.3	643.7	645.5	97.61	117.12
0.80400	610.7	612.7	611.7	92.50	115.05
1.07024	799.4	791.3	795.3	120.26	112.37
1.04280	786.7	795.6	791.1	119.63	114.72
1.03986	784.4	785.8	785.1	118.72	114.17
1.20583	911.2	910.2	910.7	137.70	114.20
1.21963	918.4	922.7	920.6	139.20	114.13
1.21525	914.5	920.2	917.4	138.71	114.14
				Mean	**114.6**
				SD	**1.2**
				% RSD	**1.1**

The obtained % RSD of 1.1 was within acceptable range. Therefore, the precision of vitamin A was satisfactory.

Table A.23: Intra-day precision for methylparaben

				Concentration	
Mass	**Peak area 1**	**Peak area 2**	**Mean**	**(µg/ml)**	**%**
0.80835	611.7	617.4	614.6	37.01	114.46
0.83340	624.1	629.1	626.6	37.73	113.19
0.80400	609.0	610.2	609.6	36.71	114.15
1.07024	812.7	813.2	812.9	48.96	114.36
1.04280	795.3	791.9	793.6	47.79	114.58
1.03986	782.6	784.8	783.7	47.19	113.46
1.20583	908.8	904.3	906.6	54.60	113.19
1.21963	909.8	906.3	908.0	54.68	112.09
1.21525	902.7	904.9	903.8	54.43	111.97
				Mean	**113.5**
				SD	**0.9**
				% RSD	**0.8**

The obtained % RSD of 0.8 was within acceptable range. Therefore, the precision of methylparaben was satisfactory.

Table A.24: Intra-day precision for propylparaben

				Concentration	
Mass	**Peak area 1**	**Peak area 2**	**Mean**	**(µg/ml)**	**%**
0.80835	93.2	96.7	94.9	6.84	105.78
0.83340	98.1	100.6	99.4	7.16	107.38
0.80400	95.5	96.6	96.0	6.92	107.59
1.07024	126.8	130.7	128.8	9.28	108.37
1.04280	124.6	126.2	125.4	9.04	108.31
1.03986	121.9	126.8	124.3	8.96	107.70
1.20583	144.3	144.7	144.5	10.41	107.94
1.21963	147.6	148.5	148.0	10.67	109.33
1.21525	145.5	144.1	144.7	10.43	107.27
				Mean	**107.7**
				SD	**0.9**
				% RSD	**0.8**

The obtained % RSD of 0.8 was within acceptable range. Therefore, the precision of propylparaben was satisfactory.

Table A.25: Intra-day precision for BHA

				Concentration	
Mass	**Peak area 1**	**Peak area 2**	**Mean**	**(µg/ml)**	**%**
0.80835	322.5	325.0	323.7	20.02	123.85
0.83340	331.2	334.7	333.0	20.59	123.55
0.80400	323.1	318.6	320.8	19.84	123.40
1.07024	418.2	422.9	420.5	26.01	121.52
1.04280	410.4	414.0	412.2	25.50	122.25
1.03986	406.6	410.7	408.6	25.27	121.53
1.20583	469.2	475.8	472.5	29.22	121.17
1.21963	476.1	480.2	478.1	29.57	121.23
1.21525	479.2	473.6	476.4	29.47	121.24
				Mean	**122.2**
				SD	**1.0**
				% RSD	**0.9**

The obtained % RSD of 0.9 was within acceptable range. Therefore, the precision of BHA was satisfactory.

Table A.26: Intra-day precision for BHT

				Concentration	
Mass	**Peak area 1**	**Peak area 2**	**Mean**	**(µg/ml)**	**%**
0.80835	414.1	409.2	411.6	19.35	119.70
0.83340	427.0	424.2	425.6	20.01	120.05
0.80400	408.0	401.3	404.7	19.03	118.32
1.07024	531.1	538.3	534.7	12.14	117.44
1.04280	528.1	532.2	530.2	24.93	119.51
1.03986	524.2	524.3	524.3	24.65	118.51
1.20583	613.7	609.2	611.5	28.75	119.20
1.21963	614.9	618.7	616.8	29.00	118.88
1.21525	608.3	613.2	610.7	28.71	118.13
				Mean	**118.9**
				SD	**0.8**
				% RSD	**0.7**

The obtained % RSD of 0.7 was within acceptable range. Therefore, the precision of BHT was satisfactory.

Table A.27: Intra-day precision for dl-α-tocopherol

				Concentration	
Mass	**Peak area 1**	**Peak area 2**	**Mean**	**(µg/ml)**	**%**
0.80835	472.2	478.8	475.5	31.32	96.86
0.83340	489.8	494.4	492.1	32.41	97.22
0.80400	470.8	473.6	472.2	31.10	96.71
1.07024	633.2	635.5	634.3	41.78	97.60
1.04280	626.3	620.7	623.5	41.07	98.46
1.03986	611.9	617.3	614.6	40.48	97.32
1.20583	711.0	712.2	711.6	46.87	97.17
1.21963	728.0	721.7	724.9	47.74	97.86
1.21525	713.6	718.0	715.8	47.15	96.99
				Mean	**97.4**
				SD	**0.5**
				% RSD	**0.5**

The obtained % RSD of 0.5 was within acceptable range. Therefore, the precision of dl-α-tocopherol was satisfactory.

A.2.5.3.2 Inter-day precision

Samples were prepared in triplicate, as described in Section A.2.3, on the three different days to determine the between-day variability of the method. The samples were transferred into amber HPLC vials and injected in duplicate into the HPLC. According to pharmaceutical standards for inter-day precision, a % RSD of ≤ 5, is accepted. Results are shown in Tables A.28 to A.33.

Table A.28: Inter-day precision for vitamin A

	Day 1	Day 2	Day 3	Between days
	112.17	107.58	112.73	
	112.34	108.32	115.30	
	112.72	106.97	114.16	
Mean	112.41	107.63	114.06	**111.37**
SD	0.23	0.55	1.05	**2.73**
% RSD	0.20	0.51	0.92	**2.45**

Inter-day precision for vitamin A complied with the pharmaceutical standards (% RSD = 2.45). The assay would therefore perform well when carried out under standard laboratory conditions.

Table A.29: Inter-day precision for methylparaben

	Day 1	Day 2	Day 3	Between days
	111.24	112.79	114.37	
	112.49	112.13	114.36	
	111.14	112.76	113.46	
Mean	111.62	112.56	114.06	**112.75**
SD	0.61	0.30	0.42	**1.01**
% RSD	0.55	0.27	0.37	**0.89**

Inter-day precision for methylparaben complied with the pharmaceutical standards (% RSD = 0.89). The assay would therefore perform well when carried out under standard laboratory conditions.

Table A.30: Inter-day precision for propylparaben

	Day 1	Day 2	Day 3	Between days
	102.94	107.57	108.79	
	103.44	106.54	109.60	
	101.78	106.76	108.14	
Mean	103.19	107.06	109.20	**106.48**
SD	0.25	0.52	0.41	**2.48**
% RSD	0.24	0.48	0.37	**2.33**

Inter-day precision complied with the pharmaceutical standards (% RSD = 2.33). The assay would therefore perform well when carried out under standard laboratory conditions.

Table A.31: Inter-day precision for BHA

	Day 1	Day 2	Day 3	Between days
	122.86	117.19	121.66	
	122.22	116.10	121.80	
	122.60	116.05	121.97	
Mean	122.54	116.65	121.73	**120.31**
SD	0.32	0.55	0.07	**2.61**
% RSD	0.26	0.47	0.06	**2.17**

Inter-day precision complied with the pharmaceutical standards (% RSD = 2.17). The assay would therefore perform well when carried out under standard laboratory conditions.

Table A.32: Inter-day precision for BHT

	Day 1	Day 2	Day 3	Between days
	115.94	119.43	117.99	
	116.82	120.33	119.74	
	115.44	118.96	118.51	
Mean	116.07	119.57	118.75	**118.13**
SD	0.57	0.57	0.73	**1.56**
% RSD	0.49	0.48	0.62	**1.27**

Inter-day precision complied with the pharmaceutical standards (% RSD = 1.27). The assay would therefore perform well when carried out under standard laboratory conditions.

Table A.33: Inter-day precision for dl-α-tocopherol

	Day 1	Day 2	Day 3	Between days
	99.87	95.70	97.52	
	98.21	97.37	98.22	
	98.67	97.18	97.48	
Mean	98.92	96.75	97.74	**97.80**
SD	0.70	0.75	0.34	**0.89**
% RSD	0.71	0.77	0.35	**0.91**

Inter-day precision complied with the pharmaceutical standards (% RSD = 0.91). The assay would therefore perform well when carried out under standard laboratory conditions.

A.2.5.4 RUGGEDNESS

Ruggedness of a sample solution can be divided into two categories, namely sample stability and system repeatability.

A.2.5.4.1 Stability of sample solutions

A sample was prepared as described in Section A.2.3. The sample was left in the autosampler tray and analysed at one hour time-intervals over a period of 12 h. Due to the inherent instability of vitamin A, only a 12 h study was performed on the samples. If sample solutions degrade by 2%, it may not be used from that time-interval forward. Stability values are shown in Tables A.34 to A.39.

Table A.34: Stability values of vitamin A

Time (h)	Peak area	% remaining
0	768.2	100.0
1	768.2	100.0
2	769.6	100.2
3	768.4	100.0
4	768.6	100.0
5	764.6	99.5
6	764.6	99.5
7	763.8	99.4
8	760.5	99.0
9	757.1	98.6
10	755.2	98.3
11	750.4	97.7
12	751.3	97.8
Mean	762.3	99.2
SD	6.5	0.9
% RSD	0.9	0.9

The stability of vitamin A proved to be acceptable, with an average degradation of 0.8% during the 12 h-period. The average degradation is acceptable. Interestingly the stability of vitamin A degrades more than 2% from the eleventh hour of analysis. If vitamin A needs to be used for a period longer than 12 h, special precautions will be followed to compensate for the loss, for example, sample bracketing. In a cyclic calibrated sequence with sample bracketing, the calibration table used to calculate the unknown quantitative results is generated by averaging the results of the current calibration with those of the previous calibration. This new calibration table is a more accurate representation of the instrument response at the time the sample was analysed (Agilent Chemstation, 2000:213).

Table A.35: Stability values of methylparaben

Time (h)	Peak area	% remaining
0	789.7	100.0
1	789.6	100.0
2	789.6	100.0
3	789.7	100.0
4	789.8	100.0
5	787.1	99.7
6	788.0	99.8
7	789.1	99.9
8	786.2	99.6
9	785.5	99.5
10	784.7	99.4
11	784.3	99.3
12	784.2	99.3
Mean	787.5	99.7
SD	2.2	0.3
% RSD	0.3	0.3

The stability of methylparaben proved to be acceptable, with an average degradation of 0.3% during the 12 h-period.

Table A.36: Stability values of propylparaben

Time (h)	Peak area	% remaining
0	131.3	100.0
1	131.4	100.1
2	131.5	100.2
3	131.3	100.0
4	131.3	100.0
5	131.4	100.1
6	131.7	100.3
7	130.7	99.5
8	130.5	99.4
9	129.9	99.0
10	130.4	99.3
11	130.6	99.5
12	130.0	99.0
Mean	130.9	99.7
SD	0.6	0.4
% RSD	0.4	0.4

The stability of propylparaben proved to be acceptable, with an average degradation of 0.3% during the 12 h-period.

Table A.37: Stability values of BHA

Time (h)	Peak area	% remaining
0	415.9	100.0
1	416.7	100.2
2	417.0	100.3
3	417.7	100.4
4	416.5	100.1
5	413.6	99.4
6	414.0	99.5
7	413.1	99.3
8	412.0	99.1
9	413.0	99.3
10	412.0	99.1
11	412.5	99.2
12	411.6	99.0
Mean	414.3	99.6
SD	2.1	0.5
% RSD	0.5	0.5

The stability of BHA proved to be acceptable, with an average degradation of 0.4% during the 12 h-period.

Table A.38: Stability values of BHT

Time (h)	Peak area	% remaining
0	414.2	100.0
1	414.2	100.0
2	415.0	100.2
3	415.5	100.3
4	414.4	100.0
5	412.3	99.5
6	408.7	98.7
7	404.6	97.7
8	404.8	97.7
9	404.5	97.6
10	403.6	97.4
11	402.1	97.1
12	403.1	97.3
Mean	409.0	98.7
SD	5.1	1.2
% RSD	1.3	1.3

The stability of BHT proved to be acceptable, with an average degradation of 1.3% during the 12 h-period.

Table A.39: Stability values of dl-α-tocopherol

Time (h)	Peak area	% remaining
0	610.2	100.0
1	610.2	100.0
2	610.0	100.0
3	609.4	99.9
4	609.5	99.9
5	608.5	99.7
6	607.6	99.6
7	604.5	99.1
8	602.2	98.7
9	603.1	98.8
10	602.6	98.7
11	602.0	98.7
12	601.6	98.6
Mean	606.3	99.4
SD	3.5	0.6
% RSD	0.6	0.6

The stability of dl-α-tocopherol proved to be acceptable, with an average degradation of 0.6% during the 12 h-period.

A.2.5.4.2 System repeatability

To determine the repeatability of the peak area and retention time, a sample was injected into the HPLC for six consecutive times on the same day. The sample was prepared as described in Section A.2.3. For system repeatability, peak area and retention times should have a % RSD of ≤ 2. The repeatability values of the system are shown in Tables A.40 to A.45.

Table A.40: System repeatability of vitamin A

Injection	Peak area	Retention time (min)
1	774.6	12.71
2	773.2	12.74
3	773.9	12.73
4	773.1	12.73
5	773.8	12.72
6	773.6	12.71
Mean	773.7	12.72
SD	0.5	0.0
% RSD	0.1	0.1

Acceptable system repeatability for vitamin A was obtained and can be seen in Table A.40. The % RSD for the peak area was 0.1%, as was the % RSD for retention time.

Table A.41: System repeatability of methylparaben

Injection	Peak area	Retention time (min)
1	771.8	5.70
2	770.0	5.70
3	771.2	5.70
4	773.3	5.72
5	773.1	5.70
6	772.9	5.70
Mean	772.1	5.71
SD	1.2	0.00
% RSD	0.2	0.1

Acceptable system repeatability for methylparaben was obtained and can be seen in Table A.41. The % RSD for the peak area was 0.2%, and the % RSD for retention time was 0.1%.

Table A.42: System repeatability of propylparaben

Injection	Peak area	Retention time (min)
1	124.2	7.74
2	124.5	7.74
3	123.7	7.74
4	124.0	7.75
5	124.4	7.73
6	122.9	7.72
Mean	123.9	7.74
SD	0.5	0.00
% RSD	0.4	0.1

Acceptable system repeatability for propylparaben was obtained and can be seen in Table A.42. The % RSD for the peak area was 0.4%, and the % RSD for retention time was 0.1%.

Table A.43: System repeatability of BHA

Injection	Peak area	Retention time (min)
1	403.7	8.62
2	403.5	8.62
3	405.2	8.62
4	406.6	8.62
5	406.9	8.61
6	405.5	8.59
Mean	405.2	8.61
SD	1.3	0.00
% RSD	0.3	0.1

An acceptable system repeatability for BHA was obtained and can be seen in Table A.43. The % RSD for the peak area was 0.3%, and the % RSD for retention time was 0.1%.

Table A.44: System repeatability of BHT

Injection	Peak area	Retention time (min)
1	509.7	10.91
2	507.0	10.93
3	506.0	10.92
4	506.5	10.92
5	508.4	10.92
6	506.3	10.91
Mean	507.3	10.92
SD	1.3	0.00
% RSD	0.3	0.1

Acceptable system repeatability for BHT was obtained and can be seen in Table A.44. The % RSD for the peak area was 0.3%, and the % RSD for retention time was 0.1%.

Table A.45: System repeatability of dl-α-tocopherol

Injection	Peak area	Retention time (min)
1	605.1	17.29
2	604.5	17.35
3	604.0	17.32
4	605.2	17.32
5	606.8	17.30
6	607.1	17.24
Mean	605.4	17.30
SD	1.1	0.00
% RSD	0.2	0.2

Acceptable system repeatability for dl-α-tocopherol was obtained and can be seen in Table A.45. The % RSD for the peak area was 0.2%, and the % RSD for retention time was 0.2%.

A.2.5.5 SPECIFICITY

Specificity of a sample is the ability to assess unequivocally the ingredient in the presence of components that may interfere with the detection thereof. The method is specific when no interfering peaks, with the same retention time as the ingredient under investigation, are detected.

A standard solution was prepared as described in section A.2.3. 4 x 1 ml standard solution was withdrawn and placed in four different test tubes. The standard solutions in the test tubes were diluted 1:1 with 10% hydrogen peroxide, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and HPLC grade water respectively. Samples were transferred into amber HPLC vials and immediately injected in duplicate into the HPLC, in order to examine if any other peaks were formed.

None of the components present in the Pheroid™ cream formulation or solvents used, interfered with the peaks of the tested ingredients. Vitamin A is prone to completely degrade in the presence of sodium hydroxide and less prone to degradation in hydrochloric acid. Vitamin A tends to be relatively stable in the presence of water and hydrogen peroxide.

A.2.6 CONCLUSION

The HPLC method developed demonstrated satisfactory results for accuracy, precision and specificity. Therefore, it could be said that the method is sensitive, accurate and consistent enough for the determination of the six ingredients, namely vitamin A, methylparaben, propylparaben, BHA, BHT and dl-α-tocopherol present in the formulated Pheroid™ cream.

This method could therefore be considered as an appropriate method to analyse the ingredients in the formulations for stability testing.

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APPENDIX B

FORMULATION OF COSMECEUTICAL EMULSIONS

B.1 INTRODUCTION

Topical formulations can be divided into two categories, namely cosmetic products and pharmaceutical products. According to McNamara (quoted by Magdassie & Touitou, 1999:2), a cosmetic is a product that is applied topically, but has no systemic effect. It is usually used by healthy people to enhance appearance and personal hygiene. In contrary, a pharmaceutical product could affect the structure and function of the body, due to their use for the treatment and prevention of illnesses (Mitsui, 1997:4).

Twenty five years ago, Dr. Albert Kligman added a third group of products to topical formulations that met the definition of both cosmetics and pharmaceutical products. This combination category is called cosmeceuticals, and is commonly used to describe a cosmetic product with pharmaceutical therapeutic benefit, but not necessarily a biologic therapeutic benefit (Choi & Berson, 2006:163).

In this study, cosmeceutical products were formulated, which include a cream, Pheroid™ cream, gel, and Pheroid™ gel. These products contained vitamin A and zinc as active ingredients and were also compared to a commercial product containing vitamin A acetate.

B.2 PREFORMULATION

The preformulation phase included studies that were performed before the formulation process started. In this phase all the physical and chemical properties of the drug substances were determined by means of a complete literature study. Preformulation should provide guidance in the compatibility of potential excipients and active ingredients, possible instabilities and solubility problems, which may occur (Wells & Aulton, 2007:337).

B.2.1 VEHICLE SELECTION

To be able to deliver drugs successfully to the skin, a compatible vehicle is needed. A vehicle is a carrier system with specific pharmaceutical, biopharmaceutical and cosmetic characteristics (Surber & Smith, 2000:17). The vehicle must promote the healing capability of the skin and must be able to release the active ingredient to the stratum corneum. Pharmaceutically used vehicle systems include liquid preparations, gels, powders, ointments, creams, pastes and aerosols (Barry, 2007:593-595). The formulations were designed to treat acne, therefore it was

decided to use a vehicle with predominantly hydrophilic properties, rather than a vehicle with lipophilic properties, to ensure that the properties of the vehicle will not influence the oiliness of the skin. A cream and a gel formulation were chosen as the vehicles.

B.2.2 PROBLEMS DURING PREFORMULATION

B.2.2.1 STABILISATION OF VITAMIN A

Formulations that contain both oils and vitamins are sensitive to oxidation, of which vitamin A is the perfect example. This happens due to the formation of lipid peroxides through auto-oxidation and consequently, a decrease in the vitamin A content. To prevent the auto-oxidation reaction, the initiation and chain transfer reaction phases should be suppressed. Compounds that are able to break these chain reactions are known as chain-breaking anti-oxidants, i.e. tocopherol (vitamin E), BHT and BHA. The anti-oxidative effect is elevated by anti-oxidant promoters which include phosphoric acid, ascorbic acid, citric acid and ethylene-diamine-tetra-acetic acid (EDTA), just to name a few (Mitsui, 1997:144-145).

The instability problem was detected during a 24 h stability test through the HPLC, where the quantity of the vitamin A was reduced with 10.90% during the first 12 h.

In an attempt to stabilise the vitamin A, different anti-oxidants were added, for example sodium-bisulphite, EDTA and ascorbic acid. Table B.1 contains the different anti-oxidants used in this study as well as the quantities they were used in and percentage decrease after 12 h.

Table B.1: Anti-oxidants, their quantities and results on the stability of vitamin A

Anti-oxidant(s)	Quantity	% Decreased
Sodium-bisulphite	0.5%	7.18%
EDTA	0.1%	7.02%
Ascorbic acid	0.5%	6.14%
Sodium-bisulphite + EDTA	0.5% and 0.1%, respectively	6.04%
Ascorbic acid + EDTA	0.5% and 0.1%, respectively	6.34%
BHA + BHT (preservatives)	0.1% respectively	6.10%

Different quantities of the anti-oxidants were weighed in a 100 ml volumetric flask and filled to volume with ethanol:PBS (50:50) after adding 5 ml of a 12.5 μg/ml vitamin A solution. The different mixtures were transferred to HPLC vials and injected into the HPLC. The percentage decreased after 12 h are reflected in table B.1.

According to these results, the minimum degradation was visible in the sodium-bisulphite and EDTA combination with degradation of 6.04%. Normally, a sample is allowed a 5% reduction in

concentration during a standard stability test (MCC, 2006:13). These results were unacceptable according to the acceptance criteria.

A Pheroid™ cream was formulated, as a final attempt, to establish whether the characteristics and components of the cream would be able to stabilise the vitamin A. A positive result was depicted, with a percentage degradation of 0.9% over a 12 h-period.

B.2.2.2 CONCLUSION

Vitamin A was stabilised when incorporated into a Pheroid™ cream formula, which included various ingredients, for example tocopherol, BHA, BHT, methylparaben and propylparaben. A factor that might have contributed, and is of significant importance in the stabilisation process of vitamin A, is the role of the fatty acids present in the Pheroid™ formulation. Loss of vitamin A during a 12 h-period was reduced from 10.90%, without any stabilising agents, to 0.9%, when formulated into a cream with stabilising agents. These results proved acceptable.

B.3 FORMULATION OF A CREAM

The purpose of a cream is to release incorporated active ingredients onto the skin surface, in the epidermis or in the stratum corneum, to protect and soften the skin (Pons *et al.,* 1999:169). Therefore, the main ingredients have to be carefully selected. According to Mitsui (1997:121), there are specific conditions that need to be considered before the selection of the materials start. The selected raw materials need to:

- have excellent functions, that matches the usage purpose,
- be safe,
- have good oxidation stability, and
- be of high quality.

Creams are defined as viscous semisolid emulsions for external use (Flynn, 2002:215). Emulsions consist of two immiscible phases (lipids and water), which remain in a metastable state due to the effects of an emulsifier. Two types of emulsions could be distinguished according to the different surfactants and oily ingredients used. It is either a water-in-oil (w/o) emulsion or an oil-in-water (o/w) emulsion (Mitsui, 1997:343). The o/w-emulsion is frequently used in cosmeceutical products and was formulated during this study.

An o/w-emulsion is where oily ingredients in the internal phase are dispersed through the external aqueous phase (Marriott *et al*., 2006:125). O/w-emulsions consist of many advantages, which include a non-greasy feeling when applied to the skin, good skin

spreadability and penetration, hydration of the skin by the external water phase and a cooling effect on the skin surface due to evaporation of the water phase (Buchmann, 2006:106-107). The aforementioned advantages were the reason for deciding to formulate an o/w-emulsion, which will fulfil the purpose of this study.

B.3.1 CREAM FORMULA

The final formula for a cream containing vitamin A and zinc, is given in Table B.2. Section A in Table B.2, represents the ingredients of the oil phase of the cream, and Section B the aqueous phase.

Table B.2: Formula of vitamin A and zinc cream

Raw material	% w/w
A: Liquid paraffin	12.00
Cetyl alcohol	10.00
Tween-80	1.50
Span-60	1.50
Methylparaben	0.20
Propylparaben	0.04
BHA	0.10
BHT	0.10
Vitamin A	0.50
B: Zinc	1.20
Distilled H_2O	to 100.00

B.3.2 MAIN INGREDIENTS OF A CREAM

The principle materials used to manufacture a cosmeceutical o/w-cream are listed in Table B.2 as previously stated. In the following sections, discussions follow on reasons for incorporating certain ingredients.

