Formulation, *in-vitro* release and transdermal diffusion of Alpha-Lipoic Acid

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ABSTRACT

Acne is a common disease characterised by follicular hyperkeratinisation, bacterial hipercolonisation as well as immune reactions and inflammation. In acne, reactive oxygen species (ROS) may be released from the damaged follicular walls, which could cause the advancement of inflammation in the pathogenesis of the disease. The topical application of antioxidants is a promising approach to support the endogenous antioxidant defence and avoid oxidative injury that may lead to acne.

The skin provides a painless and patient-friendly approach for systemic drug administration. Transdermal drug delivery not only improves patient compliance, but also avoids the first-pass effect. The major hurdle to penetration of matter through the skin is provided by an outward layer of the skin, the stratum corneurm (SC). Overcoming this barrier safely and reversibly is a fundamental problem in the field of transdermal drug delivery.

Alpha-lipoic acid was utilised as the cosmeceutical active and can be classified in a mixed category of compounds that lie between cosmetics and drugs. Alpha-lipoic acid and its reduced form, dihydrolipoic acid, have been described as the "universal antioxidants" because of their capacity to quench a number of free radicals in both aqueous and lipid environments, their metal-chelating properties and ability to restore other antioxidants from their inactive form.

The Pheriod™ system is a new manner of drug delivery aimed at overcoming the barrier function of the skin. It consists of vesicular structures, the sizes of which vary from 200-440 nm. These vesicles, prepared from customised essential fatty acids, were found to advance the efficacy of topically administered compounds. The aim of this study was to determine whether the Pheroid™ delivery system would enhance the transdermal delivery of formulations containing alpha-lipoic acid to the target site by performing Franz cell diffusion studies over a 12 hour period, followed by tape-stripping experiments. The results of the formulations containing Pheroid™ were compared to those of the formulations without Pheroid™.

Experimental determination of transdermal flux of the alpha-lipoic acid formulations revealed that Pheroid™ improved the transdermal delivery of alpha-lipoic acid. The average flux of Pheroid™ cream from 0 to 2 hours wass 58.01 ± 6.63 μg/cm^2.h. The average flux of Pheroid™ gel from 4 to 12 hours was 22.18 ± 3.33 μg/cm^2.h. Tape-stripping experiments proved that the concentrations of alpha-lipoic acid in Pheroid™ cream and cream that remained in the epidermis after application to

the skin were 569.10 µg/ml and 764.93 µg/ml respectively. The concentrations of alpha-lipoic acid in Pheroid gel and gel that diffused into the dermis were 23.62 µg/ml and 61.06 µg/ml respectively.

Aqueous solubility and log D partition coefficient of alpha-lipoic acid were determined. Inspection of the log D value of -0.78 indicated that the compound was unfavourable to penetrate the skin, whereas the aqueous solubility of 8.602 mg/ml in PBS at a temperature of 32 °C indicated favourable penetration.

Keywords : Alpha-lipoic acid, acne, transdermal diffusion, Pheroid™, formulation.

OPSOMMING

Aknee is 'n algemene siektetoestand wat gekenmerk word deur follikulêre hiperkeratinisasie, bakteriële hiperkolonisasie asook imuunreaksies en inflammasie. Reaktiewe suurstof spesies (ROS) kan moontlik vrygestel word uit beskadigde follikulêre wande tydens aknee. Hierdie proses kan moontlik aanleiding gee tot die bevordering van inflammasie in die patogenese van die toestand. Die topikale aanwending van anti-oksidante is 'n belowende benadering om die endogene anti-oksidantverdediging te ondersteun en oksidatiewe skade wat tot aknee mag lei, te vermy.

Die vel bied 'n pynlose en pasiëntvriendelike benadering vir sistemiese geneesmiddelaanwending. Transdermale geneesmiddelaflewering verhoog nie net meewerkendheid van die pasiënt nie, maar vermy ook die eerste deurgangseffek. Die belangrikste struikelblok vir penetrasie van stowwe deur die vel is die buitenste laag van die vel, die stratum corneum (SC). Om hierdie struikelblok veilig en omkeerbaar te oorkom, is 'n fundamentele probleem in transdermale geneesmiddelaflewering.

Alfa-lipoësuur is as die kosmeseutiese aktiewe bestanddeel gebruik, omdat dit geklassifiseer kan word in 'n gemengde kategorie van verbindings wat tussen kosmetika en geneesmiddels lê. Alfa-lipoësuur en die gereduseerde vorm daarvan, dihidrolipoësuur, is beskryf as die "universele antioksidante" weens hul metaal-chelerings eienskappe en hul kapasiteit om vrye radikale in beide waterige en vetterige omgewings te reduseer. Hul beskik ook oor die vermoë om ander antioksidante vanaf hul onaktiewe vorme te herstel.