B.3.2.1 OILY INGREDIENTS

Liquid paraffin was part of the oil phase of the cream (see Table B.2, Section A). It is colourless, odourless and chemically inactive. Liquid paraffin is also a very desirable ingredient due to its moisturising effect and ability to easily form emulsions (Mitsui, 1997:124). It is furthermore used as a solvent (Kibbe, 2000:345).

B.3.2.2 THICKENING AGENT

Cetyl alcohol can be identified as waxy, white flakes, with a faint characteristic odour (Kibbe, 2000:117). It was used as thickening agent to alter the viscosity of the product as well as to maintain the stability thereof (Mitsui, 1997:138). Although insoluble in water, cetyl alcohol possesses good water absorptive and emulsifying properties (Mahalingam *et al.*, 2008:274). It is also used to stabilise emulsions in emulsified products (Mitsui, 1997:138).

B.3.2.3 EMULSIFIERS

Tween-80 and span-60 were used in combination as emulsifying agents to form a complex condensed film at the o/w interface. Tween-80 is a hydrophilic emulsifier and span-60 is a lipophilic emulsifier (Magdassie & Garti, 1999:156).

B.3.2.4 PRESERVATIVES

Methylparaben and propylparaben (commonly known as parabens) are used in conjunction as preservatives in cosmeceutical products, due to their complimentary effect on each other (Bühler, 1988:81). The function of a preservative is to suppress the proliferation of microorganisms and kill them in time, which in turn prevent the deterioration of the cream (Mitsui, 1997:201).

B.3.2.5 ANTI-OXIDANTS

Creams are composed of many different ingredients, some of which contain unsaturated bonds. It is generally presumed that fats and oils with two or more unsaturated bonds are easily oxidised, and consequently cause changes in the quality of the cream. To prevent oxidation from taking place, anti-oxidants need to be added, so that they may be oxidised first, before any other components of the cream are oxidised (Mitsui, 1997:144-145). Both BHA and BHT were used in their maximum concentration allowed. The anti-oxidant action of BHT for vitamin A was higher than that of BHA, but even higher if used in combination (Bühler, 1988:18).

B.3.2.6 SOLVENTS

In formulations, water is usually the most important solvent and occupies a significant volume percentage of the total cream. In this study, zinc was dissolved in the water phase and the oil phase acted as a solvent for vitamin A.

B.3.3 PACKAGING

Selection of packaging materials is very important and plays a crucial role in the stability of a product. Vitamin A is sensitive to light (photosensitive), and adsorbs to the wall of polyvinyl chloride containers (Bühler, 1988:80). Zinc on the other hand, may not be stored in metal containers. Therefore the ideal packaging material for vitamin A and zinc will be amber glass containers with screw caps, which will protect the product form the external atmosphere. Due to the scarcity of amber glass containers, normal glass containers with screw caps were used and covered in brown paper to protect the product from light during stability testing.

B.3.4 PREPARATION PROCESS OF THE CREAM

The cream formulation was prepared as follows:

- Weigh the distilled water (see Section B.3.1, Table B.2) and heat to 40 ± 2 °C.
- Weigh zinc and dissolve it in the heated water, while continue heating to 80 ± 2 °C.
- Weigh all the ingredients of A (see Section B.3.1, Table B.2), mix together and heat to 80 ± 2 °C.
- Add A (oil phase) to B (water phase) while homogenising at 13 500 rpm.
- Homogenise until a temperature of exactly 40 ± 2 °C is reached.
- Cool with ice water.
- Stir with a glass rod until room temperature (25 ± 2 °C) is reached.
- Transfer to glass containers.

B.3.5 PREPARATION OF PHEROID™ CREAM

The same procedure was followed as described in Section B.3.4, except for the Pheroid™ ingredients i.e., dl-α-tocopherol was added to the oil phase, and instead of distilled water, water saturated with nitrous oxide was used.

B.3.6 RESULTS

Both creams depicted a homogenous texture; it applied easily to the skin and were not too oily. Both were of a light yellowish colour, due to the yellow colour of the vitamin A.

B.4 FORMULATION OF AN EMULGEL

A gel can be defined as a solid or semisolid system, which consists of at least two constituents. Small discrete particles are dispersed throughout the dispersion medium (aqueous).

Gels consist over a few advantages, which include:

- Stability over a long time period.
- Acceptable appearance.
- Suitability as vehicles.
- A high rate of release of the active ingredient, and rapid absorption (Marriott *et al*., 2006:159).

Therefore, gels are popular vehicles, easy to manufacture and practical for topical application. Gelling or thickening agents provide the consistency that is an outstanding characteristic of gels (Buchmann, 2006:110).

An emulgel can be described as an equivalent to an emulsion/hydrogel combination. The aqueous phase is the hydrogel in which the lipophilic phase is immobilised (Müller-Goymann, 2004:352). Hydrogels consist of 85-95% water or an aqueous-alcoholic mixture, together with gelling agents. Emulgel formulations have a cooling effect after application, caused by evaporation of the solvent. For this reason, an emulgel was formulated to effectively treat acne (Buchmann, 2006:110).

B.4.1 EMULGEL FORMULA

The final formula for an emulgel containing vitamin A and zinc is presented in Table B.3. Section A in Table B.3, represents the ingredients of the oil phase of the emulgel, and Section B the aqueous phase.

Table B.3: Formula of vitamin A and zinc emulgel

Raw material	% w/w
A: Liquid paraffin	20.00
Tween-80	4.50
Span-60	0.50
Methylparaben	0.40
Propylparaben	0.08
BHA	0.10
BHT	0.10
Vitamin A	0.50
B: Xanthan gum	1.50
Zinc	1.20
Distilled H_2O	to 100.00

B.4.2 MAIN INGREDIENTS OF AN EMULGEL

The emulgel consisted of all the ingredients listed in Table B.3, with xanthan gum as the only difference in comparison to the cream. The other ingredients were fully discussed in Section B.3.2.

B.4.2.1 GELLING AGENT

Gelling agents are compounds with high molecular weight that are water dispersible. They have swelling properties and function mainly to improve the viscosity of the dispersion (Mahalingam *et al*., 2008:293). Xanthan gum was used as a gelling agent due to its low temperature dependence and stability over a wide pH range of the material (Mitsui, 1997:140).

B.4.3 PACKAGING

The same packaging material was used for the emulgel as for the cream formulations. Glass containers with screw caps were used and covered in brown paper to protect the product from light.

B.4.4 PREPARATION PROCESS OF THE EMULGEL

The emulgel formulation was prepared as follows:

- Weigh the distilled water (see Section B.4.1, Table B.3) and heat to 40 ± 2 °C.
- Weigh zinc and dissolve it in the heated water.

- Weigh xanthan gum and add slowly to heated water while homogenising at 777 rpm, until dissolved and homogenous.
- Heat the mixture consisting of water, zinc and xanthan gum to 80 ± 2 °C.
- Weigh all the ingredients of A (see Section B.4.1, Table B.3), mix together and heat to 80 ± 2 °C.
- Add A (oil phase) to B (water phase) while homogenising at 13 500 rpm, making sure that the propeller is at the bottom of the container to minimise the entrapment of bubbles.
- Homogenise until a temperature of 40 ± 2 °C is reached.
- Cool with ice water.
- Stir with a glass rod until room temperature (25 ± 2 °C) is reached.
- Transfer to glass containers.

B.4.5 PREPARATION OF PHEROID™ EMULGEL

The same procedure was followed as described in Section B.4.4, except for the Pheroid™ ingredients i.e., dl-α-tocopherol that was added to the oil phase, and instead of distilled water, water saturated with nitrous oxide was used.

B.4.6 RESULTS

Both emulgels depicted a homogenous texture; it applied easily to the skin and were not to oily. Both were of a light yellowish colour, due to the yellow colour of the vitamin A.

B.5 INGREDIENTS USED IN FORMULATIONS

All the ingredients used during formulation were up to standard and of high quality. The suppliers as well as the batch numbers of the used ingredients are listed in Table B.4.

Table B.4: Ingredients used in formulations

Ingredient	Supplier	Batch number
Liquid paraffin	Merck chemicals	1034040
Cetyl alcohol	Merck chemicals	S5428004951
Tween-80	Merck chemicals	1032991
Span-60	Brunel manufacturers	00259
Methylparaben	Merck chemicals	GBG000137
Propylparaben	Merck chemicals	GBGA032949
BHA	Merck chemicals	115K0028
BHT	Sigma-Aldrich	04416 KD-076
Xanthan gum	Warren Chem Specialities	4450902790
Vitamin A	Sigma-Aldrich	047K07092
Zinc	Merck chemicals	A892302835

B.6 CONCLUSION

Four formulations were prepared, which included a cream, Pheroid™ cream, emulgel and Pheroid™ emulgel, all containing 0.5% vitamin A and 1.2% zinc. They were all prepared in sufficient quantities (2000 g) and stored at different temperatures (25 °C, 30 °C and 40 °C) for stability testing. The formulations were inspected for appearance and texture before stability testing was commenced. The stability studies that were performed will be discussed in detail in appendix C.

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APPENDIX C

STABILITY TESTING OF COSMECEUTICALS

C.1 INTRODUCTION

Stability testing is the process of evaluating products, under the influence of a variety of environmental conditions, to ensure that the functional qualities stay within acceptable guidelines over a certain period of time. It is important to know the critical characteristics of the product to be able to measure the change over time and the degree of change that is considered acceptable. In the case of cosmeceutical products, it is not only the change over time that is important, but another contributing factor is the appearance that has to be consistent and attractive, each time that the consumer uses the product (Romanowski & Schueller, 2006:655). Since emulsions were formulated in this study, which represent a mixture of two or more materials that are not always miscible with each other, stability considerations are crucial. According to the second law of thermodynamics, emulsions tend to be inherently unstable. This law states that the phases of emulsions will eventually separate. Only the speed and the degree of instability vary between different types of emulsions (Romanowski & Schueller, 2006:661). Vitamin A (all-*trans*-retinol) for example, changes configuration on the double bond at the thirteenth position of the molecule, when subjected to heat in order to form 13-*cis*-retinol which has no activity as a vitamin (Barnes, 2007:654).

To ensure product stability, changes in the physicochemical properties of the product has to be controlled. Inducing rapid chemical and physical changes through temperature variation, is the main parameter to determine product stability (Guaratini *et al.*, 2006:12). Physical changes which may occur, include separation, sedimentation, unevenness, evaporation, softening or cracking. Chemical changes on the other hand, include colour change, colour fading, fragrance change, staining and crystallisation (Mitsui, 1997:191).

The aim of stability testing was to determine the stability of four different formulations, which included a cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. Active ingredients in theseformulations were vitamin A and zinc.

C.2 STABILITY OF COSMETIC FORMULATIONS

C.2.1 FORMULATION QUANTITIES AND STORAGE CONDITIONS

A total amount of 2000 g of each formulation were manufactured for stability studies. The products were divided into equal amounts and stored in different containers at

25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH. Labcon™ humidity chambers were implemented. The stability studies were performed on months 0, 1, 2 and 3 of which the data will be included in this Appendix.

C.2.2 PACKAGING MATERIAL

Selection of packaging material is of utmost importance in many vitamin containing products, contributing to the stability thereof. Vitamin A is sensitive to light, heat and oxidation by atmospheric oxygen (Olson, 2001:7) and may be adsorbed onto polyvinyl chloride containers (Bühler, 1988:80). The ideal container for the vitamin A and zinc formulation would therefore be an amber glass container, with a screw cap, which will protect the product from the external atmosphere. Due to the scarcity of amber glass containers, normal 100 ml glass containers with screw caps were used and covered in brown paper to protect the products from light. For the rheology study, Consol® glass jars with a capacity of 125 ml were used.

All containers were sterilised before use as follow:

- Containers were washed in hot water and Extran® antibacterial soap, followed with distilled water.
- To sterilise the containers, a 70% ethanol solution were used to rinse the containers and they were then dried in laminar flow hoods.
- The containers were covered with two layers of brown paper and sterilised in a Labcon® oven at 180° C for 30 min.

C.2.3 STABILITY TESTS

Instability is typically identified by evaluating various characteristics of a product, either by the subjective observation of properties which include colour, odour and appearance, or by objective instrumental evaluation of the concentration, rheology, pH and particle size (Romanowski & Schueller, 2006:659). In this study subjective and objective instrumental evaluations were performed.

Eight different stability tests were conducted:

- Assay
- Rheology (viscosity)
- pH
- Particle size

- Zeta-potential
- Mass loss
- Particle size distribution
- Physical assessment

According to Knowlton & Pearce (1993:439), a useful rule of thumb is that the measured deviation of the formulated products, may not deviate by more than 20% of their original value. The tested products should still perform as it did when first manufactured.

C.3 METHODS USED DURING STABILITY TESTING

C.3.1 ASSAY

A stability-indicating assay can be described as a validated quantitative analytical procedure that can detect the changes in the relevant properties of the drug substance and the product as time pursues. The active ingredient is accurately measured without intrusion of degradation products, excipients and process impurities.

Measurements for vitamin A were performed on an Agilent® 1200 series HPLC system, equipped with an Agilent® 1200 quaternary pump, UV detector, autosampler, thermostat and degasser. The instrument is designed with Chemstation Rev. A.10.02 software, for analysis and data acquisition. A Venusil XBP C18 (2) (4.6 mm x 150 mm) silica column, with a 5 µm particle size was used.

Zinc present in the formulations could not be analysed by HPLC due to the fact that it is too polar. The samples of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel were tested with a standard validated and accredited method by Eco-Analytica from the North-West University, Potchefstroom Campus. A Varian SpectrAA - 250 Plus Atomic Absorption Spectrometer was used and the zinc was detected by the use of an air and acetylene flame. Varian hollow cathode lamps were employed at a wavelength of 213.9 nm.

An assay was also conducted on a commercial product containing vitamin A acetate, in order to compare its stability with the stability of vitamin A in the formulated products in this study. The same system and column were used as with the assay of vitamin A, with the chromatographic conditions described in Appendix A.1.2. Standard laboratory conditions were managed during the experiment.

An HPLC analysis was performed after each month to determine the concentration of the six analytes present in the formulations. The validation method for the assay is described in Appendix A.

C.3.1.1 ASSAY OF VITAMIN A

C.3.1.1.1 Standard preparation

A standard solution was prepared for each of the four formulations, which were used as a control for the analysis. For each stability assay done on the different time intervals (0, 1, 2, and 3 months), new samples were prepared. Different analytes for the four formulations were weighed, as shown in Table C.1.

Table C.1: Standard solution preparation for the four formulations

Substance	Cream	Pheroid™ cream	Emulgel	Pheroid™ emulgel
Methyl paraben	2 mg	2 mg	4 mg	4 mg
Propyl paraben	5 mg	5 mg	5 mg	5 mg
BHA	1 mg	1 mg	1 mg	1 mg
BHT	1 mg	1 mg	1 mg	1 mg
Vitamin A	5 mg	5 mg	5 mg	5 mg
dl-α-tocopherol	-	2 mg	-	2 mg

The weighed analytes were transferred to a 50 ml volumetric flask and filled to volume with ethanol. The amount of propyl paraben was too small to weigh accurately; therefore 5 mg was weighed in a 50 ml volumetric flask and diluted with ethanol. For the cream and emulgel formulations, with and without Pheroid™, 4 ml and 8 ml of the aforementioned solution were extracted and added to the standard solution to ensure a concentration of 8 μg/ml and 16 μg/ml, respectively.

The standard solution was sonicated on the ultrasonic bath for approximately 5 min to ensure that all the analytes were completely dissolved. An amber HPLC sample vial was filled with the standard solution and analysed on the HPLC, in duplicate.

C.3.1.1.2 Sample preparation

Different samples for the formulations were prepared by weighing (in duplicate) approximately 1 g of each in a 50 ml volumetric flask. Ethanol was added to ensure that the base dissolved. All the sample were sonicated and vigorously shaken repeatedly. Samples were transferred to amber HPLC sample vials and injected into the HPLC.

C.3.1.2 ASSAY OF ZINC

C.3.1.2.1 Sample preparation

Nitric acid (65%) (Merck Chemicals) was used as solvent for the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel, to dissolve the fatty base. The nitric acid was weighed and diluted with HPLC grade water to ensure a concentration of 1.6%.

Samples for the zinc analysis were prepared by weighing approximately 0.5 g of each of the formulations in a 100 ml volumetric flask. 1.6% nitric acid was used to fill the volumetric flask to volume. In order to obtain a concentration of 6 µg/ml; 10 ml of the sample was extracted and filled to 100 ml with the 1.6% nitric acid. The samples were sonicated for approximately 15 min to ensure that all the particles dissolved. Before analysis on the Atomic Absorption Spectrometer, the samples were thoroughly shaken.

C.3.1.3 ASSAY OF COMMERCIAL PRODUCT CONTAINING VITAMIN A

C.3.1.3.1 Standard preparation

Preparing a standard solution, 5 mg of the commercial product was weighed in a 50 ml volumetric flask and made up to volume with ethanol. The solution was sonicated for 5 min and transferred to an amber HPLC vial, which was then injected into the HPLC and analysed in triplicate.

C.3.1.3.2 Sample preparation

Samples of the commercial product, that were stored in the three different humidity chambers, were prepared by weighing (in duplicate) approximately 1 g in a 50 ml volumetric flask. Ethanol was used as solvent to ensure that the bas dissolved. Sample were sonicated and vigorously shaken repeatedly. The samples were transferred to amber HPLC sample vials and injected into the HPLC in duplicate.

C.3.2 RHEOLOGY OF THE FORMULATIONS

The term rheology is a description of the flow characteristics of a liquid and the deformation of solid formulations. Therefore, viscosity can be described as an expression of the resistance of a fluid to flow. Rheological properties are crucial in any cosmeceutical formulation, because they determine the properties of the product during mixing, production and before use, as well as sensory properties which include the consistency, spreadability and smoothness when applied. There are various factors that can influence the rheological properties of emulsions, such as viscosity of the internal and external phases, phase volume ratio, particle size

distribution, type and concentration of the emulsifying system, and viscosity-modifying agents (Buchmann, 2006:116-117).

Rheological determinations were performed on a model DV-II+ Brookfield viscometer (Brookfield, United States of America), which was connected to a Helipath D20733. Viscosity of the four formulations was determined initially; and at months 1, 2 and 3. The formulations were stored in glass containers with a volume of 100 ml each. Rheological parameters were only determined on the batches that were stored at 25 °C/60% RH, using different Helipath spindles. The direct temperatures of the samples (25 °C) were controlled by a Brookfield circulating water bath with a temperature controller. The viscometer took one reading every 10 seconds for 5 min, in order to determine a total of 32 data points. Different spindles and speed used are shown in Table C.2.

Table C.2: Viscosity parameters

Formulation	Spindle	rpm
Cream	E - F	0.3 - 6
Pheroid™ cream	A - E	1.5 - 60
Emulgel	D - E	0.3 - 0.5
Pheroid™ emulgel	D - E	0.3 - 0.6

C.3.3 pH MEASUREMENTS OF THE FORMULATIONS

To measure the pH means to measure the concentration of the hydrogen ions present in the formulation (Buchmann, 2006:121). The pH of a topical formulation is one of the most important factors in the stability of vitamins, due to decomposition of the vitamins that depend on the pH in most of cases. The optimum pH of vitamin A is between 5.5 and 6.0 (Törmä & Vahlquist, 1990:132). However, the stratum corneum is extremely resistant to changes in pH, tolerating a range of only 3 – 9 (Barry, 2007:576).

A Mettler Toledo pH meter (Switzerland) and a Mettler Toledo Inlab® 410 electrode were used to measure the pH of the formulations. The apparatus was calibrated before use with buffer solutions of pH 4.0, 7.0 and 10.0.

C.3.4 PARTICLE SIZE AND ZETA-POTENTIAL DETERMINATION

C.3.4.1 DT-1200

The DT-1200 acoustic and electro-acoustic spectrometer, developed by Dipsersion Technology Inc. was used to measure the droplet size and zeta-potential of the formulated products.

It has the advantage over other methods that no dilution is necessary. When a sample is diluted, it causes modification in the thermodynamic equilibrium and affects the rheological properties, consequently causes variation in the droplet size and surface chemistry of the system (Dukhin *et al.*, 2000:127).

The DT-1200 combines acoustic and electro-acoustic spectroscopy with separate sensors to measure both signals. An acoustic spectrometer produces sound impulses which then pass through a sample system to be measured by a receiver. When passing through the sample system, the sound energy changes in intensity and phase, due to the interaction with the particles and liquid. The acoustic spectroscopy is therefore used to measure the acoustic properties such as attenuation (sound energy losses) and sound speed. Ultrasound in the frequency range of 1 – 100 MHz is used to determine droplet size distribution (Dukhin *et al.*, 2000:128).

On the other hand, electro-acoustic spectroscopy measures the colloid vibration, which can determine both droplet size distribution and zeta-potential (Dukhin & Goetz, 1998:49). Electro-acoustics involve an additional electric field, which makes it more complex than acoustic spectroscopy.

C.3.4.1.1 Theory of acoustics

The Epstein and Carhart, Allegra and Hawley (ECAH) theory, is the most familiar acoustic theory for heterogeneous systems. This theory reflects the multiple mechanisms of the interaction between ultrasound and colloids, which include, viscous, thermal, scattering and intrinsic. Attenuation for a monodisperse system (particles with the same diameter) of spherical particles is described through this theory. Unfortunately, it is only valid for diluted systems, which indicates, that there are no interactions between the particles. This limitation prevents the theory form being applied to dispersions with a volume fraction of less than a few volume percent, which consequently makes the ECAH theory far form ideal (Dukhin *et al.*, 2000:129-130).

C.3.4.1.2 Theory of electro-acoustics

Electro-acoustic spectroscopy is more complicated than acoustic spectroscopy, because it deals with the coupling between electric and acoustic fields of the dispersion. Colloid vibration potential (CVP) is measured with electro-acoustic spectroscopy (Dukhin *et al.*, 2000:135). The stability of emulsions depends on two key parameters, such as particle/droplet size and zeta-potential (Hsu & Nacu, 2002:374).

C.3.4.2 DROPLET SIZE

The droplet size of the formulations was determined (without dilution), with the use of the DT-1200. This method is most desirable, since dilution of an emulsion could cause changes in the emulsion drop size and the surface charge. Droplet size is important to accurately calculate zeta-potential. According to Barry (2002:36), particles smaller than 3 µm penetrate the stratum corneum readily. Particles between 3 µm and 10 µm enter the skin via follicles and those larger than 10 µm stay on the surface of the skin.

To be able to determine the droplet size of the emulsion, the density should first be determined. This was done by weighing 10 ml of each formulation in a volumetric flask. The total volume of the formulations required were 120 ml, which were transferred to the sample chamber. The sample chamber consisted of a special magnetic stirrer, which prevented sedimentation and ensured a homogenous emulsion. The DT-1200 consisted of an analysis program that calculated particle size distribution from the attenuation spectra. To be able to measure the size of the oil droplets in the oily phase of the emulsion, the cream, Pheroid™ cream, emulgel or Pheroid™ emulgel formulations were used as the liquid media. An extra dispersed phase, the vitamin A, was added. An error analysis (fitting error) was used to search for the best particle size distribution (Dukhin & Goetz, 1998:50). Three consecutive measurements were taken and an average droplet size was determined. For emulsions to posses acceptable physical stability and texture, a mean globule diameter of between 0.5 and 2.5 µm is needed (Billany, 2007:404). The standard deviation should be smaller than 1 – 2% to be acceptable (Wells & Aulton, 2007:355).

C.3.4.3 ZETA-POTENTIAL

Zeta-potential could be defined as the electrical potential that exists across the interface of all solids and liquids, also known as electrokinetic potential (Kosmulski *et al*., 1999:200). Any particle in a colloidal system exhibits zeta-potential, and is an indication of the physical stability of the system. An electrical double layer consists of a solid that exhibits an electrical charge and an aqueous solution that consists of positive and negative ions. The electrical surface charge influences the distribution of ions in the aqueous medium, which could be ions of opposite charge of that of the surface (counter-ions), or ions with similar charge (co-ions). Counter-ions are attracted towards the surface, whereas co-ions are repelled away from the surface. The double-layer is formed through the increased concentration of counter-ions close to the surface of the solid. This double layer is divided into two parts; an inner region, named the Stern layer, and the outer diffusive region. Counter-ions are strongly bound in the Stern layer, where they are less firmly associated in the diffusive region. A notional boundary exists within the diffusive layer, where the particles and ions form a stability entity.

This boundary is named the slipping plane of the surface, or hydrodynamic shear. It represents the boundary of relative movement between the solid and the liquid. The potential at the plane of shear is termed the zeta-potential, as seen in Figure C.1 (Attwood, 2007:76-77).

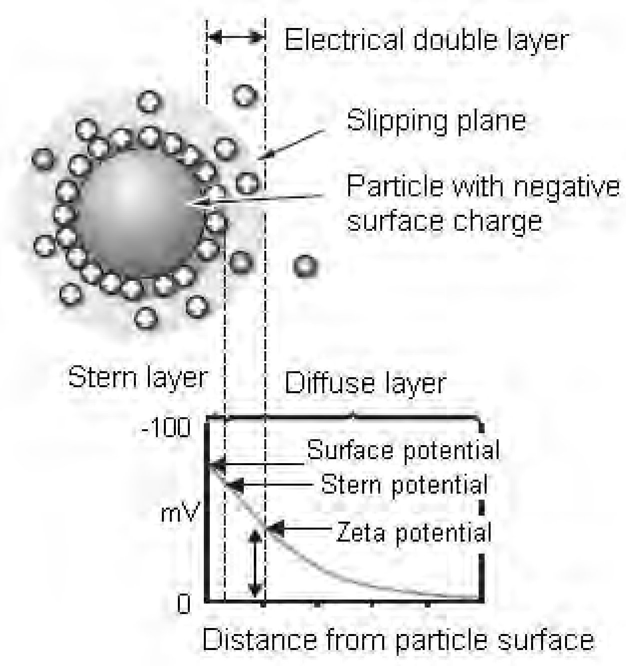

Figure C.1: Illustration of electrical double layer and zeta-potential (Bioresearch Online, 2005)

Zeta-potential plays a crucial part in the stability and rheology of colloids. Higher pH-values result in more negative zeta-potential values, whereas lower pH-values cause the zeta-potential to be more positive (Kosmulski *et al*., 1999:200). Particles with the same electrical charge ward each other off; where particles with different electrical charges attract each other. The general guide line to keep the system in a stable dispersed state, is that the zeta-potential needs to consist of a force smaller than -30 mV and bigger than 30 mV (Malvern Instruments, 2010).

C.3.5 MASS LOSS OF THE FORMULATIONS

The mass of each formulation was determined on a Shimadzu AUW 120D scale (Shimadzu, Japan) during storage in the 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH humidity chambers. The same container was used to weigh the mass initially and for the following three months, in order to compare the change during stability testing. For accuracy, the scale was calibrated every time before use.

C.3.6 MORPHOLOGY

Morphology was visually evaluated by a Nikon eclipse TE300 light microscope. The samples were prepared by weighing 0.1 g of a formulation in an Eppendorf vial. Nile-red (2 µl) and distilled water (500 µl) were added in every Eppendorf vial. Microscope plates were prepared by placing 25 µl of the sample on the plate whilst incubating it in the dark for 15 min. The samples were studied under the light microscope where a photograph was taken of the samples and inspected for variations.

C.3.7 PHYSICAL ASSESSMENT

As the colour, odour and texture of cosmeceutical products are of utmost importance for the consumer‟s approval, physical assessment studies are an important part of the stability testing program.

Stability of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel were evaluated through the subjective observation of colour, texture and odour of the formulations over three months during stability testing. Photos were taken and compared to photos of the previous months, using paint colour cards as indicators. The different formulations were stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH, and were visually assessed initially and at 1, 2 and 3 months.

C.4 RESULTS AND DISCUSSION

C.4.1 ASSAY

The stability indicating assay was performed initially, and at 1, 2 and 3 months to measure the percentage of the ingredients present in the different formulations. The four formulations were stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH for a three month period.

C.4.1.1 ASSAY OF VITAMIN A

Maximum reduction in concentration of a drug that is acceptable during a stability indicating assay is a reduction of not more than 20% of the initial concentration (Knowlton & Pearce, 1993:439). Initial stability testing was only performed at one temperature and humidity (25 °C/60% RH) before commencement of vitamin A stability testing for the three month period (section C.4.1).

C.4.1.1.1 Cream

Table C.3: The percentage values of the ingredients in the cream formulation

	Month			
Ingredients measured in %	**0**	**1**	**2**	**3**
	25 °C/60% RH			
Vitamin A	99.27	95.76	93.40	89.53
Methyl paraben	100.26	100.67	101.12	101.33
Propyl paraben	99.71	98.23	99.42	96.24
BHA	99.92	92.55	89.70	88.80
BHT	106.20	95.16	90.67	75.17
	30 °C/60% RH			
Vitamin A		95.89	90.11	85.28
Methyl paraben		98.10	99.30	100.03
Propyl paraben		99.11	99.05	94.03
BHA		95.95	92.25	87.29
BHT		97.84	93.6	77.91
	40 °C/75% RH			
Vitamin A		95.51	75.16	71.82
Methyl paraben		95.53	98.54	100.06
Propyl paraben		99.40	98.64	96.19
BHA		93.65	95.44	90.39
BHT		94.71	82.86	78.85

Vitamin A in the cream formulation depicted an overall decrease in concentration over the three months. From the results obtained (Table C.3), it was clear that the highest reduction in concentration was for the cream formulation stored at 40 °C/75% RH (27.65%), followed by the cream formulation stored at 30 °C/60% RH (14.09%). The cream formulation stored at 25 °C/60% RH was therefore the most stable formulation, with a decrease in concentration of only 9.81%. It was thus clear that the formulation stored at either 25 °C/60% RH or 30 °C/60% RH, still depicted concentrations within the acceptable range; however, the formulation stored at 40 °C/75% RH was not within the acceptable range and therefore considered unstable.

The anti-oxidant, BHT, in the cream formulation did not remain in the accepted range (Table C.3). BHT concentrations in the cream formulations, stored in the different humidity chambers, decreased with 29.22%, 26.64% and 25.75%, in the 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH chambers, respectively.

One of the reasons for the significant decrease in the BHT concentrations might be that the BHT was first oxidised, before any other ingredient (including vitamin A) could be oxidised, as BHT is an anti-oxidant. All the other ingredients in the cream formulation showed no significant decrease in concentration and these concentrations remained within the acceptable range for the duration of the study.

C.4.1.1.2 Pheroid™ cream

Table C.4: The percentage values of the ingredients in the Pheroid™ cream formulation

	Month			
Ingredients measured in %	**0**	**1**	**2**	**3**
	25 °C/60% RH			
Vitamin A	100.34	98.91	82.59	80.02
Methyl paraben	105.68	98.38	97.59	91.60
Propyl paraben	109.50	108.61	96.78	98.57
BHA	102.50	100.90	99.64	97.59
BHT	99.30	89.85	91.61	90.76
dl-α-tocopherol	96.01	88.37	82.78	80.88
	30 °C/60% RH			
Vitamin A		97.88	80.42	68.26
Methyl paraben		102.04	97.39	91.82
Propyl paraben		107.95	96.22	96.83
BHA		98.25	98.86	96.78
BHT		68.84	70.81	62.41
dl-α-tocopherol		86.87	82.93	75.37
	40 °C/75% RH			
Vitamin A		84.45	59.65	45.29
Methyl paraben		100.07	101.42	93.24
Propyl paraben		100.05	99.15	100.34
BHA		102.86	101.68	101.66
BHT		40.49	34.88	30.51
dl-α-tocopherol		92.99	73.29	66.89

Vitamin A in the Pheroid™ cream formulation depicted an overall decrease in concentration over the three months, and was therefore not in the accepted degradation range of 20% after stability testing ceased. Therefore the Pheroid™ cream could be considered as unstable. From the results obtained (Table C.4), it was clear that the highest reduction in concentration was for the Pheroid™ cream formulation stored at 40 °C/75% RH (54.86%).

The anti-oxidant, BHT, and dl-α-tocopherol concentrations in the Pheroid™ cream formulation stored at 30 °C/60% RH and 40 °C/75% RH chambers, did not remain in the accepted range (Table C.4). However, all the other ingredients in the Pheroid™ cream formulation showed no significant decrease in concentration and these concentrations remained within the acceptable range for the duration of the study. One of the reasons for the significant decrease in the BHT concentrations might be that the BHT was first oxidised, before any other ingredient (including vitamin A) could be oxidised, as BHT is an anti-oxidant.

C.4.1.1.3 Emulgel

A reduction in the vitamin A concentration formulated in the emulgel was within the acceptable range during stability testing (Table C.5). However, at 40 °C/75% RH, the vitamin A concentration decreased by 58.68% and the BHT concentration decreased by 29.10%, which were unacceptable and could be considered as unstable. All of the other ingredients in the emulgel formulation still depicted concentrations within the acceptable range for the duration of the study.

Table C.5: The percentage values of the ingredients in the emulgel formulation

	Month			
Ingredients measured in %	**0**	**1**	**2**	**3**
	25 °C/60% RH			
Vitamin A	101.87	100.90	95.46	84.21
Methyl paraben	100.79	92.38	91.74	93.77
Propyl paraben	101.25	98.01	92.09	91.95
BHA	102.10	86.03	85.48	84.57
BHT	110.08	102.28	96.83	93.72
	30 °C/60% RH			
Vitamin A		99.64	85.04	82.45
Methyl paraben		98.20	99.12	98.11
Propyl paraben		99.66	95.68	96.90
BHA		99.98	98.89	95.68
BHT		102.72	90.39	88.74
	40 °C/75% RH			
Vitamin A		78.85	49.36	43.19
Methyl paraben		89.27	86.16	84.06
Propyl paraben		93.68	90.19	91.92
BHA		90.16	90.47	87.12
BHT		106.35	86.75	78.05

C.4.1.1.4 Pheroid™ emulgel

The ingredients of the Pheroid™ emulgel formulation exposed to the 25 °C/60% RH and 30 °C/60% RH were relatively stable and within the accepted range, except for the anti-oxidant BHT (Table C.6). The reason for the decrease in the anti-oxidant concentrations might have been due to the BHT being oxidised before the vitamin A or other ingredients in the cream were oxidised. The Pheroid™ emulgel formulation stored at 40 °C/75% RH depicted concentrations of vitamin A and BHT which decreased to such an extent that it was no longer within the acceptable range after three months and could be considered as unstable. All of the other ingredients in the Pheroid™ emulgel formulation still depicted concentrations within the acceptable range for the duration of the study.

Table C.6: The percentage values of the ingredients in the Pheroid™ emulgel formulation

	Month			
Ingredients measured in %	**0**	**1**	**2**	**3**
	25 °C/60% RH			
Vitamin A	103.62	100.02	98.86	95.71
Methyl paraben	99.09	97.43	97.15	96.47
Propyl paraben	100.56	99.81	100.06	98.81
BHA	101.74	96.84	93.31	94.00
BHT	100.01	83.75	72.42	52.02
dl-α-tocopherol	101.19	94.68	92.24	90.06
	30 °C/60% RH			
Vitamin A		96.67	89.60	83.35
Methyl paraben		99.89	93.94	94.68
Propyl paraben		98.67	95.03	96.95
BHA		95.22	90.43	89.30
BHT		87.08	78.86	58.76
dl-α-tocopherol		95.74	91.35	88.93
	40 °C/75% RH			
Vitamin A		77.29	69.23	58.50
Methyl paraben		98.77	92.25	88.10
Propyl paraben		91.80	83.14	85.99
BHA		95.03	87.00	82.19
BHT		94.69	86.61	54.39
dl-α-tocopherol		90.39	81.11	84.53

C.4.1.1.5 SUMMARY

All ingredients present in the various formulations showed a decrease in concentration during the three months stability testing. However, only the vitamin A and BHT concentrations decreased significantly in some of the formulations. Due to the complexity of the different formulation matrices, analysis on the HPLC was complicated, and therefore the results obtained during the three month stability testing could not be used quantitatively, but rather comparatively.

The cream formulation proved to be more stable than the other formulations. This might have been due to insufficient protection of the other formulations. Vitamin A concentrations in all the formulations were largely reduced especially in the 40 °C /75% RH chamber. This indicated that as the temperature and humidity increased, the vitamin A concentration decreased. Thus care should be taken to store vitamin A-containing products at temperatures not higher than 25 °C and humidity not higher than 60% RH.

C.4.1.2 ASSAY OF ZINC

The assay values obtained from the zinc present in the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel are given in Table C.7.

Table C.7: The percentage values of the zinc present in the four formulations

Ingredients measured in %	Month			
	0	1	2	3
	25 °C/60% RH			
Cream	98.83	91.86	98.63	101.79
Pheroid™ cream	98.77	91.95	98.38	100.25
Emulgel	99.76	90.29	100.82	96.31
Pheroid™ emulgel	100.75	99.02	94.68	96.49
	30 °C/60% RH			
Cream		97.71	98.91	104.78
Pheroid™ cream		95.33	95.74	103.19
Emulgel		88.96	90.06	99.28
Pheroid™ emulgel		86.17	101.02	99.68
	40 °C/75% RH			
Cream		98.29	93.65	99.22
Pheroid™ cream		97.92	97.67	100.28
Emulgel		86.60	93.17	97.78
Pheroid™ emulgel		90.07	105.32	96.94

Stability indicating assays were conducted, initially and at 1, 2 and 3 months to measure the percentage zinc present in the different formulations. Four formulations were stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH for the period of three months. The detected zinc values were measured in µmol/L and then diverted to µg/ml.

Zinc concentrations of all the formulations stored in the 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH were relatively stable during the three months. This indicated that the zinc concentration was not significantly influenced by elevated temperatures.

C.4.1.3 ASSAY OF THE COMMERCIAL PRODUCT

Vitamin A concentrations obtained from the commercial product are expressed as percentage values, and displayed in Table C.8. The product was stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH. Stability testing was also performed as for the other formulations.

Table C.8: The percentage values of the vitamin A present in the commercial product

Month			
0	**1**	**2**	**3**
25 °C/60% RH			
125.43	120.39	114.70	102.16
30 °C/60% RH			
	122.60	118.41	79.81
40 °C/75% RH			
	107.95	59.19	24.54

The decrease of vitamin A concentration during the three months in the different humidity chambers were respectively 18.55%, 36.37% and 80.44% for 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH chambers. The formulation stored in the 25 °C/60% RH was stable and within the acceptable range. However, vitamin A concentrations decreased with more than 20% in the 30 °C/60% RH and 40 °C/75% RH, which was unacceptable.

Stability of the vitamin A in the commercial product was not comparable to the stability profiles of vitamin A in the formulations in this study, as it did not contain the same concentration vitamin A and its degradation was not within the same range. It was however clear, that the vitamin A in the commercial product, was also not stable when exposed to elevated temperatures. The decrease in vitamin A concentration was more significant than the decrease in the cream formulation (23.27% at 25 °C/60% RH, 45.62% at 30 °C/60% RH and 100.89% at 40 °C/75% RH).

It could therefore be concluded that vitamin A is not resistant to elevated temperatures, and an effective stabilisation method is needed to stabilise vitamin A in topical formulations.

C.4.2 RHEOLOGY OF THE FORMULATIONS

Results obtained from the viscosity measurements of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel during stability testing are presented in Table C.9.

Table C.9: Average viscosity (cP) of determined for the four formulations

25 °C/60% RH				
Formulations measured in cP	**Initial**	**Month 1**	**Month 2**	**Month 3**
Cream	170×10^4	54×10^4	20×10^4	5×10^4
Pheroid™ cream	4×10^4	0.0016×10^4	0.0041×10^4	*
Emulgel	33×10^4	23×10^4	20×10^4	12×10^4
Pheroid™ emulgel	33×10^4	22×10^4	21×10^4	14×10^4

*No value were obtained, it was below the viscosity range of the Brookfield viscometer

The formulations exposed to 25°C/60% RH were analysed for changes in viscosity. Viscosity decreased significantly from month 0 to month 3 with approximately 96.93%, 61.46% and 57.58% for the cream, emulgel and the Pheroid™ emulgel formulations, respectively. The viscosity of the Pheroid™ cream at month 3 could not be determined due to the fact that it was below the viscosity range of the Brookfield viscometer. None of the formulations therefore, met the acceptance criteria.

The cause for the decrease in viscosity might have been due to chemical changes in the formulated products. This is usually a sign of chemical and/or physical instability (Zatz *et al.,* 1989:174). Another contributing reason might have been due to broken van der Waals forces between the molecules that occurred during the previous viscosity measurements.

C.4.3 pH OF THE FORMULATIONS

The four formulations were stored at temperatures and relative humidity of 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH for three months. Each month the change in pH was measured and documented.

A pH of between 3 and 9 should be maintained for formulations, in order to obtain ideal transdermal delivery. Even though the pH decreased slightly over the period of stability testing, the changes were not significant enough to disregard the formulations.

C.4.3.1 pH-VALUES OF THE CREAM FORMULATION

Results of the pH measurements for the cream formulation are shown in Table C.10.

Table C.10: pH-values for cream formulation over the period of three months

Storage temperatures	Initial pH	Month 1	Month 2	Month 3
25 °C/60% RH	6.02	5.61	5.61	5.57
30 °C/60% RH		5.33	5.40	5.38
40 °C/75% RH		5.30	5.36	5.30

The pH of the cream formulation decreased from the initial time taken to month one. This could have been due to settling of the cream. However, no significant change in pH was observed for the cream formulation during the rest of stability testing. The largest pH change was detected at 40 °C/75% RH (± 0.7), with the slightest decrease of ± 0.45 at a temperature of 25 °C/60% RH. According to this data, the stability of a cream containing vitamin A will be ensured for a longer period, if stored at conditions, or below 25 °C/60% RH. The creams stored at 25 °C/60% RH were within the accepted pH range of vitamin A, whereas the creams stored at 30 °C/60% RH and 40 °C/75% RH consisted of a pH below 5.5. According to Bühler (1988:84) this could be an indication of commencement of isomerisation and could thus reduce the activity of vitamin A.

C.4.3.2 pH-VALUES OF THE PHEROID™ CREAM FORMULATION

Results of the pH measurements for the Pheroid™ cream formulation are shown in Table C.11.

Table C.11: pH-values for Pheroid™ cream formulation over the period of three months

Storage temperatures	Initial pH	Month 1	Month 2	Month 3
25 °C/60% RH	5.49	5.44	5.53	5.33
30 °C/60% RH		5.39	5.44	5.28
40 °C/75% RH		5.34	5.41	5.23

The pH of the Pheroid™ cream formulation decreased from the initial time taken to month one. This could have been due to settling of the cream. However, no significant change in pH was observed for the cream formulation during the rest of stability testing. The largest pH change was detected at 40 °C/75% RH (± 0.26), with the slightest decrease of ± 0.28 at a temperature of 25 °C/60% RH. None of the formulations were in the accepted range and consisted of a pH below 5.5. According to Bühler (1988:84) this could be an indication of commencement of isomerisation and could thus reduce the activity of vitamin A. Thus, the Pheroid™ cream is unstable.

C.4.3.3 pH-VALUES OF THE EMULGEL FORMULATION

Results of the pH measurements of the emulgel formulation are shown in Table C.12.

Table C.12: pH-values for emulgel formulae over the period of three months

Storage temperatures	Initial pH	Month 1	Month 2	Month 3
25 °C/60% RH	6.04	5.55	5.55	5.48
30 °C/60% RH		5.39	5.44	5.36
40 °C/75% RH		5.16	5.28	5.16

The pH of the emulgel formulation decreased from the initial time taken to month one. This could have been due to settling of the emulgel. However, no significant change in pH was observed for the emulgel formulation during the rest of stability testing. The largest pH change was detected at 40 °C/75% RH (± 0.88), with the slightest decrease of ± 0.56 at a temperature of 25 °C/60% RH. According to this data, the stability of an emulgel containing vitamin A will be ensured for a longer period, if stored at conditions, or below 25 °C/60% RH. The emulgels stored at 25 °C/60% RH, for the first two months, were within the accepted pH range of vitamin A, whereas the emulgels stored at 30 °C/60% RH and 40 °C/75% RH consisted of a pH below 5.5. According to Bühler (1988:84) this could be an indication of commencement of isomerisation and could thus reduce the activity of vitamin A.

C.4.3.4 pH-VALUES OF THE PHEROID™ EMULGEL FORMULATION

Results of the pH measurements of the Pheroid™ emulgel formulation are given in Table C.13.

Table C.13: pH-values for Pheroid™ emulgel formulae over the period of three months

Storage temperatures	Initial pH	Month 1	Month 2	Month 3
25 °C/60% RH	6.07	5.53	5.57	5.51
30 °C/60% RH		5.38	5.48	5.38
40 °C/75% RH		5.15	5.26	5.19

The pH of the Pheroid™ emulgel formulation decreased from the initial time taken to month one. This could have been due to settling of the formulation. However, no significant change in pH was observed for the Pheroid™ emulgel formulation during the rest of stability testing. The largest pH change was detected at 40 °C/75% RH (± 0.88), with the slightest decrease of ± 0.56 at a temperature of 25 °C/60% RH. According to this data, the stability of a Pheroid™ emulgel containing vitamin A will be ensured for a longer period, if stored at conditions, or below 25 °C/60% RH.

The emulgels stored at 25 °C/60% RH were within the accepted pH range of vitamin A, whereas the emulgels stored at 30 °C/60% RH and 40 °C/75% RH consisted of a pH below 5.5. According to Bühler (1988:84) this could be an indication of commencement of isomerisation and could thus reduce the activity of vitamin A.

C.4.3.5 SUMMARY

Overall, all formulations depicted an initial decrease in pH to month one. This phenomenon might have been due to the formulations first having to settle. From month one to three no significant change in pH could be observed for the different formulations stored at a specific condition (i.e., 25 °C/60% RH, 30 °C/60% RH, 40 °C/75% RH). However, significant decrease in pH could be observed for all the formulations as the temperatures and humidity increased.

Considering the decrease in pH-values, the cream formulation could have been described as most stable. The following rank order for stability according to the pH-values, was established: cream formulation >> emulgel formulation > Pheroid™ emulgel formulation >> Pheroid™ cream formulation.

The Pheroid™ cream, emulgel and Pheroid™ emulgel showed pH-values that were not in the accepted range of 5.5 – 6. This may lead to further instability problems of the formulations.

According to the literature, vitamin A is subjected to isomerisation below a pH of 5.5, which then minimises the activity of vitamin A. This aspect has to be kept in mind if changes in pH enlarge over a longer period. In this study, it was clear that vitamin A was very sensitive to elevated temperatures, thus only the formulations exposed to 25 °C/60% RH were stable and within the accepted pH range of 5.5 – 6.0.

C.4.4 DROPLET SIZE

Droplet size measurement, standard deviation and fitting error of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel are represented in Tables C.14 to C.17, after storing the formulations for three months in the different stability chambers.

C.4.4.1 DROPLET SIZE OF THE CREAM FORMULATION

The droplet size of the cream formulation did not vary significantly over the period of three months, when exposed to the different temperatures, as seen in Figure C.2. The decrease in size during the first month might have been attributed to the cream still having to settle. The mean diameter of the droplets in the cream was between 0.5 and 2.5 μm, which proved it physical stable. The standard deviation remained constant (0.05) during stability testing. Creams exposed to 40 °C/75% RH showed a higher standard deviation, but were still in the

acceptable range of less than 1 to 2%. However, the fitting errors varied slightly, which could be due to inadequate homogeneity of the cream.

Table C.14: Droplet size, standard deviation and fitting error of the cream during a three month period

	Month			
	0	1	2	3
	25 °C/60% RH			
Size (µm)	1.09	0.18	1.03	1.05
Standard Deviation	0.05	0.05	0.05	0.05
Fitting Error (%)	96.30	70.27	75.4	68.67
	30 °C/60% RH			
Size (µm)		0.47	1.03	1.07
Standard Deviation		0.05	0.05	0.05
Fitting Error (%)		82.33	62.57	70.77
	40 °C/75% RH			
Size (µm)		1.01	1.03	1.01
Standard Deviation		0.10	0.10	0.10
Fitting Error (%)		91.97	91.00	93.5

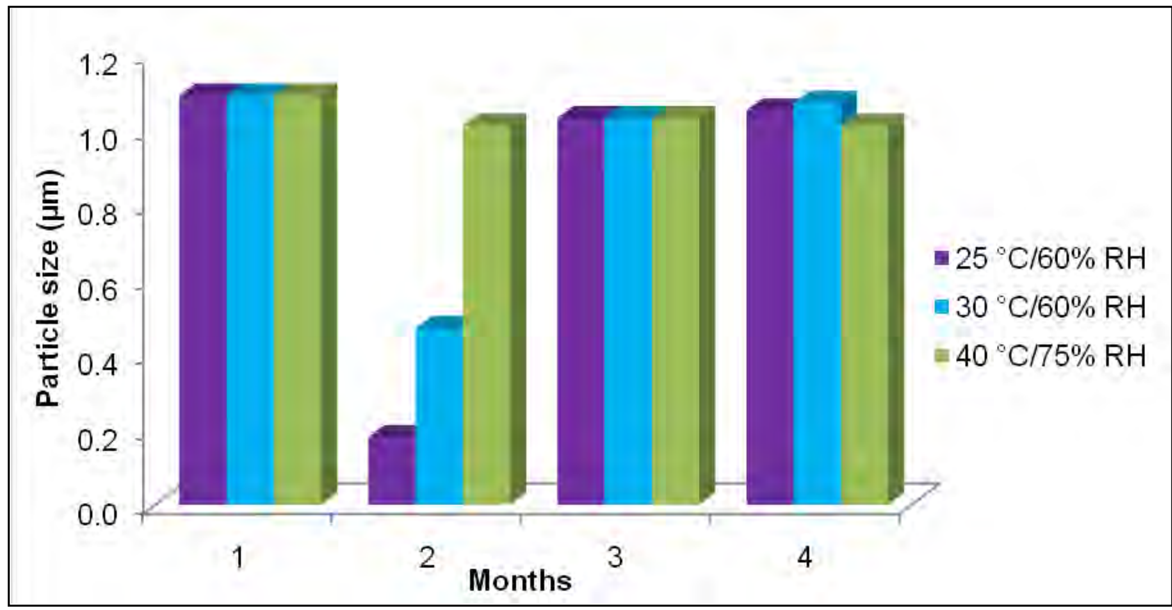

Figure C.2: Illustration of droplet size of cream during a three month period.

C.4.4.2 DROPLET SIZE OF THE PHEROID™ CREAM FORMULATION

The droplet size, standard deviation and fitting error of the Pheroid™ cream formulation (Table C.18) remained relatively constant when, exposed to the different stability chambers as

seen in Figure C.3. Difference in size during the first month might have been attributed to the cream still having to settle. The mean diameter of the droplets in the cream was not in the accepted range, and therefore it was physically unstable. However, the standard deviation was in the accepted range of less than 1 to 2%, throughout the study in the different humidity chambers.

Table C.15: Droplet size, standard deviation and fitting error of the Pheroid™ cream during a three month period

	Month			
	0	1	2	3
	25 °C/60% RH			
Size (µm)	0.18	0.12	0.19	0.18
Standard Deviation	0.05	0.05	0.05	0.05
Fitting Error (%)	99.4	98.83	97.33	97.27
	30 °C/60% RH			
Size (µm)		0.12	0.15	0.19
Standard Deviation		0.05	0.05	0.05
Fitting Error (%)		98.1	98.63	97.90
	40 °C/75% RH			
Size (µm)		0.21	0.18	0.19
Standard Deviation		0.05	0.05	0.05
Fitting Error (%)		99.7	99.43	98.00

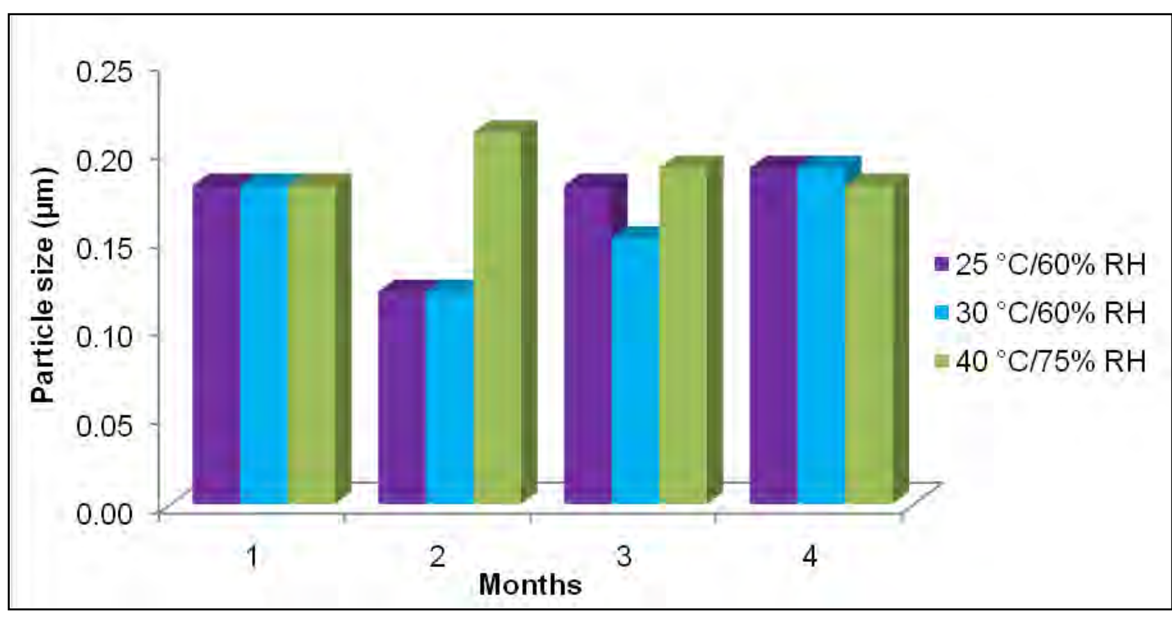

Figure C.3: Illustration of droplet size of Pheroid™ cream during a three month period.

C.4.4.3 DROPLET SIZE OF THE EMULGEL FORMULATION

The droplet size, standard deviation and fitting error of the emulgel formulation remained relatively constant when exposed to the different stability chambers as seen in Figure C.4. The standard deviation was in the accepted range of less than 1 to 2%, throughout the study in the different humidity chambers.

The mean emulgel droplet size (mean droplet diameter of ± 89 μm) was bigger than that of the cream and Pheroid™ cream. These size values were not in the acceptable range and therefore the emulgel formulation was considered physical unstable.

Table C.16: Droplet size, standard deviation and fitting error of the emulgel during a three month period

	Month			
	0	1	2	3
	25 °C/60% RH			
Size (μm)	89.59	89.15	89.30	89.15
Standard Deviation	0.05	0.05	0.05	0.05
Fitting Error (%)	34.83	35.07	27.9	33.23
	30 °C/60% RH			
Size (μm)		89.15	89.15	89.36
Standard Deviation		0.05	0.05	0.05
Fitting Error (%)		28.3	33.13	24.93
	40 °C/75% RH			
Size (μm)		88.69	89.15	89.44
Standard Deviation		0.06	0.05	0.05
Fitting Error (%)		24.03	28.7	24.00

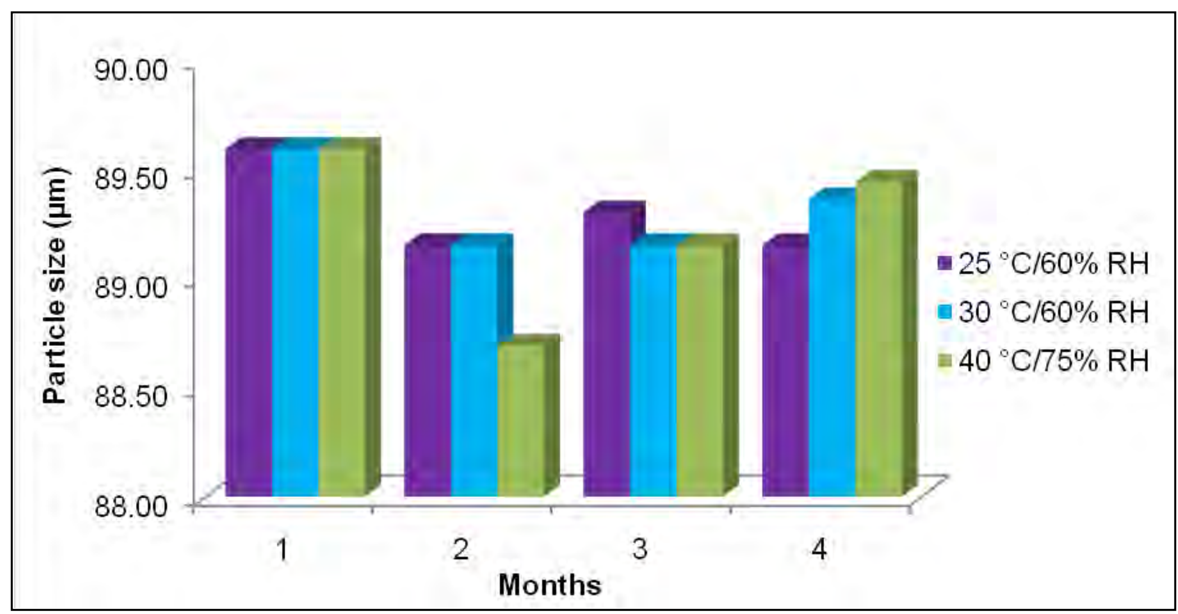

Figure C.4: Illustration of droplet size of emulgel during a three month period.

C.4.4.4 DROPLET SIZE OF THE PHEROID™ EMULGEL FORMULATION

The droplet size of the Pheroid™ emulgel decreased significantly during stability testing. The most significant decrease in droplet size could be observed from the initial measurement to month two (Table C.17 and Figure C.5), for all three stability conditions. From month two to month three no significant change could be observed. The standard deviation remained relatively constant during the initial measurement, as well as during the first, second and third month at 25 °C/60% RH and 30 °C/60% RH. However, decrease in the fitting errors from the initial measurement to month 1 could be attributed to the Pheroid™ emulgel still having to settle. The fitting errors remained relatively constant during the second and third month.

Table C.17: Droplet size, standard deviation and fitting error of the Pheroid™ emulgel during a three month period

	Month			
	0	1	2	3
	25 °C/60% RH			
Size (µm)	89.45	30.35	1.50	1.51
Standard Deviation	0.05	0.15	0.05	0.05
Fitting Error (%)	20.33	17.4	78.77	79.10
	30 °C/60% RH			
Size (µm)		68.00	1.50	1.51
Standard Deviation		0.06	0.05	0.05
Fitting Error (%)		21.13	80.83	79.50
	40 °C/75% RH			
Size (µm)		82.69	1.49	1.50
Standard Deviation		0.05	0.05	0.05
Fitting Error (%)		20.83	81.83	79.27

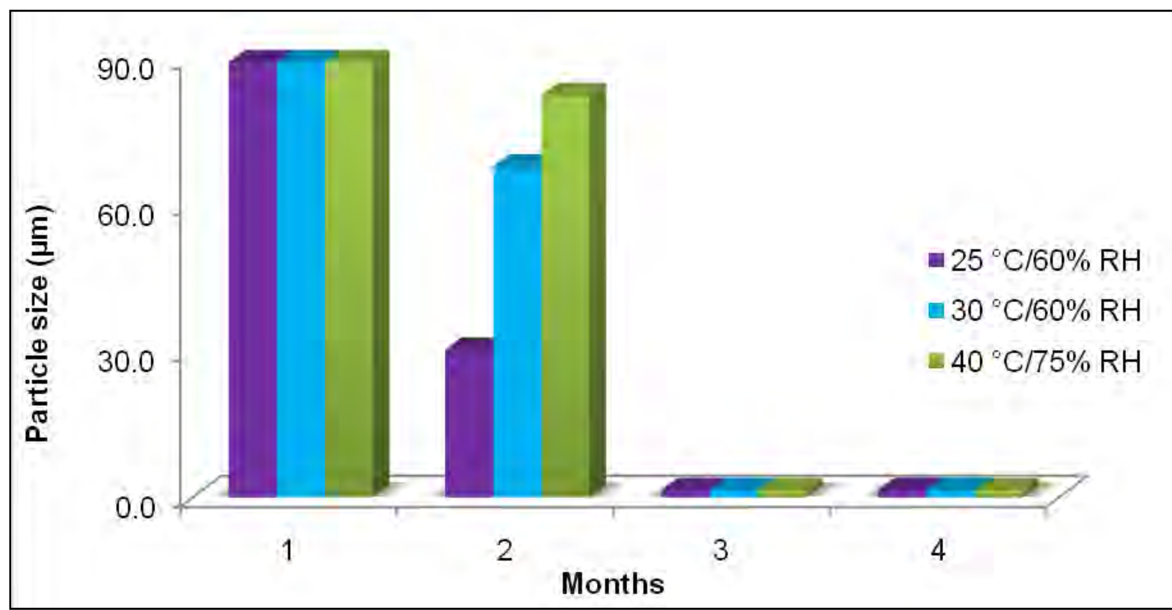

Figure C.5: Illustration of droplet size of Pheroid™ emulgel during a three month period.

Considering penetration of the stratum corneum, the size of the droplets in the cream and Pheroid™ cream were all below 3 µm, which indicated that they would penetrate the stratum corneum readily (Barry, 2002:36). The droplets in emulgel and Pheroid™ emulgel were not smaller than 3 µm, thus the prediction could be made that it would not penetrate the stratum corneum readily.

C.4.5 ZETA-POTENTIAL

The colloid vibration potential (CVP) is measured with electro-acoustic spectroscopy which subsequently supply zeta-potential values. The DT-1200 was also used to determine zeta-potential, after storage of all the formulations in the different humidity chambers for three months. Three readings were taken each month and the average was calculated. The zeta-potential values of the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel are represented in Tables C.18 to C.21.

C.4.5.1 ZETA-POTENTIAL OF THE CREAM FORMULATION

The zeta-potential of the cream formulation remained relatively constant from the initial measurement through to the second month (Table C.18). Values taken at month three were significantly lower, but still in the accepted range of higher than 30 mV. Values measured at 40 °C/75% RH were significantly lower than the values of the other storage conditions. This revealed that zeta-potential values decrease with an increase in temperature. Overall, the cream formulation remained stable over the three months during stability testing.

Table C.18: Zeta-potential of cream

Zeta-potential (mV)				
Storage temperature	**Initial**	**Month 1**	**Month 2**	**Month 3**
25 °C/60% RH	154.27	161.79	149.98	67.18
30 °C/60% RH		156.10	136.68	58.21
40 °C/75% RH		96.90	101.13	50.35

C.4.5.2 ZETA-POTENTIAL OF THE PHEROID™ CREAM FORMULATION

The zeta-potential of the Pheroid™ cream formulation remained relatively constant from the initial measurement through to the second month (Table C.19). Values taken at month three were significantly lower, but were still in the accepted range of lower than -30 mV. Values measured at 40 °C/75% RH were significantly lower than the values of the other storage conditions. This revealed that zeta-potential values decrease with an increase in temperature. Overall, the Pheroid™ cream formulation remained stable over the three months during stability testing.

Table C.19: Zeta-potential of Pheroid™ cream

Zeta-potential (mV)				
Storage temperature	**Initial**	**Month 1**	**Month 2**	**Month 3**
25 °C/60% RH	-390.28	-365.27	-336.23	-180.40
30 °C/60% RH		-316.45	-359.30	-196.75
40 °C/75% RH		-262.10	-104.36	-160.63

C.4.5.3 ZETA-POTENTIAL OF THE EMULGEL FORMULATION

Zeta-potential values for the emulgel formulation fluctuated significantly over the three months (Table C.20). The values were however, still in the accepted range, but very unpredictable. The emulgel was subsequently viewed as unstable over the three month stability period.

Table C.20: Zeta-potential of emulgel

Zeta-potential (mV)				
Storage temperature	**Initial**	**Month 1**	**Month 2**	**Month 3**
25 °C/60% RH	-122.05	-3256.69	-3847.72	1293.51
30 °C/60% RH		-2845.82	-4112.46	1043.93
40 °C/75% RH		-3582.79	-4088.90	1629.01

C.4.5.4 ZETA-POTENTIAL OF THE PHEROID™ EMULGEL FORMULATION

Zeta-potential values for the Pheroid™ emulgel formulation fluctuated significantly over the three months. The values were not within the accepted range of lower than -30 mV and higher than 30 mV. Therefore, the Pheroid™ emulgel was subsequently viewed as unstable.

Table C.21: Zeta-potential of Pheroid™ emulgel

Zeta-potential (mV)				
Storage	**Initial**	**Month 1**	**Month 2**	**Month 3**
25 °C/60% RH	-1444.02	-518.40	-52.39	34.02
30 °C/60% RH		-415.06	-41.47	21.89
40 °C/75% RH		-457.75	-22.15	26.46

C.4.6 MASS LOSS

The mass variation of all the formulations, and the percentage loss over the three month stability testing, are shown in Table C.22. Variation in mass was tested at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH.

Table C.22: Mass variation of cream, Pheroid™ cream, emulgel and Pheroid™ emulgel over 3 months

	Month				
	0	1	2	3	Percentage loss (%)
	25 °C/60% RH				
Cream	92.81 g	92.59 g	90.47 g	90.27 g	2.74
Pheroid™ cream	94.79 g	93.79 g	92.94 g	92.56 g	2.35
Emulgel	100.51 g	99.99 g	98.78 g	98.58 g	1.92
Pheroid™ emulgel	101.67 g	101.14 g	100.87 g	99.95 g	1.69
	30 °C/60% RH				
Cream	93.37 g	93.27 g	91.42 g	91.15 g	2.38
Pheroid™ cream	93.60 g	93.57 g	91.97 g	91.40 g	2.35
Emulgel	102.53 g	101.51 g	101.05 g	100.32 g	2.16
Pheroid™ emulgel	101.12 g	101.01 g	100.21 g	99.68 g	1.42
	40 °C/75% RH				
Cream	93.40 g	92.46 g	91.48 g	90.53 g	3.07
Pheroid™ cream	94.80 g	94.03 g	93.41 g	92.50 g	2.43
Emulgel	100.76 g	97.89 g	96.68 g	96.16 g	4.57
Pheroid™ emulgel	102.64 g	102.16 g	101.70 g	100.00 g	2.57

All the formulations showed a decrease in mass during the three months, but were still in the accepted range, with a decrease of not more that 20% from the initial mass. The cream, Pheroid™ cream, emulgel and Pheroid™ emulgel stored at 40 °C/75% RH, showed the highest mass loss of 3.07%, 2.43%, 4.57% and 2.57%, respectively. This might have been due to the exposure of the formulations to higher temperatures; and consequently evaporation of the aqueous-phase (Romanowski & Schueller, 2006:662). Overall, the formulations containing Pheroid™ seemed to have a smaller percentage loss than the formulations without Pheroid™. The Pheroid™ delivery system comprised mainly of essential fatty acids and plant fatty acids (Grobler, 2004:4). Therefore, the aqueous phase of the formulation was of a smaller percentage, with subsequent smaller percentage evaporation.

C.4.7 MORPHOLOGY

The following images are representations of the differences in morphology between the formulations, stored for three months in the different stability chambers. The images were taken with a Nikon digital camera DXM 1200 and will be illustrated in Figures C.6 to C.9. A stable emulsion may be defined as a system in which the particles retain their initial character and size, and remain uniformly distributed throughout the continuous phase (Attwood, 2007:96).

C.4.7.1 MORPHOLOGY OF THE CREAM FORMULATION

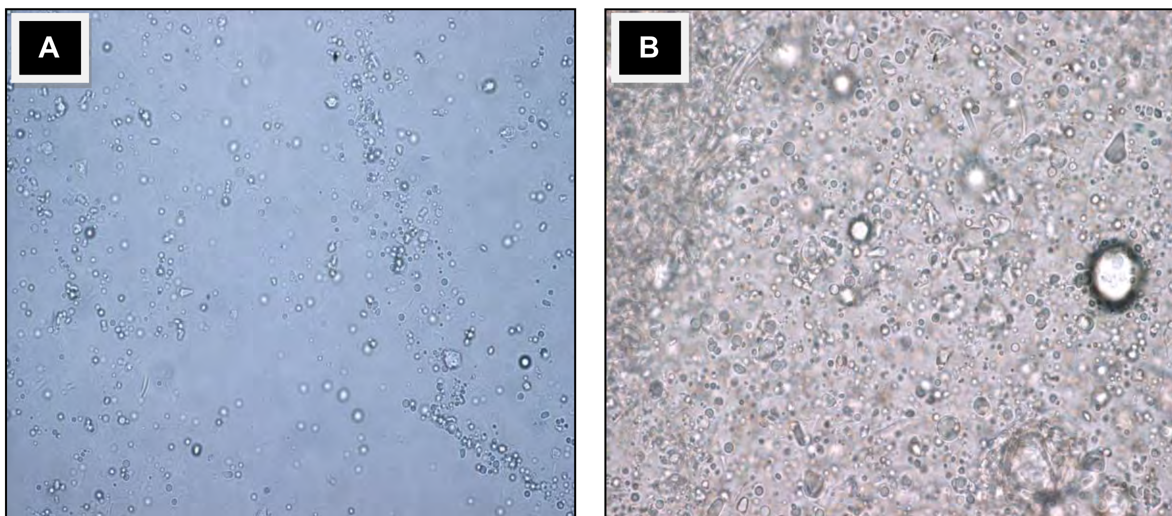

Figure C.6: Representation of light microscope micrographs of cream formulations containing vitamin A and zinc with A) month 0 and B) month 3 at 40 °C/75% RH.

As could be seen in Figure C.6, the morphological characteristics of the cream formulation did not remain constant throughout the study. The most significant change in morphology of the particles in the cream formulation could be seen for the formulation stored at 40 °C/75% RH. The particles did not consist of a spherical shape after three months and were no longer uniformly distributed, which can propose application problems. Thus, it can be said that the cream formulation were not stable during stability testing.

C.4.7.2 MORPHOLOGY OF THE PHEROID™ CREAM FORMULATION

From the micrograph in Figure C.7, “diamond-like” crystals were present from the initial time the micrographs were taken (month 0) to the end of stability testing (month 3). A small amount of the Pheroid™ cream was subsequently tested in order to establish the identity of the crystals. It was found that the crystals were probably vitamin A due to their solubility in ethanol. Vitamin A tends to crystallise in an unfavourable pH-environment, especially when the pH is decreased as described in Section C.4.3.2. The fatty acids present in the Pheroid™ formulation might have caused a decrease in pH; lowering the pH below the optimum environment for vitamin A (5.5 - 6), which in turn caused it to crystallise (Törmä & Vahlquist, 1990:132). Crystallisation of the vitamin A may cause problems with the application and delivery at the site of action. Therefore, the Pheroid™ cream was considered instable from the time it was manufactured to the end of stability testing, due to incompatibilities between Pheroid™ and ingredients in the formulation.

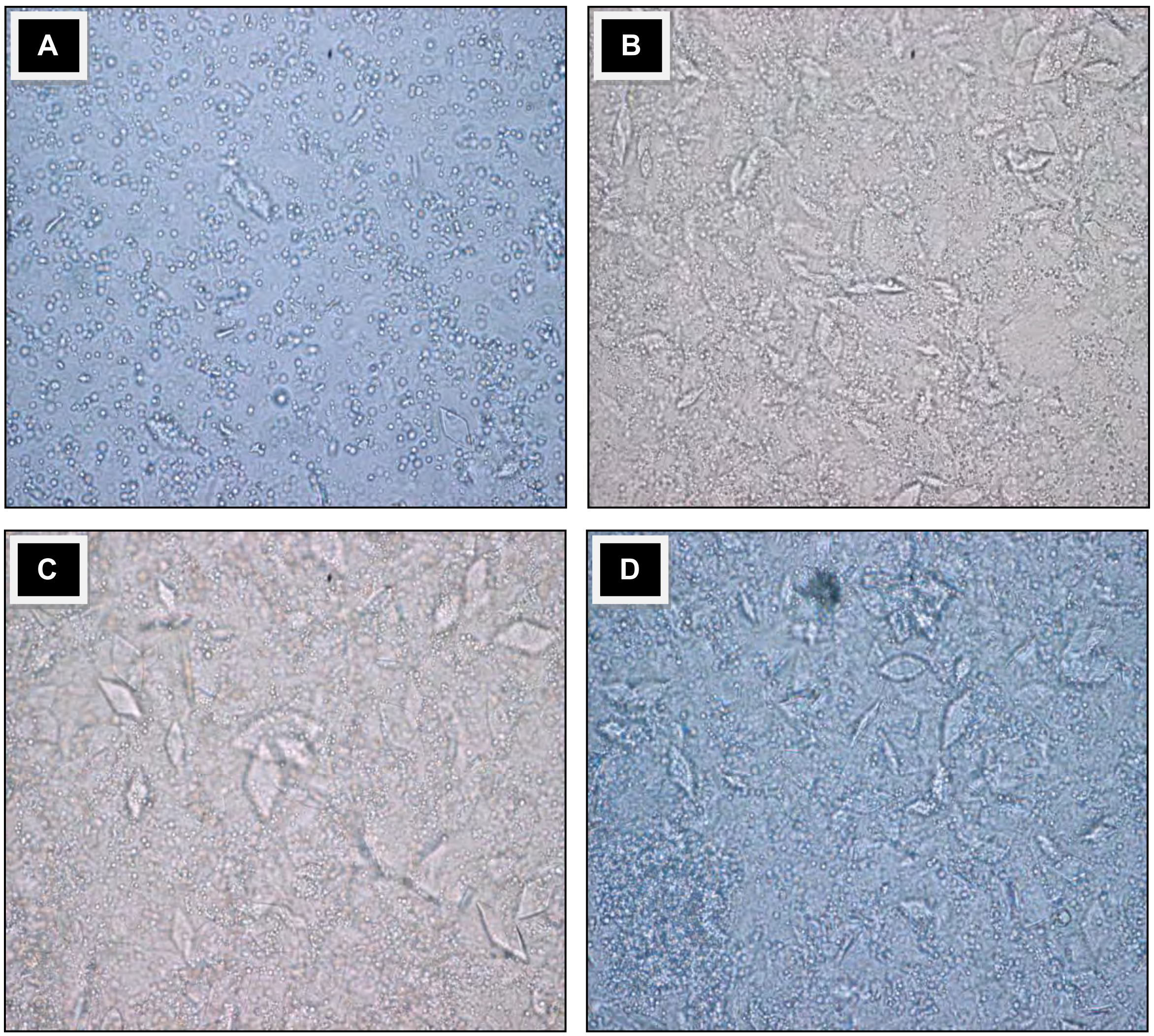

Figure C.7: Representation of light microscope micrographs of the Pheroid™ cream formulation containing vitamin A and zinc with A) month 0, B) month 3 at 25 °C/60% RH, C) month 3 at 30 °C/60% RH and D) month 3 at 40 °C/75% RH.

C.4.7.3 MORPHOLOGY OF THE EMULGEL FORMULATION

From Figure C.8, it was clear that the emulgel formulation did not depict significant changes in morphology during the stability study. The particles were uniformly distributed after three months. Thus, it could be conducted that the emulgel formulation remained stable, when considering the morphology of the emulgel formulation.

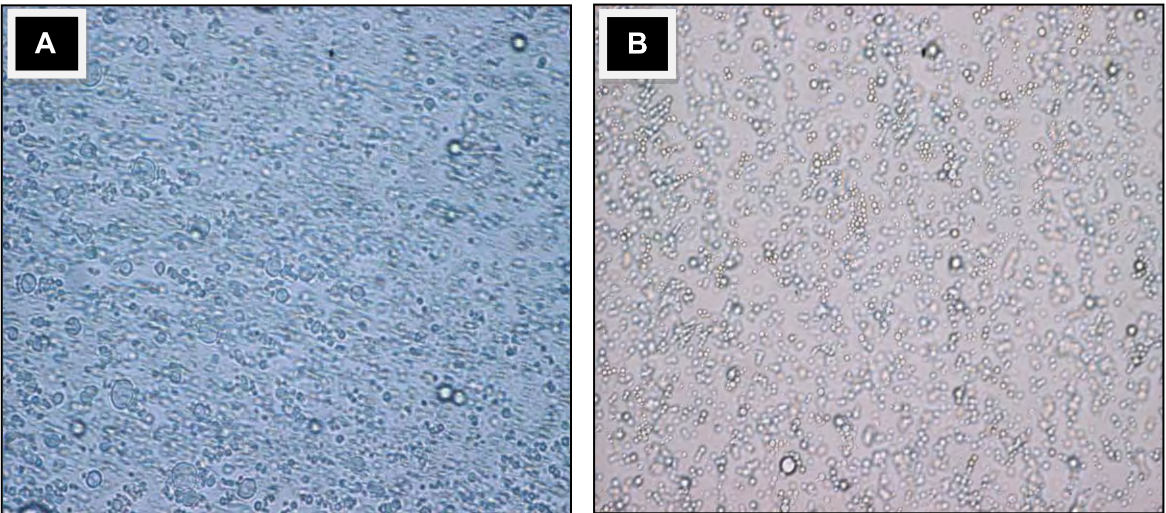

Figure C.8: Representation of light microscope micrographs of emulgel formulation containing vitamin A and zinc with (A) month 0 and B) month 3 at 40 °C/75% RH.

C.4.7.4 MORPHOLOGY OF THE PHEROID™ EMULGEL FORMULATION

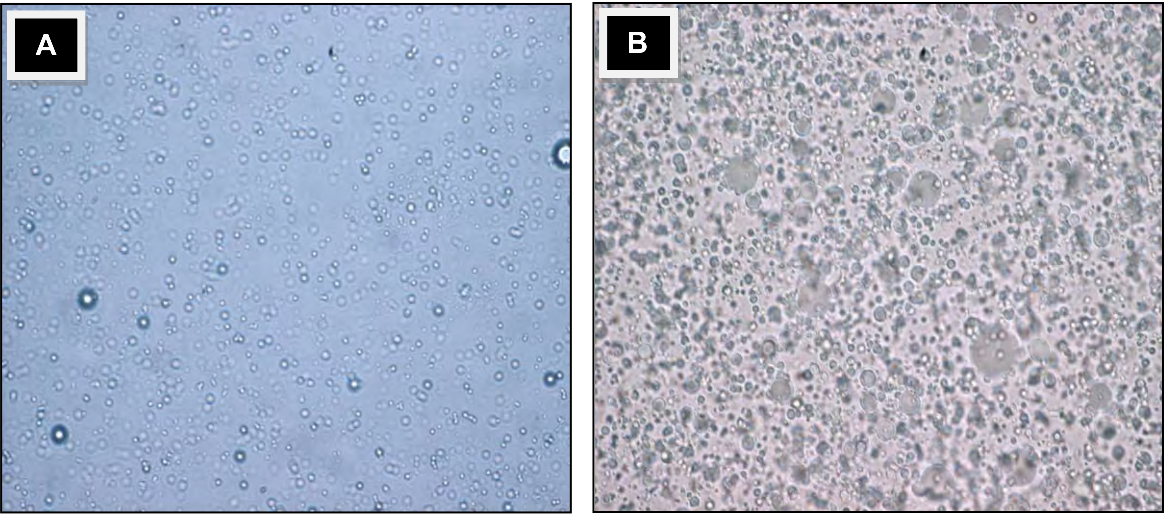

Figure C.9: Representation of light microscope micrographs of the Pheroid™ emulgel formulation containing vitamin A and zinc with A) month 0 and B) month 3 at 40 °C/75% RH.

The Pheroid™ emulgel (Figure C.9) was considered instable after stability testing due to the fact that the particles were no longer uniformly distributed. Particle droplet size varied significantly, which was probably due to coalescence. Coalescence is a process where the thin liquid film between the droplets is removed, and they are united into a larger droplet (Friberg *et al.,* 1988:57). A representation of this phenomenon can be observed in Figure C.10, and may lead to stability problems, which in turn will change the physical properties of the formulation. Stabilisation of the formulations could be enhanced if stored at 25 °C/60% RH or lower.

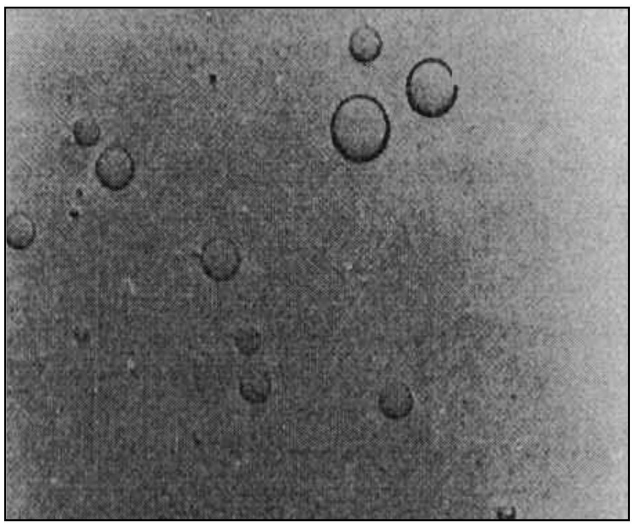

Figure C.10: Representation of a coalesced system (Friberg *et al.,* 1988:59)**.**

C.4.8 PHYSICAL ASSESSMENT

The cream, Pheroid™ cream, emulgel and Pheroid™ emulgel, were all a light yellowish colour (Vanilla Mist 4) with the initial visual assessment. This could be due to the yellowish colour of vitamin A. In Figures C.11 to C.14 the change in colour of each formulation during stability testing can be observed.

C.4.8.1 PHYSICAL ASSESSMENT OF THE CREAM FORMULATION

No significant change in colour was observed form the initial colour assessment (Figure C.11.A) to the assessment after three months of the cream exposed to 25 °C/60% RH (Figure C.11.B). The colour of the creams exposed to 30 °C/60% RH (Figure C.11.C) and 40 °C/75% RH (Figure C.11.D), changed to a deeper yellow colour after three months. When compared to the colour chart, the cream stored at 30 °C/60% RH changed from vanilla mist 4 to spring breeze 5 and the cream stored at 40 °C/75% RH changed to spring breeze 4. The slight change in colour might have been due to the oxidation of oils and other ingredients in the formulation (Barry, 2007:596).

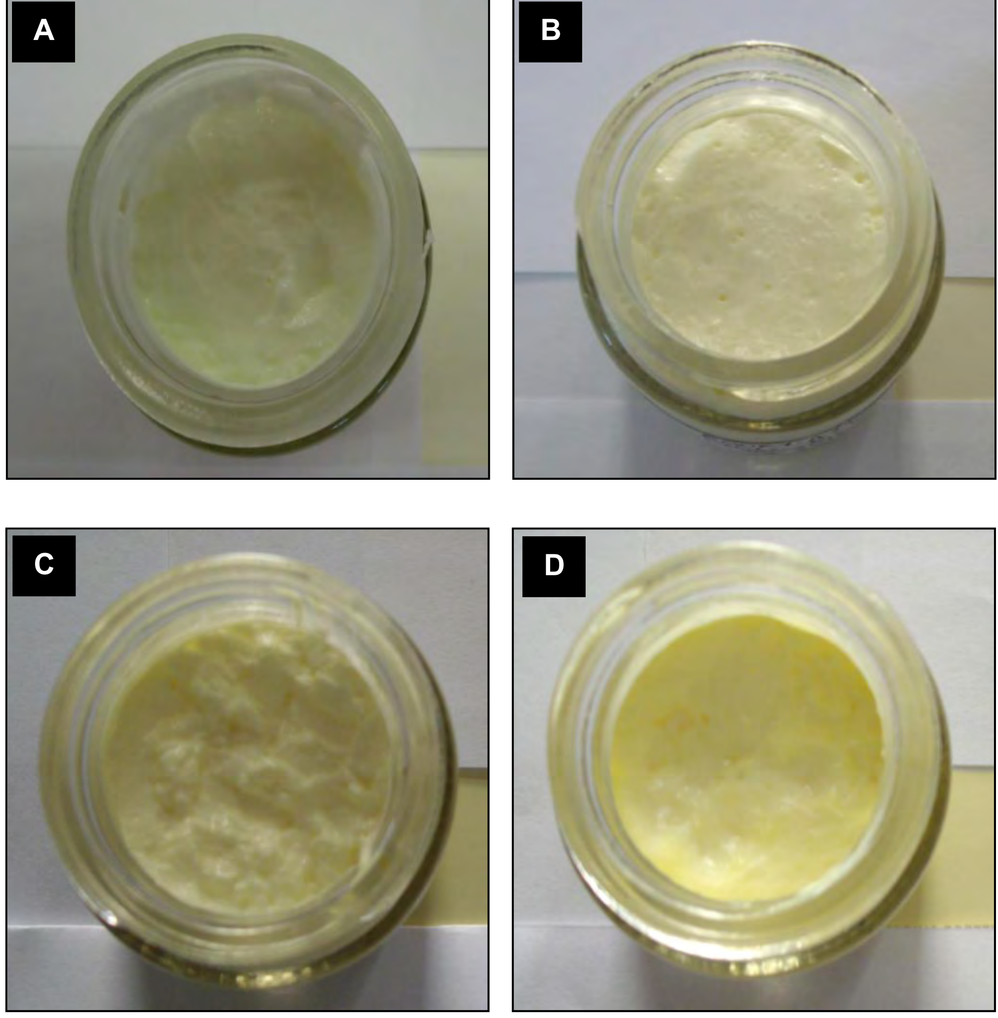

Figure C.11: Change in colour of cream from A) the initial visual appearance to month 3 at B) 25 °C/60% RH, C) 30 °C/60% RH and D) 40 °C/75% RH.

Small amounts of water were present at the bottom of the cream containers stored at 40 °C/75% RH. This is a sign of phase separation, and may be a result of variation in relative density between the two phases of the cream (Knowlton & Pearce, 1993:98). The cream formulations produced a rancid odour at the third month of stability testing, which may be a result of oxidation by atmospheric oxygen or by the actions of micro-organisms (Billany, 2007:401).

C.4.8.2 PHYSICAL ASSESSMENT OF THE PHEROID™ CREAM FORMULATION

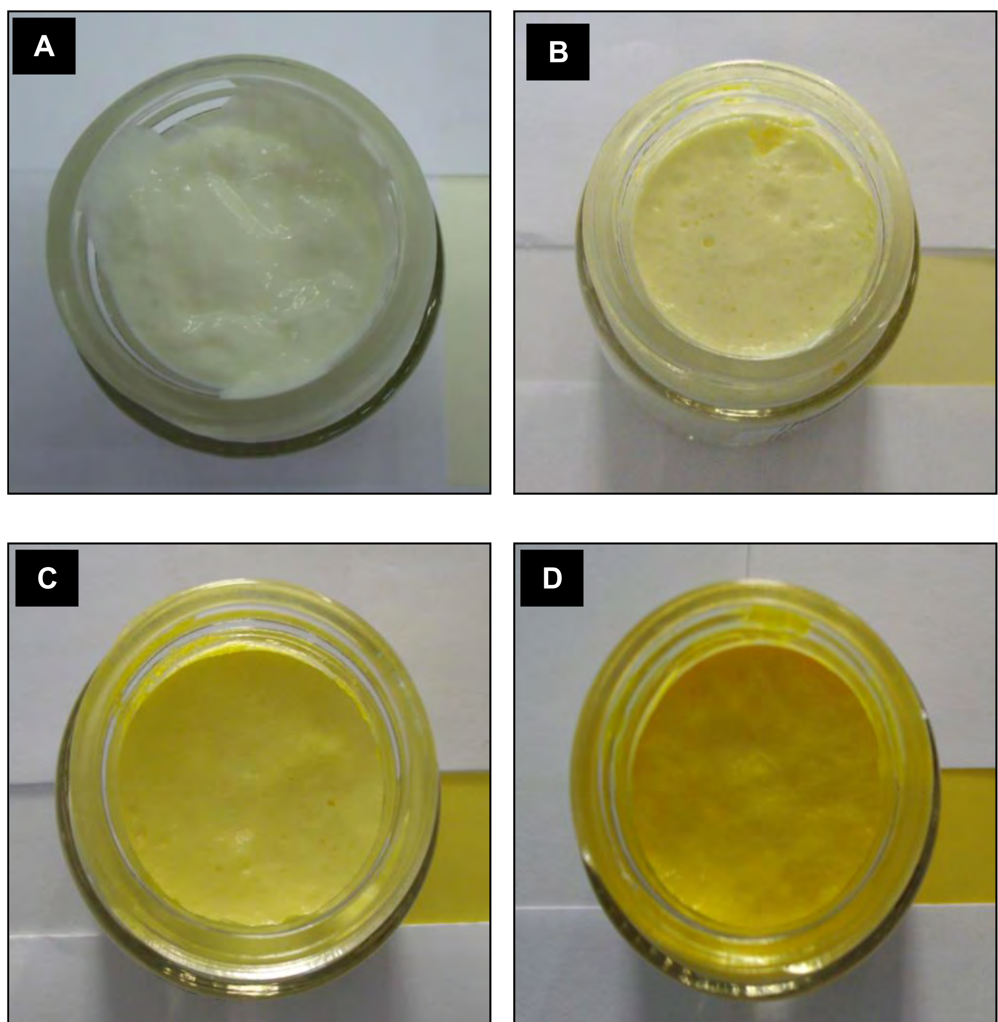

Figure C.12: Change in colour of Pheroid™ cream from A) the initial visual appearance to month 3 at B) 25 °C/60% RH, C) 30 °C/60% RH and D) 40 °C/75% RH.

Only the cream formulation containing Pheroid™ changed drastically in colour. Therefore, the conclusion could be made that some of the Pheroid™ ingredients for example dl-α-tocopherol, played an important role in the discolouration of the Pheroid™ creams. Small amounts of water were present at the bottom of the Pheroid™ cream containers stored at 40 °C/75% RH. This is a sign of phase separation, and may be a result of variation in relative density between the two phases of the cream (Knowlton & Pearce, 1993:98). The Pheroid™ cream formulations also produced a rancid odour at the third month of stability testing, which may be a result of oxidation by atmospheric oxygen or by the actions of micro-organisms (Billany, 2007:401).

The abovementioned factors proved that the formulation was unstable and the change in colour could pose a problem with consumer acceptability.

C.4.8.3 PHYSICAL ASSESSMENT OF THE EMULGEL FORMULATION

A slight change in colour of the Pheroid™ cream, stored at 25 °C/60% RH, was observed from the initial colour assessment (Figure C.12.A) to the assessment after the first, second and third month (Figure C.12.B). The colour of the creams exposed to 30 °C/60% RH (Figure C.12.C) and 40 °C/75% RH (Figure C.12.D), changed more drastically after three months. Compared to the colour chart, the cream stored at 30 °C/60% RH changed from vanilla mist 4 to spring breeze 5, and the cream stored at 40 °C/75% RH changed to banana dream 3. Therefore, the Pheroid™ cream stored at 40 °C/75% RH showed the most significant change in colour; from yellow to a bright yellow.

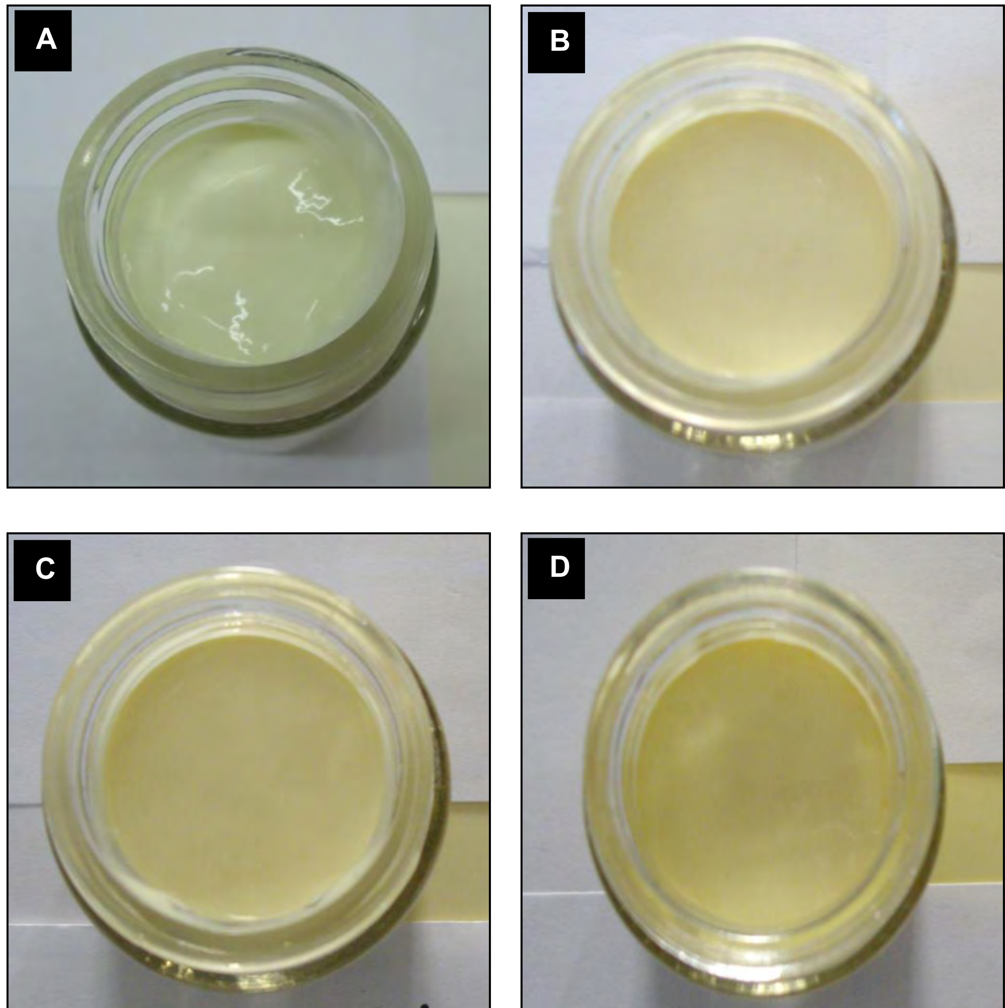

Figure C.13: Change in colour of emulgel from A) the initial visual appearance to month 3 at B) 25 °C/60% RH, C) 30 °C/60% RH and D) 40 °C/75% RH.

No significant change in colour was observed form the initial colour assessment (Figure C.13.A) to the assessment after three months of the emulgel exposed to 25 °C/60% RH (Figure C.13.B). The colour of the emulgel formulations exposed to 30 °C/60% RH (Figure C.13.C) and 40 °C/75% RH (Figure C.13.D), respectively; changed slightly to a more yellow colour after

three months. Compared to the colour chart, the emulgel stored at 30 °C/60% RH changed from vanilla mist 4 to eastern morn 5 and the emulgel stored at 40 °C/75% RH also changed to eastern morn 5. The slight change in colour might have been due to the oxidation of oils and other ingredients in the formulation (Barry, 2007:596). The emulgel formulation produced a rancid odour at the third month of stability testing, which may be a result of oxidation by atmospheric oxygen or by the actions of micro-organisms (Billany, 2007:401).

C.4.8.4 PHYSICAL ASSESSMENT OF THE PHEROID™ EMULGEL FORMULATION

A slight change in colour of the Pheroid™ emulgel stored at 25 °C/60% RH was observed from the initial colour assessment (Figure C.14.A) to the assessment after the third month. The colour of the Pheroid™ emulgel exposed to 30 °C/60% RH (Figure C.14.B) and 40 °C/75% RH (Figure C.14.C) however, changed more drastically over the three months.

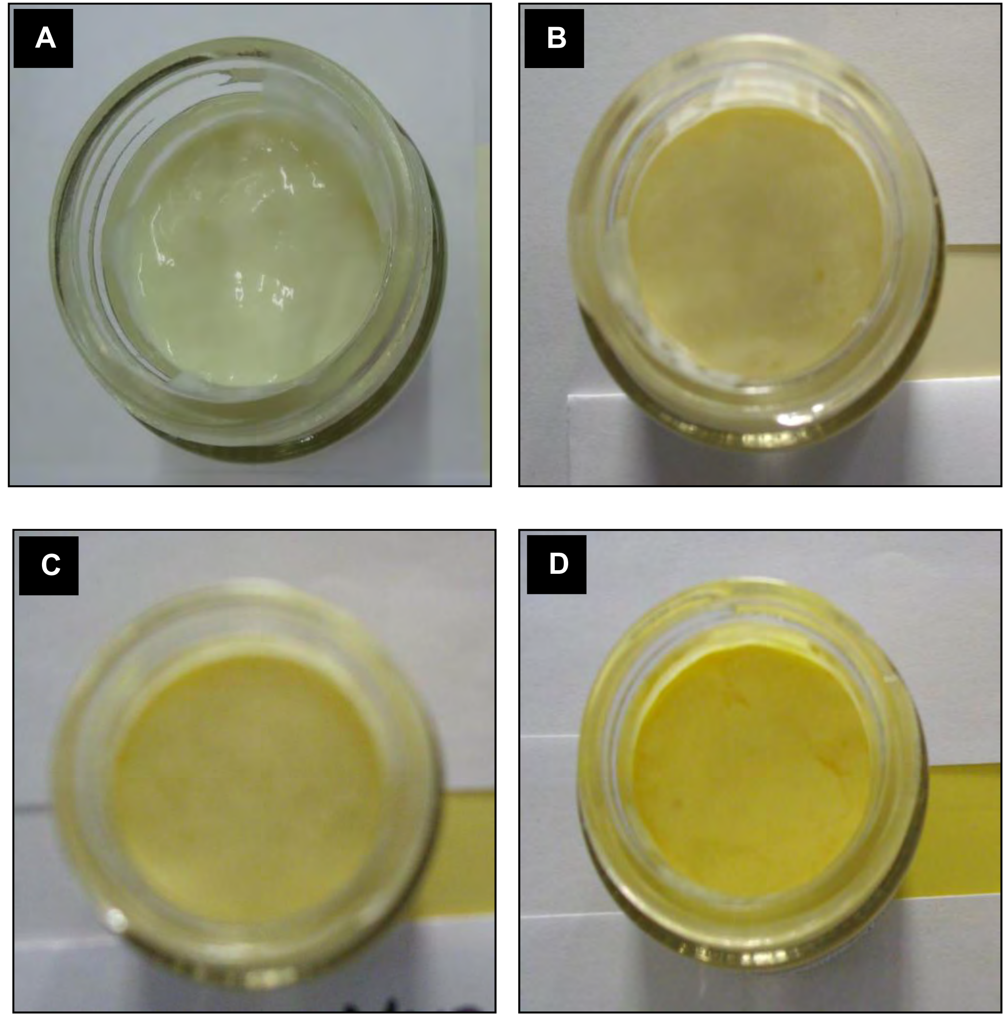

Figure C.14: Change in colour of Pheroid™ emulgel from A) the initial visual appearance to month 3 at B) 25 °C/60% RH, C) 30 °C/60% RH and D) 40 °C/75% RH.

Compared to the colour chart, the Pheroid™ emulgel stored at 30 °C/60% RH changed from vanilla mist 4 to spring breeze 4 and the Pheroid™ emulgel stored at 40 °C/75% RH changed to spring breeze 3. The Pheroid™ emulgel stored at 40 °C/75% RH showed the most significant change in colour, from yellow to a bright yellow; but this change was not as drastic as the chance in colour of the Pheroid™ cream formulation.

Only the formulations containing Pheroid™ changed drastically in colour. Therefore, the conclusion could be made that some of the Pheroid™ ingredients for example dl-α-tocopherol, played an important role in the discolouration of the Pheroid™ formulations. The Pheroid™ emulgel formulations produced a rancid odour at the third month of stability testing, which might have been a result of oxidation by atmospheric oxygen or by the actions of micro-organisms (Billany, 2007:401).

Small amounts of water were present at the bottom of the Pheroid™ emulgel containers stored at 40 °C/75% RH. This is a sign of phase separation, and may be a result of variation in relative density between the two phases of the emulgel (Knowlton & Pearce, 1993:98).

The abovementioned factors proved that the formulation was unstable and the change in colour could pose a problem with consumer acceptability.

C.5 CONCLUSION

Stability testing was performed on the four formulated products for a period of three months. Table C.23 is a summary of all the stability tests performed, and the results obtained from the three different humidity chambers. According to the summary, the cream formulation, stored at 25 °C/60% RH and 30 °C/60% RH were the most stable of all the formulations throughout the study. On the other hand, the Pheroid™ emulgel formulation showed the most significant degradation and therefore, was most unstable.

Stability testing conducted on the commercial product could not be compared to any of the formulations in this study, as it was clear that the product was very unstable. Stabilisation experiments were performed in attempt to stabilise vitamin A during preformulation studies (see Appendix B.2.2.1).

The emulgel containing Pheroid™ proved to be the most stable formulation when considering the zinc concentration. Zinc concentrations in the formulations remained relatively stable during stability testing.

A suggestion for future vitamin containing product formulations is to prepare an overage of 110% when the product is manufactured. This will ensure a product with the correct amount of active ingredient with an extended shelf life. It is important to keep in mind that this strategy is only possible where the dose of the product is not critical and the degradation products are not toxic (Barnes, 2007:650).

Table C.23: Summary of different stability tests performed, and the results after three months at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH

Stability test	Acceptance criteria	Cream			Pheroid™ cream			Emulgel			Pheroid™ emulgel		
		25° C	30° C	40° C	25° C	30° C	40° C	25° C	30° C	40° C	25° C	30° C	40° C
Assay (vitamin A)	≤ 20% decrease	√	√	×	×	×	×	√	√	×	√	√	×
Assay (zinc)	≤ 20% decrease	√	√	√	√	√	√	√	√	√	√	√	√
Assay (commercial)	≤ 20% decrease	√	×	×									
Rheology	≤ 20% decrease	×			×			×			×		
pH	5.5 - 6	√	×	×	×	×	×	×	×	×	√	×	×
Droplet size	0.5 - 2.5 µm	√	√	√	×	×	×	×	×	×	×*	×*	×*
Zeta-potential	≤ -30 mV and ≥ 30 mV	√	√	√	√	√	√	√*	√*	√*	√	×	×
Mass loss	≤ 20% decrease	√	√	√	√	√	√	√	√	√	√	√	√
Morphology	Constant morphology			×			×			√			×
Physical assessment	Colour change or not	√	√	×	×	×	×	√	√	×	×	×	×

X * The droplet size values of the Pheroid™ emulgel were in the acceptable range, due to degradation, therefore this formulation can be stated as unstable in terms of droplet size.

√ * The zeta-potential values of the emulgel were in the accepted range, but very precarious, therefore this formulation can be stated as unstable in terms of zeta-potential.

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APPENDIX D

TRANSDERMAL DIFFUSION STUDIES

D.1 INTRODUCTION

Transdermal drug delivery has been a challenging topic in the pharmaceutical and cosmeceutical industries for the past thirty years, with increasing successes and achievements being reached. Delivery of drugs across the skin has its advantages, such as limited metabolic activity, compared to that of oral drugs in the liver (Bouwstra, 2003:3), controlled plasma levels of potent drugs, acceptability and improved compliance by patients (Delgado-Charro & Guy, 2001:216). Despite its many advantages, various factors influence / limit the diffusion of drugs across the skin.

For the effective delivery of drugs, various factors should be taken into account, including:

- Identification of the disease or condition to be treated.
- The site where drug action is needed.
- Choice of a formulation that will be compatible with the characteristics of the drug(s).
- Identification of rate-limiting steps (Barry, 2007:596-597).

Various skin conditions occur at different sites of the skin and require therapeutic drug levels within a particular region. Acne, for example, requires drug targeting of the pilosebaceous structures in the dermis. Transdermal application of a drug may thus enhance the delivery thereof and minimise systemic side effects (Singh, 1999:598).

In vitro diffusion studies were conducted during this study, using Franz diffusion cells. To determine whether the two active ingredients, vitamin A acetate and zinc acetate, were being released from the two formulated creams and two emulgels, membrane diffusion studies were performed. Full thickness human skin was used to determine the transdermal delivery of vitamin A and zinc in the four formulated topical products. The Pheroid™ was incorporated into both types of formulations, in order to compare the permeation results with the formulations without Pheroid™. Transdermal diffusion studies were also performed on a commercial product, containing vitamin A acetate, in order to compare its diffusion through the skin with that of the formulated products of this study. Tape stripping techniques were further utilised to determine the dermal delivery of the active ingredients.

D.2 METHODS

D.2.1 ANALYSIS OF SAMPLES

Vitamin A was analysed, using a validated, high performance liquid chromatography (HPLC) method, whereas zinc was analysed on an atomic absorption (AA) spectrometer, as described in Appendix A.1. Analyses were performed in a controlled laboratory environment at 25°C. Both methods are described in the next sections.

D.2.1.1 HPLC ANALYSIS OF VITAMIN A

In order to accurately determine the concentration of vitamin A in the samples, an Agilent® 1200 series HPLC system was used. It was equipped with an Agilent® 1200 quaternary pump, ultraviolet (UV) detector, autosampler, degasser and thermostat, with the temperature set at 5°C. Data analysis was performed by Chemstation Rev. A.10.02 analysis software. A Verusil XBP C_{18} (2) (4.6 mm x 150 mm) silica column with 5 µm particle size was used during the analysis.

The mobile phase consisted of 100% analytical HPLC grade methanol. The flow rate was set at 1 ml/min, with an injection volume of 25 µl and a runtime of 10 min. Vitamin A was detected at 349 nm and it eluted at 4.7 min.

D.2.1.2 ANALYSIS OF ZINC

The zinc concentration was determined, using a standard, validated and accredited method by Eco-Analytica from the North-West University, Potchefstroom Campus. A Varian SpectrAA - 250 Plus AA spectrometer was used during this study. Zinc was detected by the use of an air and acetylene flame. Varian hollow cathode lamps were employed at a wavelength of 213.9 nm.

Samples were prepared by withdrawing 1 ml from the Franz diffusion cell, or politop with tape strips, or politop containing dermis samples and transferred into a 10 ml volumetric flask. Nitric acid (65%) was used as solvent and diluted with HPLC grade water to attain a concentration of 1.6%. 9 ml of the nitric acid (1.6%) was added to the 1 ml sample in the volumetric flask, to prepare a 10 ml solution.

D.2.2 DONOR AND RECEPTOR PHASE PREPARATION

The donor phases used in this study for both the membrane release and skin diffusion studies, contained the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel, all containing vitamin A and zinc as active pharmaceutical ingredients (APIs). A commercial product containing

vitamin A acetate was used for diffusion studies, to compare the transdermal diffusion with the formulated products, containing vitamin A, in this study.

A mixture of ethanol (50%) and 50% phosphate buffer solution (PBS) was used as receptor phase for both the membrane and skin diffusion studies. The solution of the receptor phase must be able to dissolve the ingredients that require analysis, therefore an organic solvent, ethanol, was added to dissolve the lipophilic vitamin A and other oily ingredients. PBS was prepared by dissolving potassium orthophosphate crystals (13.62 g) in 500 ml of HPLC grade water. Subsequently, sodium hydroxide pearls (3.174 g) were dissolved in 786.8 ml HPLC grade water. The two solutions were mixed and the pH was adjusted to 7.4 with 10% phosphoric acid. A 50:50 (v/v) ethanol:PBS solution and a fresh receptor phase were prepared before every diffusion study, in order to prevent the formation of crystals.

D.2.3 STANDARD PREPARATION

Vitamin A (5 mg) was transferred into a 100 ml volumetric flask and filled to volume with 50:50 (v/v) ethanol:PBS. A fresh standard sample was prepared before every diffusion study.

D.2.4 SKIN PREPARATION

Abdominal skin of Caucasian female patients, who had undergone abdominoplastic surgery, was obtained from various medical institutions. The Research Ethics Committee of the North-West University granted ethical approval for acquiring and utilising the donated skin under reference number 04D08. Informed consent had been obtained from all donors before surgery and their identities kept anonymous.

The skins had been frozen at -20°C within 24 h after surgery. Full thickness skin was used during this study, consisting of the stratum corneum, epidermis and dermis. Subcutaneous fat and connective tissue were removed with a blunt scalpel, taking care not to damage the skin. The skin was placed on a flat surface for inspection for any defects, such as stretch marks, scars, or blemishes. Circles, having a diameter of approximately 15 mm, were punched from the skins, placed on Whatman® filter paper and covered with aluminium foil. These samples were then frozen at -20°C until use. According to Leveque *et al*. (2004:324), the skin circles would be stable under such conditions for a period of three to six months. Before diffusion studies were performed, the frozen skin circles were thawed at room temperature and mounted onto the diffusion apparatus.

D.2.5 FRANZ CELL DIFFUSION

Figure D.1: Illustration of instrumentation used during transdermal diffusion studies: A) amber Franz diffusion cell, B) horseshoe clamp, C) assembled Franz diffusion cell and D) Grant water bath.

Amber Franz diffusion cells were used in order to prevent photo-isomerisation of vitamin A. The Franz diffusion cells consisted of a donor and receptor compartments (Figure D.1.A). The receptor capacity of these cells was approximately 2 ml with a diffusion area of 1.075 cm^2. Five diffusion studies were performed, one for each formulation (cream, Pheroid™ cream, emulgel, Pheroid™ emulgel and commercial product). Fifteen Franz diffusion cells were used per diffusion study, of which ten cells were used for skin diffusion investigation, and five cells as controls (see Section 2.5.2). In order to compare the results, skin of the same donor was used for each individual diffusion study (Leveque *et al*., 2004:324).

Full thickness skin circles were mounted between the receptor and donor compartments, with the stratum corneum facing upwards and in contact with the donor phase. The unit was sealed

with Dow Corning® vacuum grease and fastened with a metal horseshoe clamp (Figure D.1.B) to prevent leakage. The donor compartments of the ten experimental cells were each filled with approximately 1 ml of the formulated product, i.e., cream, Pheroid™ cream, emulgel, Pheroid™ emulgel or the commercial product. Samples were submerged in a water bath set at 32°C, the same as human skin temperature. Placebo formulations (without APIs) were transferred into four of the control cells and into another cell a 50:50 (v/v) ethanol:PBS solution (pH 7.4). Care was taken to ensure that the whole diffusion area was each covered with the formulated product. In order to prevent evaporation of volatile components, the donor compartment was covered with Parafilm® and a cap. The receptor phase was magnetically stirred in order to maintain homogeneity throughout the experiment.

The receptor compartment was filled with 2 ml of the 50:50 (v/v) ethanol:PBS (pH 7.4) solution, having a temperature of 37°C. Care was taken to prevent air bubbles beneath the skin samples. The fifteen cells were each securely clamped (Figures D.1.B and D.1.C) and placed on a Variomag® magnetic stirrer plate in a Grant water bath (Figure D.1.D), with the receptor compartment covered under water. After placing the Franz cells in the water bath (37°C), it was confirmed that the mounted skin samples were at a temperature of 32°C. The content of the receptor compartments were withdrawn at specific time intervals, and immediately replaced with 2 ml of the preheated ethanol:PBS (50:50) solution, to maintain sample conditions.

1 ml each of the withdrawn receptor phase samples was transferred into a 10 ml volumetric flask and made up to volume for the analysis of zinc on the AA spectrometer. The remaining solutions was each transferred into amber HPLC vials and immediately analysed on the HPLC in order to determine the concentration of vitamin A within the receptor compartment, for each withdrawal time.

D.2.5.1 MEMBRANE DIFFUSION

The aim of the membrane diffusion study was to determine whether vitamin A and zinc were released from the formulated products. For this reason, the extraction times differed from those of the skin diffusion study. The exact same method, as described in Section D.2.5, was used for the membrane diffusion studies, except for the use of polytetrafluoroethylene (PTFE) membranes, with a thickness of 0.4 μm, instead of skin. The samples from the receptor compartments of the Franz cells were withdrawn after 0.5, 1.0, 2.0, 4.0, and 6.0 h. The receptor compartment was immediately refilled with fresh (50:50) ethanol:PBS solution (37°C). Analyses of vitamin A and zinc were performed as described in Sections D.2.1.1 and D.2.1.2.

D.2.5.2 SKIN DIFFUSION

The method, as described in Section D.2.5, was used for the skin diffusion studies. Originally, the contents of the receptor phase were withdrawn after 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 h. The generated results were worthless though, because of the degradation and inherent instability of vitamin A. Subsequently, only one extraction for both vitamin A and zinc was done after 6 h, after which the receptor compartment was filled with fresh (50:50) ethanol:PBS solution (37°C).

According to Hostýnek and Maibach (2002:34), zinc is the most abundant element in the human body, of which 6% is present in the skin. To eliminate the possibility of endogenous zinc diffusion, placebo formulations (without zinc) were prepared for use as control samples during the skin diffusion investigation. A placebo formulation was formulated for the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. A Franz cell for each of the four formulations was used and the extraction of the receptor phase was conducted after 6 h.

D.2.6 TAPE STRIPPING

Tape stripping is a basic method to remove the outermost layer of the skin. This technique was used to investigate the penetration and reservoir behaviour of topically applied formulations (Pellett *et al.,* 1997:92).

After the 6 h diffusion study, the skin samples were carefully removed from the Franz diffusion cells. The diffusion area of each skin circle was dabbed dry with clean paper towel. 3M Scotch® Magic™ tape was cut into strips, big enough to cover the diffusional area. Sixteen tape strips were needed per skin circle, of which the first strip was discarded, due to possible residue from the formulated product on the skin‟s surface (Pellett *et al*., 1997:94). The remaining 15 strips were used to remove the stratum corneum-epidermis and placed in a politop filled with 5 ml of ethanol:PBS (50:50) solution. Complete removal of the stratum corneum-epidermis was indicated by the glistening of the viable epidermis.

The non-diffusion area of each skin sample was cut off and discarded, whereas the remaining area of diffusion of the skin was cut into pieces, thus enlarging the surface area. These were placed into a politop filled with 5 ml of ethanol:PBS (50:50) solution, in order to determine the concentration of APIs being diffused into the epidermis-dermis. Politops, containing the tape strips, as well as the dermis samples, were sonicated for approximately 30 min directly after sampling. All the samples were centrifuged for 10 min with an Eppendorf centrifuge 5804R and analysed on the HPLC to determine the permeant contents.

According to Hostýnek and Maibach (2002:34), 75% of the 6% zinc present in the skin, is situated in the epidermis. To ensure accurate results, placebo formulations for the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel were prepared and diffusion studies were conducted on full thickness skin. A Franz cell was used for each of the four formulations. The same tape stripping method, as described above, was done for the placebo formulations, after completing the skin diffusion study.

D.2.7 STATISTICAL ANALYSIS

All data was statistically analysed, using quantitative statistical methods. Analyses were performed utilising the SPSS and R statistical software. Descriptive statistics in the form of median values were reported and data (with the data points superimposed) was graphically represented. Inferential statistics were performed, involving formal hypothesis testing. These tests included a two-way design, where any relationship between the application of Pheroid™ in the topical formulations (i.e. the cream and emulgel) was investigated on epidermis (stratum corneum-epidermis), dermis (epidermis-dermis) and the diffusion concentrations of vitamin A and zinc. Should a significant variation in the data be observed, it would be more accurate to use the median value (centre of data set) (Gerber *et al*., 2008:190). Hence, based on the small sample sizes and skew distributions of the data, the non-parametric test, as proposed by Brunner *et al.* (1997:1494), henceforth referred to as the BDM test, was used for this purpose.

The non-parametric Mann-Whitney test was performed to investigate the main effects of Pheroid™ and the formulations on the concentrations of vitamin A and zinc. All statistical inferences were performed at a 5% (0.05) level of significance. A p-value of less than 0.05 indicated a statistically significant difference among the groups of data, whereas a p-value of more than 0.05 was statistically insignificant, meaning no noticeable difference between the data.

It should be noted that the standard two-way analyses of variance (ANOVA), followed by T-tests, were also performed, but were not reported, due to model assumption violation, since more data points were required.

The tape stripping experiment conducted on the commercial product, delivered very consistent (no variation) epidermal and dermal results. These values were therefore only used as reference values in the graphical representations and discussions.

D.3 RESULTS AND DISCUSSION

D.3.1 OVERVIEW OF PHYSICOCHEMICAL PROPERTIES OF VITAMIN A AND ZINC

In order to permeate the skin effectively, an API must possess specific physicochemical properties. Two important physicochemical properties that may play an essential role in the prediction of transdermal diffusion of an API, are the octanol-water partition coefficient (log P) value and its aqueous solubility.

The log P value of a compound is an indication of its ability to dissolve in both oil and water. According to Williams (2003:36), a compound with a log P between 1 and 3 will permeate the skin relatively fast. Due to the high lipophilicity of vitamin A, no log P value was available in the literature. The prediction that vitamin A would penetrate the skin poorly, could be made. Contrary, the experimental log P value for zinc is 1.22 (Drugbank, 2008:1), which indicated that zinc would be able to permeate the skin effectively.

Aqueous solubility, however, needs to be more than 1 mg/ml in order to penetrate the aqueous boundary layer (viable epidermis) of the skin (Naik *et al.*, 2000:319). Vitamin A is completely insoluble in water and its aqueous solubility unknown, whilst the aqueous solubility of zinc is 430 mg/ml, thus indicative of excellent skin permeability.

D.3.2 MEMBRANE DIFFUSION

D.3.2.1 MEMBRANE DIFFUSION OF VITAMIN A

The average, cumulative concentration of vitamin A being released after 6 h from the Pheroid™ cream (49.518 μg/cm^2) was the highest, followed by the cream (18.314 μg/cm^2), emulgel (0.720 μg/cm^2) and Pheroid™ emulgel (0.554 μg/cm^2), as summarised in Table D.1.

Table D.1: Membrane diffusion results of vitamin A after 6 h

	Average %released after 6 h	Average cumulative concentration after 6 h (μg/cm^2)
Cream	0.223	18.314
Pheroid™ cream	0.555	49.518
Emulgel	0.008	0.720
Pheroid™ emulgel	0.006	0.554

The Pheroid™ cream formulation released more than two and a half times the amount of vitamin A, than the cream formulation, with an average percentage being released of 0.555% after 6 h. More vitamin A diffused through the membrane from the cream formulations, than from the emulgel formulations.

D.3.2.2 MEMBRANE DIFFUSION OF ZINC

The average, cumulative concentration of zinc being released from the Pheroid™ emulgel formulation (14.313 μg/cm^2) after 6 h was the highest, followed by the other three formulations, all with similar concentrations. The results are presented in Table D.2.

Table D.2: Membrane diffusion results of zinc after 6 h

	Average %released after 6 h	Average cumulative concentration after 6 h (μg/cm^2)
Cream	0.037	8.277
Pheroid™ cream	0.027	6.125
Emulgel	0.039	8.760
Pheroid™ emulgel	0.064	14.313

The Pheroid ™ emulgel formulation released almost twice the amount of zinc, compared to that of the emulgel, cream and Pheroid™ cream formulations.

Vitamin A and zinc were released from all the formulations, but only in small percentages. Since low concentrations were expected to diffuse during the diffusion studies, it was decided that only one extraction for the skin diffusion studies would be done at 6 h.

D.3.3 DIFFUSION STUDIES

D.3.3.1 VITAMIN A DIFFUSION STUDIES

D.3.3.1.1 Diffusion results of the commercial product

The commercial product that was used for the diffusion studies contained an unknown concentration of vitamin A acetate. This product was used to compare the transdermal diffusion concentration of its vitamin A with that of the formulated products in this study. No vitamin A was detected in the receptor compartment during these diffusion studies, therefore indicative that it did not penetrate through the skin. The high lipophilicity of vitamin A may have prevented diffusion through the skin into the Franz cell receptor. Vitamin A would therefore remain in the epidermis-dermis (Jenning *et al.*, 2000:214), which was an acceptable result, when considering the intended target area of treatment.

D.3.3.1.2 Diffusion results of vitamin A

According to the literature, vitamin A generally penetrates the skin very poorly (Bershad, 2001:157), as was confirmed by the results from this study, since no vitamin A was detected in the receptor compartment. Vitamin A did not penetrate through the thickness skin, possibly due

to its high lipophilicity, that may have formed a reservoir in the stratum corneum (Goosen *et al*., 1998:207) that prevented diffusion from the skin into the Franz cell receptor (Jenning *et al*., 2000:214). Since the target area for the treatment of acne is the dermis, these results were acceptable (Webster, 1996:241).

D.3.3.1.3 Diffusion of vitamin A from the commercial product, compared to the formulations

When the diffusion concentrations of vitamin A of the formulations were compared to those of the commercial product, it was observed that no vitamin A had diffused through the skin during the 6-h diffusion studies.

D.3.3.2 ZINC DIFFUSION STUDIES

Different salt forms show different behaviour in the transdermal diffusion of metals. The hierarchy in the behaviour between the different salt forms of zinc are as follows: acetate > nitrate > sulphate > chloride (Hostýnek, 2003:331). Therefore, the chosen zinc acetate may have contributed to the good diffusion results of zinc through the skin.

D.3.3.2.1 Determination of intrinsic zinc present in the skin by using different placebo formulations

According to the literature, zinc does not normally diffuse through the skin (Dussert *et al.,* 1997:119-129; Filipe *et al.,* 2009:266-275). However, Hostýnek and Maibach (2002:34) found that the skin inherently contains approximately 6% of zinc. The presence of zinc in the receptor phase during the transdermal diffusion study was thus not unexpected. To eliminate uncertainty, transdermal diffusion experiments were conducted on placebo (without zinc) formulations. These placebo formulations were also a cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. Zinc was once again detected in the receptor phase, thus confirming the natural presence of zinc in the skin. The zinc in the skin, however, diffused into the receptor compartment, whenever a placebo formulation was applied. The highest average zinc concentration resulted from the Pheroid™ cream (0.205 μg/cm^2), followed by the emulgel (0.096 μg/cm^2), cream (0.067 μg/cm^2) and Pheroid™ emulgel (0.059 μg/cm^2) formulations.

D.3.3.2.2 Diffusion of zinc by using different formulations

A summary of the zinc results, generated during the skin diffusion studies, are illustrated in Figure D.2.

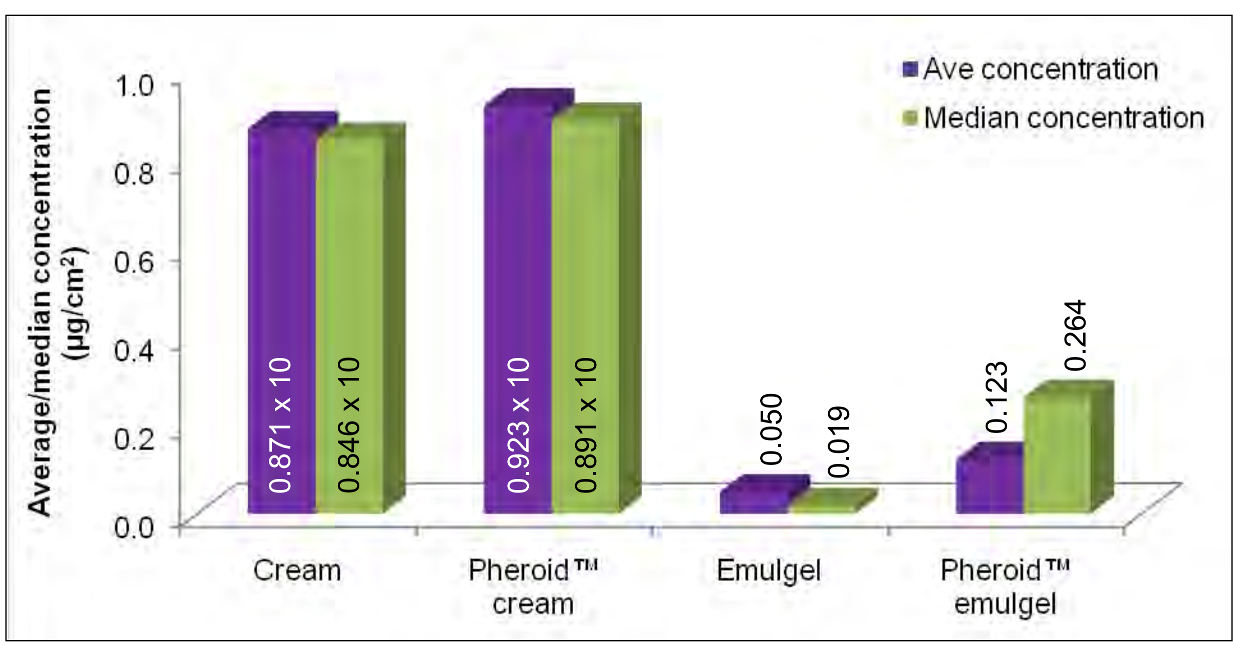

Figure D.2: Average and median concentrations (µg/cm^2) of zinc after 6 h for the four formulations.

Zinc diffused through the skin, with the cream, Pheroid™ cream, emulgel and Pheroid™ emulgel formulations used as donor phases. However, since no flux was observed, a sample was extracted only once, after 6 h. The average concentration of zinc in the receptor compartment after the diffusion studies showed that it had permeated through the skin.

The highest average zinc concentration that permeated through the skin after 6 h, was from the Pheroid™ cream (9.231 µg/cm^2), followed by the cream (8.710 µg/cm^2), Pheroid™ emulgel (0.123 µg/cm^2) and emulgel (0.050 µg/cm^2) formulations (Figure D.2). Compared to the membrane release studies, these results were unexpected. After comparing the results from the cream formulations with those of the emulgel formulations, it was observed that the cream formulations showed higher diffusion concentrations than the emulgel formulations. The cream formulations were more lipophilic than the emulgel formulations, due to the higher contents of oil ingredients that may have diffused through the lipid domain of the stratum corneum. This in turn may have resulted in the higher diffusion concentrations through the skin.

When comparing the test outcomes of the Pheroid™ formulations with the non-Pheroid™ formulations, it was established that the incorporation of Pheroid™ into the topical formulations positively affected the diffusion potential of zinc. This was probably due to the presence of the fatty acids associated with Pheroid™, which caused the formulations to be more lipophilic. The hydrophilic zinc was thus possibly entrapped into the lipophilic Pheroid™ vesicle, rendering it more permeable through the skin (Grobler *et al.,* 2008:290).

A graphical representation of the data is given in Figure D.3.

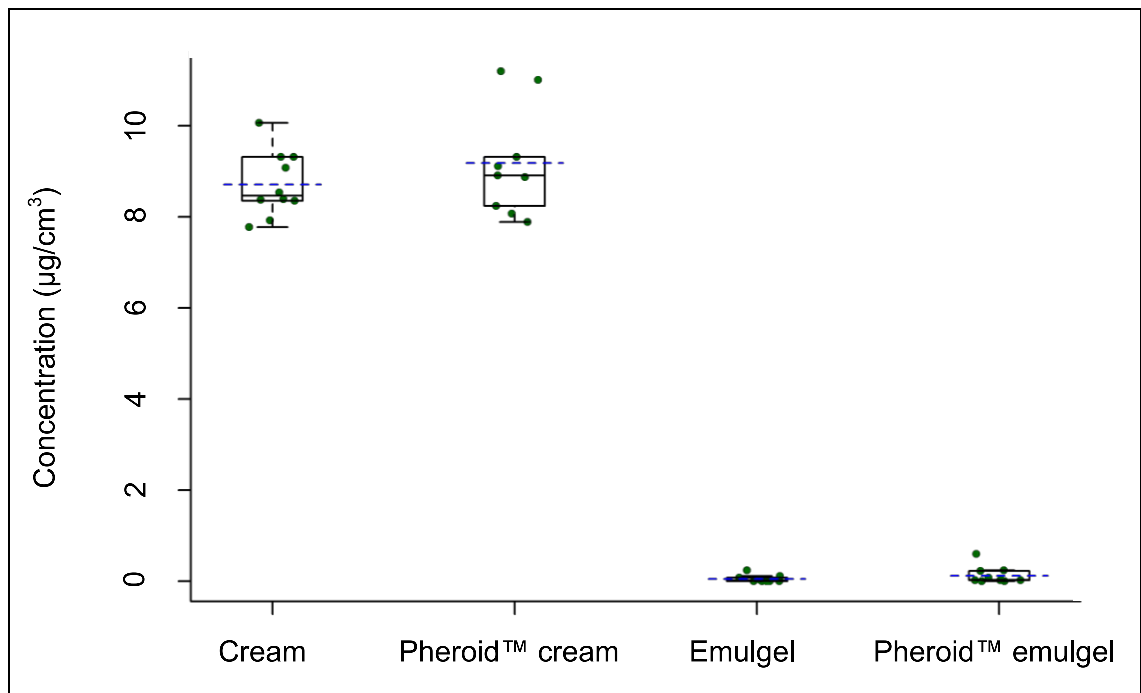

Figure D.3: Graphical representation of zinc concentrations (µg/cm^2) for the four formulations (The blue and black lines represent the average and median values, respectively)**.**

A comparison of the median (centre point of the data) and average concentrations (sum of the data divided by the number of data points) of the diffusion study outcomes showed no significant differences between the cream and Pheroid™ cream formulations. Since there was only a small variation in data points and thus a small amount of outliers among the data, the average concentration, or the median concentration values, could be used.

However, there was a significant difference between the average concentration and the median concentration values of the emulgel and Pheroid™ emulgel formulations. In order to ensure more accurate results, the median values were hence used (Gerber *et al.,* 2008:190).

D.3.3.2.3 Diffusion of zinc from the formulations, compared to the placebos

A comparison of the diffusion concentrations of the zinc from the formulations to those from the placebos, showed that the emulgel and Pheroid™ emulgel formulations, relative to the placebos, delivered insignificant concentrations of zinc transdermally. Contrary, the cream and Pheroid™ cream formulations transferred significant amounts of zinc transdermally. Figure D.4 illustrates the diffused zinc concentrations from the different formulations and placebos.

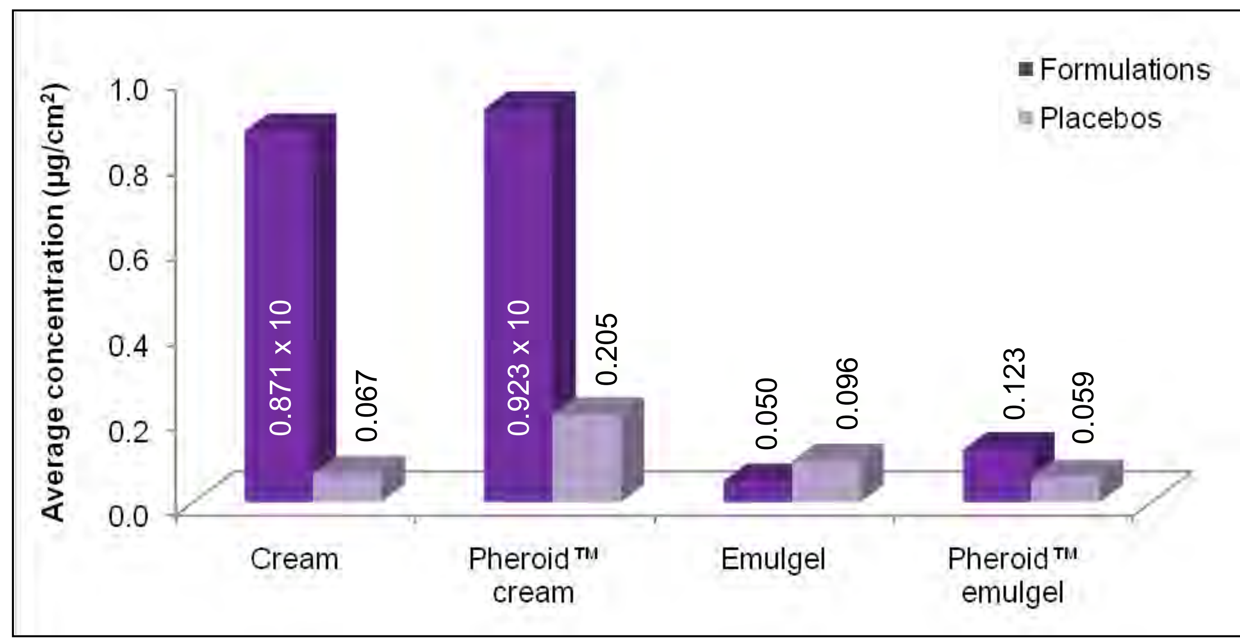

Figure D.4: Diffusion concentrations of zinc for the four formulations, compared to the placebos.

It was concluded that the incorporation of Pheroid™ into the topical formulations positively affected the diffusion potential of the zinc. This was probably due to the presence of the fatty acids associated with Pheroid™, which may have caused the formulations to be more lipophilic. The hydrophilic zinc was thus possibly entrapped into the lipophilic Pheroid™ vesicle, rendering it more permeable through the skin (Grobler *et al.,* 2008:290).

D.3.4 TAPE STRIPPING

In the following sections, stratum corneum-epidermis will be referred to as "epidermis" and the epidermis-dermis will be referred to as "dermis".

D.3.4.1 VITAMIN A CONCENTRATIONS IN THE EPIDERMIS AND DERMIS

D.3.4.1.1 Vitamin A concentrations in the epidermis and dermis being released from the commercial product

Detectable concentrations of vitamin A acetate from the commercial product (cream) were able to penetrate the epidermis and the dermis, although these concentrations were significantly low. The total concentration of vitamin A acetate in the dermis (0.0020 µg/ml) was noticeably higher than the concentration in the epidermis (0.0012 µg/ml). These results were favourable, due to the target area for acne treatment that is in the dermis.

D.3.4.1.2 Vitamin A concentrations in the epidermis and dermis being released from the formulations

Significantly low vitamin A concentrations were released from the four formulated products, as was detected in the epidermis and dermis.

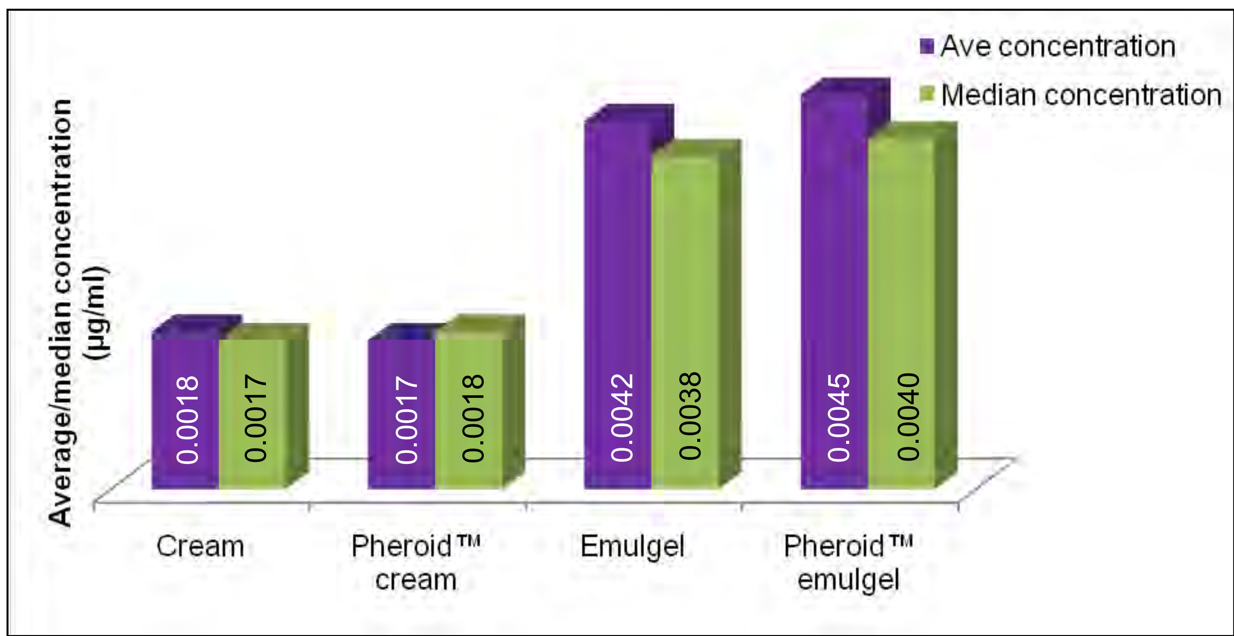

Figure D.5: Average and median concentrations (µg/ml) of vitamin A in the epidermis after 6 h.

The highest average concentration of vitamin A in the epidermis was obtained from the Pheroid™ emulgel (0.0045 µg/ml), followed by the emulgel (0.0042 µg/ml), cream (0.0018 µg/ml) and Pheroid™ cream (0.0017 µg/ml) formulations. These results are illustrated in Figure D.5. A possible explanation for this phenomenon may be the hydration effect on the stratum corneum of the emulgel formulation, which increased epidermal delivery of vitamin A (Bouwstra *et al.*, 2003:22-23). However, no significant differences were observed from a comparison of the Pheroid™ formulations with the non-Pheroid™ formulations.

The average and median concentrations were graphically summarised (Figure D.6) in order to determine which concentration would be a more accurate representation of the true concentration. From Figure D.6 it was evident that the highest median vitamin A concentration in the epidermis was obtained from the Pheroid™ emulgel (0.0040 µg/ml), followed by the emulgel (0.0038 µg/ml), Pheroid™ cream (0.0018 µg/ml) and cream (0.0017 µg/ml) formulations. Since insignificant differences between the average and median concentrations of the cream and Pheroid™ cream were observed, both the median and average concentrations could be used to determine the amount of vitamin A being released from the cream formulations.

However, there was a significant difference between the median and average concentrations of the emulgel and Pheroid™ emulgel. It was determined that the median value would be a more accurate representation of the true concentration, since it considered all the data points and would not be affected by outliers in the data (Gerber *et al.,* 2008:190).

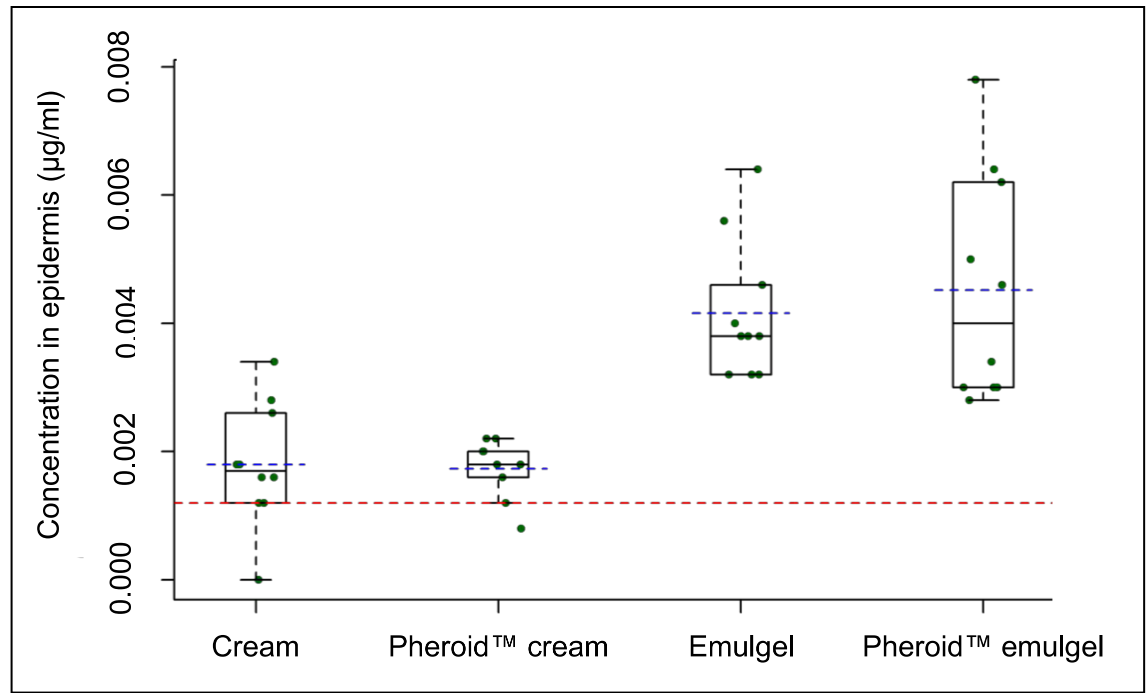

Figure D.6: Graphical representation of vitamin A concentrations (µg/ml) in the epidermis for the four formulations (The blue and black lines represent the average and median values, respectively, whilst the red dotted line represents the concentration of the commercial product)**.**

Degradation products were detected in the emulgel and Pheroid™ emulgel formulations by means of HPLC, but were unquantifiable. The emulgel showed 8.89% of degradation and the Pheroid™ emulgel 17.24%, relative to the amount of vitamin A that had diffused into the epidermis.

Figure D.7 illustrates the average and median vitamin A concentrations being measured in the dermis. From this figure it is evident that the emulgel formulation released the highest average vitamin A concentration to the dermis, followed by the Pheroid™ emulgel. The lowest vitamin A concentrations in the dermis were obtained from both the cream and Pheroid™ cream formulations. Due to the hydrophilic nature of the dermis (Williams, 2003:2), permeation into the dermis of the hydrophilic emulgel was expected to be higher than for the cream formulation, which had a more lipophilic nature. The vitamin A concentration being detected in the dermis with the Pheroid™ emulgel, was slightly lower than that of the emulgel. This may have been attributed to the more lipophilic nature of Pheroid™ ingredients, which may have increased the

lipophilicity of the emulgel formulation and subsequently decreased the delivery of vitamin A into the hydrophilic dermis. The concentration in the dermis showed that the Pheroid™ vesicles did not significantly contribute to the penetration of vitamin A through the skin.

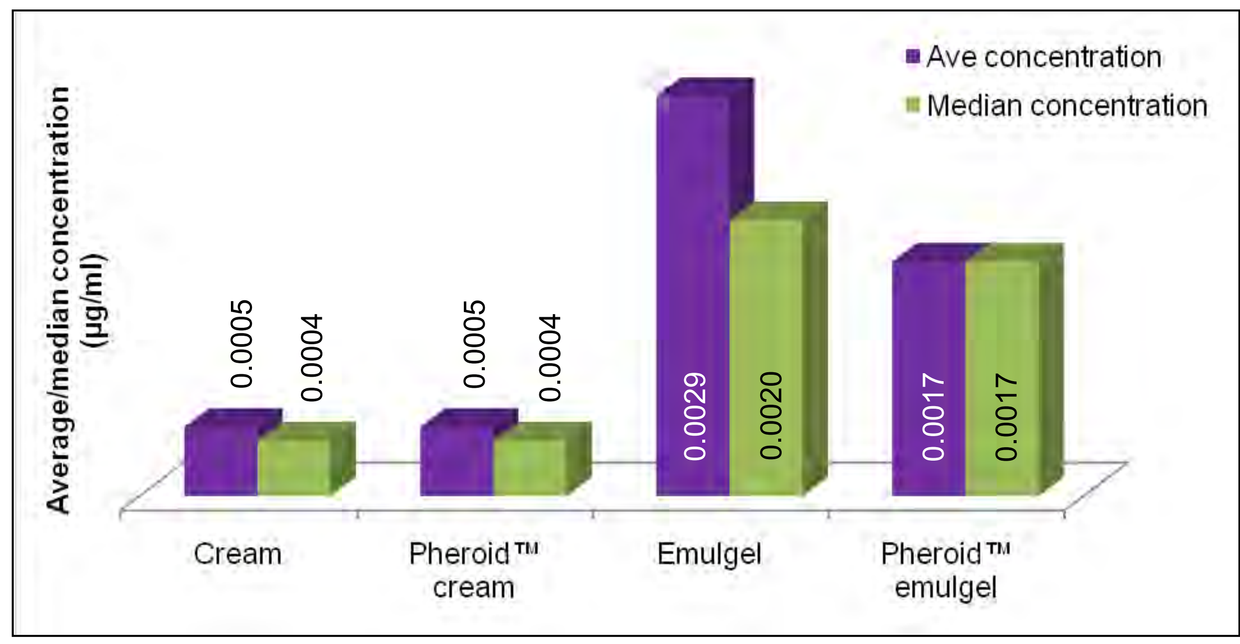

Figure D.7: Average and median concentrations (µg/ml) of vitamin A in the dermis after 6 h.

From Figure D.7 it was clear that the highest average vitamin A concentration in the dermis was obtained from the emulgel (0.0029 µg/ml), followed by Pheroid™ emulgel (0.0017 µg/ml), cream (0.0005 µg/ml) and Pheroid™ cream (0.0005 µg/ml) formulations. Since insignificant differences between the average and median concentrations of the cream, Pheroid™ cream and Pheroid™ emulgel were observed, both the median and average concentrations could be used to determine the vitamin A being released from the cream formulations. Since a significant difference was observed between the median and average concentrations of the emulgel formulation, the median concentration was a more accurate value to use, as it recognised all the data points and would not be affected by outliers in the data (Gerber *et al.,* 2008:190).

Degradation products were detected in the emulgel and Pheroid™ emulgel formulations by means of HPLC, but were unquantifiable. The emulgel showed 8.89% of degradation and the Pheroid™ emulgel 17.65%, relative to the amount of vitamin A that had diffused into the dermis.

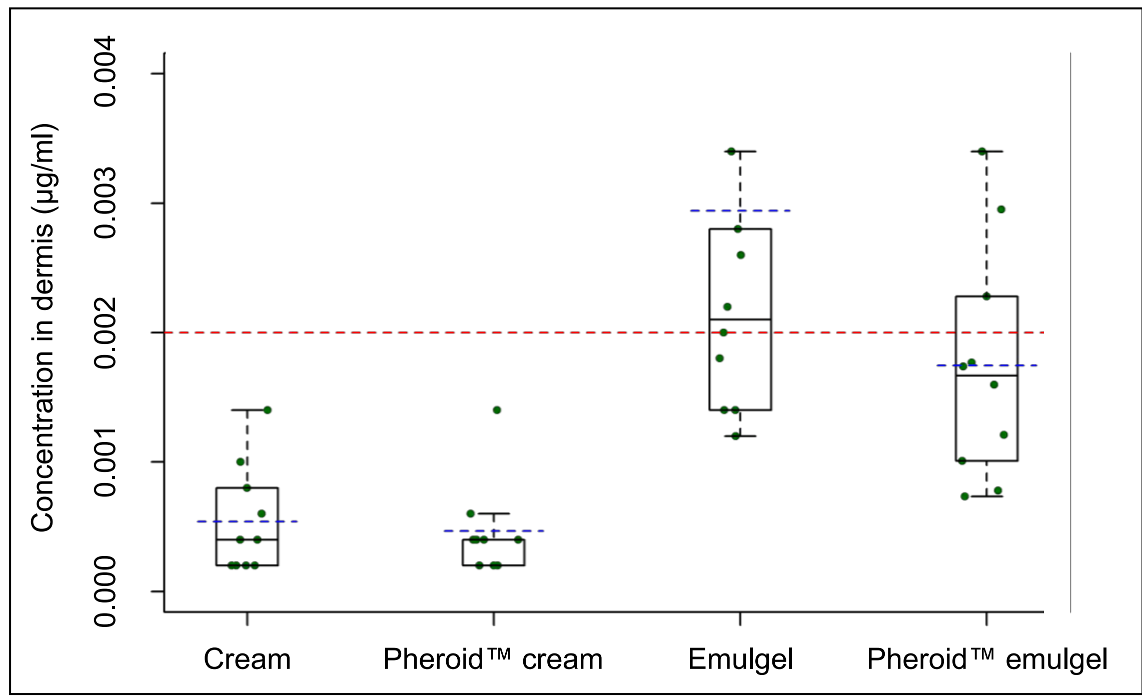

Figure D.8: Graphical representation of vitamin A concentrations (µg/ml) in the dermis for the four formulations (The blue and black lines represent the average and median values, respectively, whilst the red dotted line represents the observed concentration of the commercial product)**.**

As a result, the total concentration of vitamin A in the epidermis was noticeably higher than in the dermis, when the four topical formulations were applied. This may have been due to the hydrophobic nature of vitamin A, causing it not to partition effectively from the lipid rich stratum corneum to the underlying hydrophilic skin layers (Rieger, 1993:49). Due to acne that develops in the sebaceous follicles in the dermis (Webster, 1996:241), it was evident from the test outcomes, that these four formulations reached their intended target site. The reason for the small concentrations may have been due to the small amounts of vitamin A being released by the formulations.

D.3.4.1.3 Comparison of the commercial product to the formulations

Results obtained from the vitamin A concentrations being released into the dermis and epidermis from the commercial product, compared well with the formulated products. The vitamin A concentrations in the epidermis, released from the commercial product (0.0012 µg/ml), was, however, lower, compared to the vitamin A concentrations, released from the formulated products. Contrary, the vitamin A concentrations being released from the commercial product into the dermis (0.0020 µg/ml), was slightly higher than from the formulated Pheroid™ emulgel, cream and Pheroid™ cream. It was not higher than the concentration of vitamin A that was released from the emulgel into the dermis (0.0029 µg/ml), though.

D.3.4.2 ZINC CONCENTRATIONS IN THE EPIDERMIS AND DERMIS

D.3.4.2.1 Placebo formulations to determine the intrinsic zinc concentration in human skin

During the tape stripping experiments on the different placebo formulations, traces of zinc were detected in both the epidermis and dermis. These results indicated the natural presence of zinc in human skin, as was also observed by Hostýnek and Maibach (2002:34).

Tape stripping results revealed that the highest average concentration of zinc in the epidermis was obtained after placebo Pheroid™ cream (0.086 µg/ml) was applied to full thickness skin, followed by the application of placebo emulgel (0.069 µg/ml), placebo cream (0.045 µg/ml) and placebo Pheroid™ emulgel (0.019 µg/ml) formulations. The highest average zinc concentration was detected in the dermis after the application of placebo Pheroid™ cream (0.062 µg/ml) to full thickness skin, followed by the placebo cream (0.039 µg/ml), placebo Pheroid™ emulgel (0.024 µg/ml) and placebo emulgel (0.010 µg/ml) formulations.

D.3.4.2.2 Zinc concentrations in the epidermis and dermis being released from the formulations

Zinc was detected in the subcutaneous layer of the skin, using different formulations. However, the diffused concentrations of zinc in the epidermis and dermis were significantly low. Figure D.9 illustrates the difference between the average and the median concentrations of zinc in the epidermis.

The following rank order for the concentration of zinc being released from the formulations into the epidermis was established: the cream (0.1336 µg/ml) >> Pheroid™ cream (0.0314 µg/ml) >>> Pheroid™ emulgel (0.0009 µg/ml) >> emulgel (0.000 µg/ml). No detectable concentration of zinc diffused from the emulgel formulation into the epidermis, which may have been due to the affinity of the hydrophilic zinc for the aqueous phase of the emulgel (Rieger, 1993:49). Hostýnek and Maibach (2002:35) concluded from their studies that zinc should be delivered transdermally, if it is in its ionised form and formulated in a lipophilic vehicle, for example a cream formulation. It could therefore be concluded that the reason for the relatively high zinc concentration being delivered by the cream formulation during this study, was due to the lipophilicity of the cream formulation. This enhanced the penetration of zinc through the lipid domain of the stratum corneum (Williams, 2003:1). Zinc could also have been delivered from the cream formulation into the epidermis, due to the affinity of the zinc for the hydrophilic surroundings (Rieger, 1993:49).

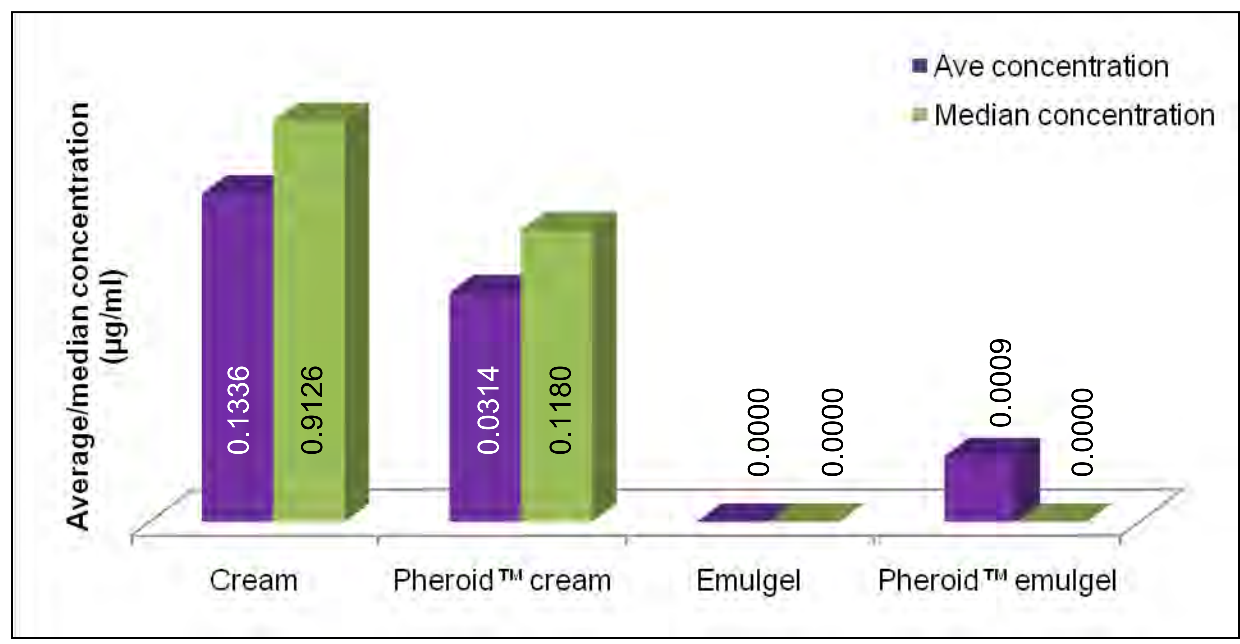

Figure D.9: Average and median concentrations (µg/ml) of zinc in the epidermis after 6 h.

A significant difference was detected between the median and average values of all the formulations, except for the emulgel formulation. This may have been due to the many outliers in the data. Hence, in this case, the median value would be more accurate in determining the concentration (Gerber *et al.,* 2008:190).

From a comparison of the average zinc concentrations being detected in the dermis, the cream formulation (0.0144 µg/ml) depicted the highest average concentration. This average concentration was more than 14 times higher than the zinc concentration delivered to the dermis by the Pheroid™ cream (0.0010 µg/ml) and 48 times higher than that of both the emulgel (0.0003 µg/ml) and Pheroid™ emulgel (0.0003 µg/ml). Zinc concentrations were slightly higher in the dermis than in the epidermis for the emulgel and Pheroid™ emulgel formulations. Contrary, zinc concentrations were higher in the epidermis than in the dermis for the cream and Pheroid™ cream formulations, which may possibly have contributed to the higher concentrations of the cream formulations in the dermis.

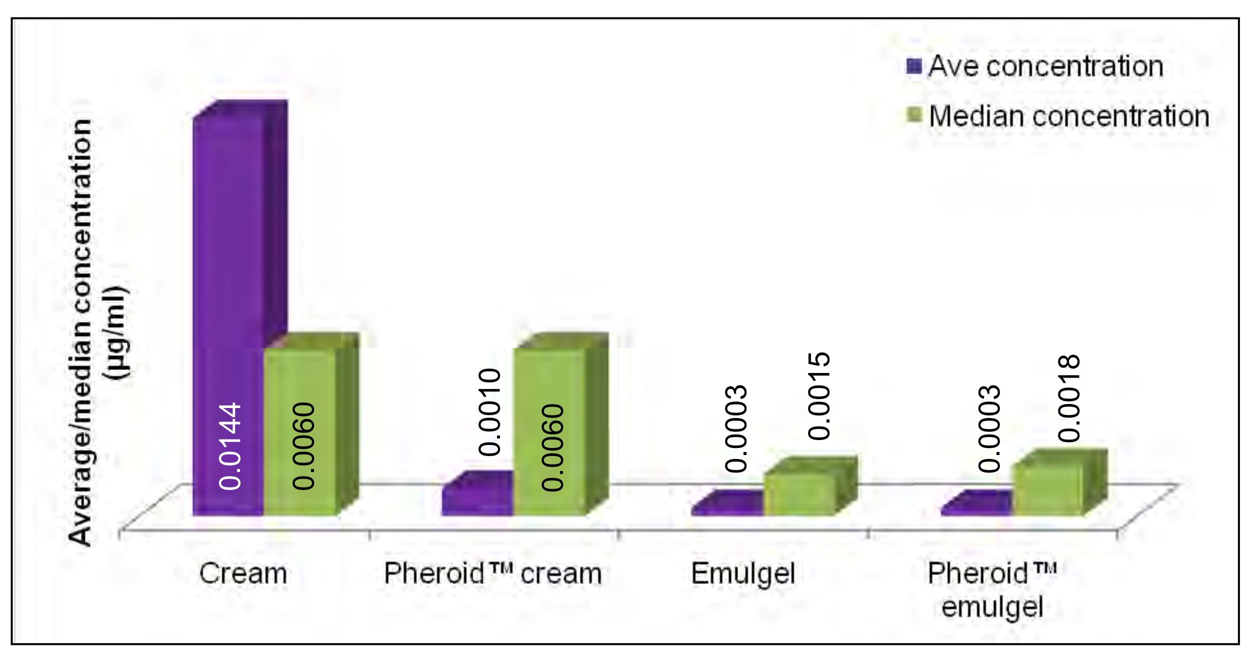

Figure D.10: Average and median concentrations (µg/ml) of zinc in the dermis after 6 h.

A significant difference was depicted between the median and average values of all the formulations (as could be seen in Figure D.10). This might have been due to the many outliers in the data. The median value will thus be more accurate to determine concentration (Gerber *et al.,* 2008:190).

D.3.4.2.3 Formulations, compared to placebos

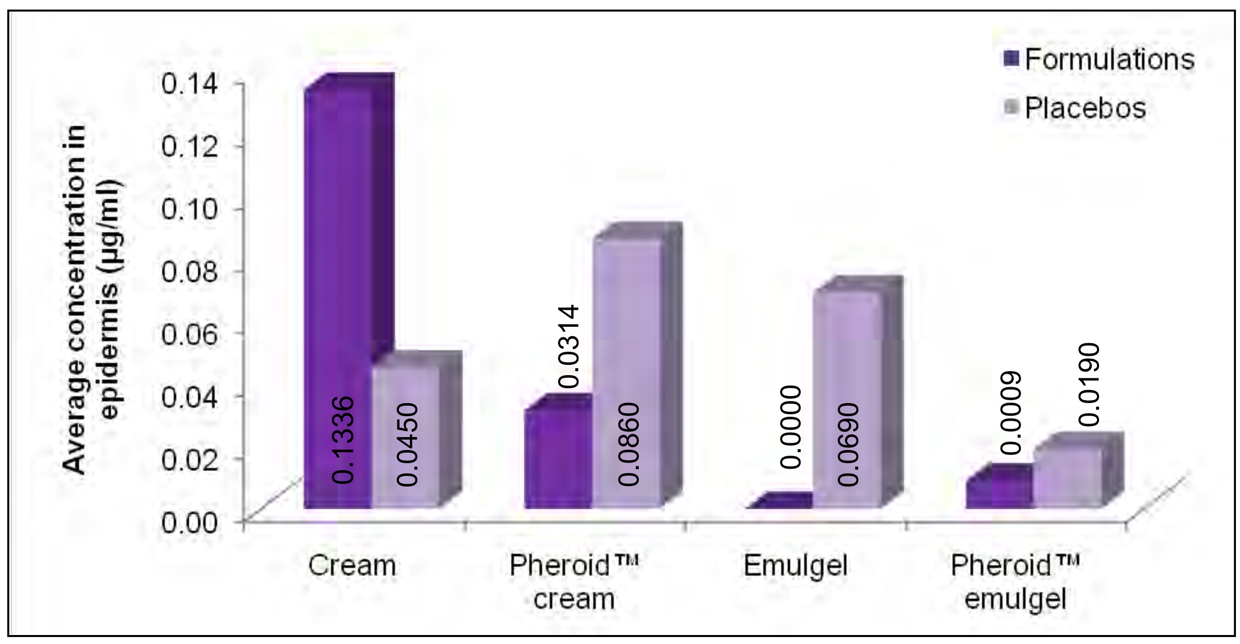

Figure D.11: Concentrations (µg/ml) of zinc in the epidermis from the four formulations, compared to the placebos.

Figure D.11 illustrates the average zinc concentrations being measured in the epidermis, after application of the four formulations and four placebo formulations on full thickness skin. Figure D.11 clearly shows that zinc concentrations in the epidermis were significantly higher when the placebo Pheroid™ cream, placebo emulgel and placebo Pheroid™ emulgel were applied. It could therefore be assumed that zinc concentrations, detected in the epidermis after individual application of the Pheroid™ cream, emulgel and Pheroid™ emulgel formulations, was not zinc that had diffused from these formulations into the epidermis, but rather represented endogenous zinc, normally present in the skin (Hostýnek & Maibach, 2002:34). However, from a comparison of the zinc concentrations in the epidermis, when the cream or placebo cream was applied, it was found that the possibility existed that zinc did indeed diffuse from the cream formulation into the epidermis.

Average zinc concentrations being measured in the dermis, after application of the four formulations and placebo formulations on full thickness skin, are illustrated in Figure D.12. Again, from this figure, it was apparent that zinc concentrations in the dermis were significantly higher when the placebo formulations were applied. The zinc concentration, as depicted in the dermis after application of the cream formulation, was significantly lower than when the placebo cream was applied. Thus, it could be assumed that the zinc concentrations present in the dermis after individual application of the four formulations containing zinc, was not zinc that had diffused from these formulations into the dermis, but rather the intrinsic zinc, normally present in the skin (Hostýnek & Maibach, 2002:34).

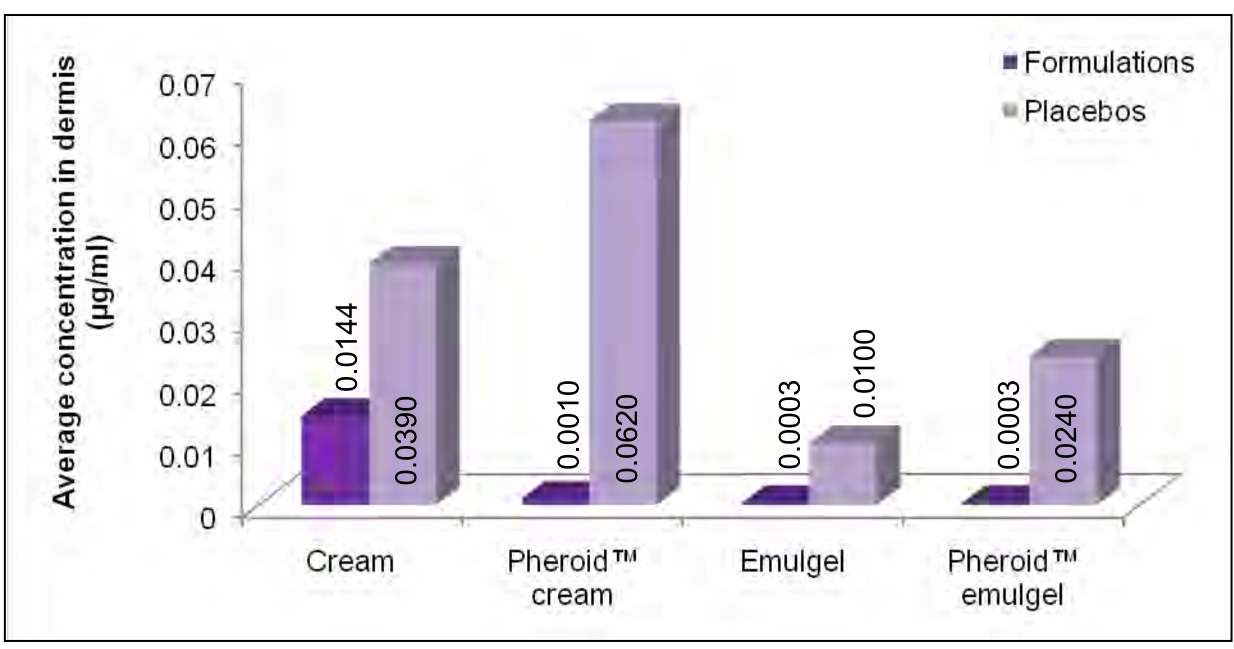

Figure D.12: Concentrations (µg/ml) of zinc in the dermis from the four formulations, compared to the placebos.

D.3.5 STATISTICAL RELATIONS

D.3.5.1 DIFFUSION CONCENTRATIONS OF VITAMIN A

Vitamin A was unable to diffuse through the skin; therefore no statistical data was available for the transdermal delivery of vitamin A.

D.3.5.2 VITAMIN A CONCENTRATIONS IN THE EPIDERMIS AND DERMIS

According to the BDM test, no significant relationship existed between the application of Pheroid™ in the topical formulations and the concentration of vitamin A in the epidermis (p-value = 0.92), nor dermis (p-value = 0.44). Similarly, the Mann-Whitney test further revealed that Pheroid™ did not have any statistically significant effect on the delivery of vitamin A to the epidermis (p-value = 0.93), nor dermis (p-value = 0.56). However, the cream and emulgel formulations showed a statistically significant effect on vitamin A concentrations in the epidermis and dermis, both having a p-value of 0.

D.3.5.3 DIFFUSION CONCENTRATIONS OF ZINC

The BDM test did not show any significant relationship (p-value = 0.84) between the application of Pheroid™ in the formulations and the zinc concentrations. Similarly, the Mann-Whitney test revealed that the Pheroid™ did not have any statistically significant effect on the delivery of zinc (p-value = 0.99). However, the cream and emulgel formulations depicted a statistically significant effect (p-value = 0) on zinc concentrations.

D.3.5.4 ZINC CONCENTRATIONS IN THE EPIDERMIS AND DERMIS

According to the BDM test, there was a significant relationship between the application of Pheroid™ in formulations and the zinc concentration in the epidermis (p-value = 4.41×10^{-5}), whilst an insignificant relationship existed with regards to zinc in the dermis (p-value = 0.78).

Since a positive relationship was observed, the effect of Pheroid™ on the zinc concentration was tested for both the cream and emulgel formulations. The effect of the cream and emulgel formulations on zinc concentrations was also determined for both the Pheroid™ and non-Pheroid™ formulations. Using the Mann-Whitney test, the results are summarised as follows:

- Effect of Pheroid™ on zinc concentrations in cream and emulgel formulations:
  - A p-value of 0.000 indicated that a statistically significant effect was observed for the cream formulation.
  - For the emulgel, no significant difference was observed (p-value = 0.087).

- Effect of cream and emulgel formulations on zinc concentrations in Pheroid™ and non-Pheroid™ formulations:

  On both levels of Pheroid™ application, a statistically significant difference in the median concentration was observed. The p-values was 0.000 and 0.002 for the Pheroid™ and non-Pheroid™, respectively. However, a more significant difference between zinc concentrations was observed when no Pheroid™ was applied.

Considering the dermis, the Mann-Whitney test revealed that the application of Pheroid™ did not have any statistically significant effect on the delivery of zinc to the dermis (p-value = 0.99). However, the cream and emulgel formulations depicted a statistically significant effect on zinc concentrations, with a p-value of 0.000 in the dermis. However, in the latter case, a more focused study would be required to investigate the repeatability of the observed distributions.

D.4 PREVIOUS STUDIES

Due to the lack of information on vitamin A acetate in literature, the results from this study were compared to another vitamin A derivative, namely vitamin A palmitate. Both the acetate and palmitate derivatives are classified as vitamin A esters. Vitamin A and its derivatives consist of similar chemical structures, therefore biological properties on the skin would be comparable (Cheng & Depetris, 1998:6).

Previous studies indicated that vitamin A palmitate (20 µg/cm^2) in an acetone vehicle showed a 0.2% penetration through excised human skin after 24 h (Boehnlein *et al.*, 1994:1155-1159). Abdulmajed and Heard (2004:119) also conducted a study on full thickness human skin, using the Franz diffusion cell method. They made use of a donor phase composed of vitamin A palmitate (100 µl of 2.5 mM) situated in a methanol:PBS (50:50) vehicle at pH 4.8. A flux value of 500 ng.cm^{-2}.h^{-1} was obtained after 4 h (Abdulmajed & Heard, 2004:119). When considering their results, vitamin A acetate should be more likely to penetrate the skin, than for example vitamin A palmitate, as vitamin A acetate has the smallest molecular weight of all of the vitamin A esters (Cheng & Depetris, 2010:7). Therefore, the reason that no transdermal diffusion results could be obtained with vitamin A acetate during this study, may have been due to incompatible vehicles or penetration enhancers being employed.

Vitamin A concentration results obtained from the tape strip studies during this study, compared well with a study performed by Yan *et al.* (2006:189). The study was performed on female hairless mice, using 0.5% and 2.0% oil in water (o/w) cream, containing vitamin A palmitate. Vitamin A concentrations were significantly higher in the epidermis than in the dermis. For the 2.0% cream, concentrations of 120.49 ±5.09 ng/ml (epidermis) and 10.28 ±0.48 ng/ml (dermis) were obtained. The 0.5% cream delivered 24.57 ±0.47 ng/ml in the epidermis and

2.15 ±0.12 ng/ml in the dermis. Therefore, it was clear that the 2.0% cream showed significantly higher results than the cream containing only 0.5% of vitamin A palmitate. When those outcomes were compared to this study outcomes, it was found that higher vitamin A concentrations were obtained in both the epidermis and dermis when vitamin A acetate was employed.

Due to the lack of information from previous studies on zinc acetate, results from this study were compared with results being obtained from studies that had been conducted on the transdermal delivery of zinc oxide. Cross *et al.*, (2007:148-154) incorporated zinc oxide into an o/w emulsion. PBS (pH 7.4) was used as the receptor phase. They found that 0.09 ±0.04 $\mu g/cm^2$ of zinc oxide had diffused from nanoparticles over a 24-h period. Cross *et al.,* (2007:148-154) also ascribed their results to the endogenous zinc being released from the epidermal reservoir. These results compared well with the diffusion studies conducted on zinc acetate during this study.

D.5 CONCLUSION

The aim of this study was to deliver vitamin A and zinc to the dermis, where therapeutic action is required for the treatment of acne.

Membrane release studies were conducted on a newly formulated topical cream, Pheroid™ cream, emulgel and Pheroid™ emulgel. Vitamin A and zinc were both released from these formulations, although the released concentrations were significantly low.

Vitamin A in the formulated products, as well as in a commercial product being tested, could not be delivered transdermally, as had been expected, due to the high lipophilicity of the compound, preventing diffusion from the skin into the Franz cell receptor, thus remaining in the epidermis-dermis (Jenning *et al*., 2000:214).

Diffusion into the different layers of the skin was further examined. Results proved that vitamin A diffused into the epidermal and dermal layers of human skin. The Pheroid™ emulgel delivered the highest average concentration of vitamin A to the epidermis, followed by the emulgel. Contrary to these results, the average vitamin A concentration being released from the emulgel into the dermis was the highest, followed by that of the Pheroid™ emulgel. Overall, the Pheroid™ and non-Pheroid™ emulgel formulations delivered vitamin A more readily into the dermis and epidermis. A possible explanation for these results was the hydrophilic nature of the underlying layers of the skin, which caused enhanced permeation of the hydrophilic emulgel. The emulgel formulations might also have hydrated the stratum corneum to a higher extent than the creams, causing it to be more permeable.

The commercial product, containing vitamin A, produced lower concentrations of the API in the epidermis than the four new formulations. Concentrations measured in the dermis, showed that the average vitamin A concentration released from the commercial product was within the concentration range delivered by the formulations. However, the emulgel without Pheroid™ delivered approximately 31% more vitamin A to the dermis, than the commercial product.

Experimentally, it was possible to deliver zinc transdermally, although no flux values were obtained. In order to determine whether zinc was actually released from the various formulations and whether it was transdermally delivered, diffusion studies with placebo formulations were conducted. It was found that the emulgel and Pheroid™ emulgel formulations were unable to deliver significant zinc concentrations transdermally. However, transdermal diffusion of zinc from both the cream and Pheroid™ cream formulations were attained. The lipophilic nature of these cream formulations may have enhanced diffusion through the lipid domain of the stratum corneum, which resulted in higher diffusion concentrations of zinc through the skin. Incorporation of the Pheroid™ technology might have achieved a positive effect on the penetration of zinc through the skin. Although zinc reached the systemic circulation, it was in sub-therapeutic concentrations that could not produce any effects of significant concern. The lethal dose for zinc salts administered subcutaneously, is 330 mg/kg (Hayes & Martin, 1994).

Zinc was delivered to the upper layers of the skin. Again, in order to determine whether zinc was actually released from the various formulations and delivered to the epidermis and dermis, tape strip studies with placebo formulations were conducted. It was observed that zinc concentrations in the epidermis and dermis were significantly higher when the placebo formulations were applied to full thickness skin, compared to when three of the new formulations were applied, with the exception of the cream formulation. The conclusion could therefore be drawn that no zinc diffused into the epidermis and dermis from the new formulations, except from the cream. It was rather the endogenous zinc that is naturally present in the epidermis and dermis, which was measured. The cream formulation, however, may have delivered zinc to the epidermis. Again, the reason may have been due to the lipophilicity of the cream formulation, which enhanced penetration through the lipid domain of the stratum corneum (Williams, 2003:1). Consequently, the penetration of zinc into the epidermis was enhanced, due to the affinity of zinc for the hydrophilic environment (Rieger, 1993:49).

Acne develops from the sebaceous follicles in the dermis. It was thus proven that vitamin A that was released from all four formulations, reached its target site of treatment. Zinc, however, did not reach the dermis and therefore its target site (Webster 1996:241).

Based on the results from this study, if transdermal zinc delivery is required, the Pheroid^{TM} cream as delivery system is recommend. Emulgel formulations would significantly enhance delivery of vitamin A into the dermis and the cream formulation would effectively deliver zinc to the dermis.

It was concluded that it would, therefore, be very difficult to formulate a product containing both vitamin A and zinc, which would be able to effectively deliver both active ingredients to their specific target areas, the dermis.

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