Die Pheroid™ stelsel is 'n nuwe manier van geneesmiddelaflewering wat gemik is op die verbetering van die hindernis wat die vel vir transdermale geneesmiddelaflewering bied. Dit bestaan uit vesikulêre strukture waarvan die grootte wissel tussen 200-400 nm. Bevindings toon dat hierdie vesikels, wat voorberei is uit gemodifiseerde essensiële vetsure, die doeltreffendheid van topikaal aangewende verbindings verbeter. Die doel van hierdie studie was om te bepaal of die Pheroid™ afleweringsisteem die transdermale aflewering van topikaal aangewende formulerings sal verbeter deur Franz-sel diffusiestudies oor 'n tydperk van 12 ure uit te voer, gevolg deur kleefbandafstropingseksperimente. Die resultate van formulerings wat Pheroid™ bevat, is vergelyk met die formulerings daarsonder.

Die eksperimentele bepaling van transdermale fluks van die alfa-lipoësuurformulerings het getoon dat Pheroid™ die transdermale aflewering van alfa-lipoësuur verbeter. Die gemiddelde fluks van

Pheroid™ room van 0 tot 2 ure was 58.01 ± 6.63 µg/cm^2.h. Die gemiddelde fluks van Pheroid™ jel van 4 tot 12 ure was 22.18 ± 3.33 µg/cm^2.h. Kleefbandafstropingseksperimente het *getoon dat die* konsentrasies van alfa-lipoësuur in Pheroid™ room en room wat in die epidermis agtergebly het na aanwending tot die vel 569.10 µg/ml en 764.93 µg/ml, onderskeidelik was. Die konsentrasies van alfa-lipoësuur in Pheroid™ jel en jel wat tot in die dermis gediffundeer het, was 23.62 µg/ml en 61.06 µg/ml, onderskeidelik.

Alfa-lipoësuur se wateroplosbaarheid en log D verdelingskoëffisiënt is bepaal. Die log D waarde van -0.78 toon dat die verbinding nie gunstig was om die vel te penetreer nie, terwyl die wateroplosbaarheidswaarde van 8.60 mg/ml in PBS by 'n temperatuur van 32 °C gunstige *penetrasie aangedui het.*

Sleutelwoorde: Alfa-lipoësuur, aknee, transdermale diffusie, Pheroid™, formulering.

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ABBREVIATIONS

$(CH_3)_2CHOH$	Isopropyl alcohol
ALA	*Alpha-lipoic acid*
BHA	Butylated hydroxyan–isole
BHT	Butylated hydroxyl toluene
CH_3CN	Acetonitrile
CLSM	Confocal laser scanning microscopy
DHLA	Dihydro lipoic acid
DMSO	Dimethylsulphoxide
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
H_3PO_4	Orhtophosporic acid
HPLC	High pressure liquid chromatography
KH_2PO_4	*Potassium dihydrogen phosphate*
LH	Lutenising hormone.
Log D	Octanol-PBS partition coefficient
Log P	Octanol-water partition coefficient
MeOH	Methanol
NF-κB	Nuclear factor kappa B
PBS	Phosphate buffered solution

PG	Propylene glycol
RAR	Retinoic acid receptors
ROS	Reactive oxygen species
SB	Stratum basale
SC	Stratum corneum
SG	Stratum granulosum
SHBG	Sex hormone-binding globulin
SHBG	Sex hormone binding globulin
SS	Stratum spinosum
TFα	Tumor necrosis factor alpha

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

The skin, the largest organ of the human body, provides a painless and patient-friendly crossing point for systemic drug administration. In addition to providing a leading edge over injections and oral routes by increasing patient compliance and avoiding first pass metabolism, respectively, the transdermal route provides continuous and controlled delivery (Mitragotri, 2004:555).

Although the skin is one of the major sites for non-invasive delivery of remedial agents into the body, this task can be fairly testing owing to the impermeability of the skin (Foldvari, 2000:417). Being the outermost layer of the human organism, separating the internal from the external environment, the skin acts as a two-way barrier, i.e., preventing the entrance of unfamiliar molecules and the egress of endogenous substances. The major hurdle to penetration of matter through the skin is provided by an outward layer of the skin, the stratum corneurm (SC) and its dense structure. The stratum corneum has a water permeability around 1000 times lower than most other biological membranes, which has been ascribed to the unique lipid composition end content of the stratum corneum and, especially, the outstanding structural arrangement of the intercellular lipid matrix and the lipid envelope surrounding the corneocytes (Suhonen *et al.*, 1999:149).

The penetration and permeation of active ingredients into the skin is crucial in the management of certain skin conditions or to achieve systemic beneficial effects. Two routes of delivery are distinguished, i.e. dermal and transdermal delivery. In the case of transdermal delivery, the active ingredient permeates through the skin into deeper tissues (muscle pain or anti-inflammatory effects) and/or into the systemic circulation (hypertension, pain, sickness, postmenopausal or withdrawal symptoms) to carry out its pharmacological outcome. Contrary to transdermal delivery, dermal (topical) delivery is aimed at the skin and minimises the transfer through the skin to support the local treatment of skin diseases (Williams, 2003:2).

There are numerous steps between a molecule's first application to the skin surface until it appears in the systemic circulation, and this complicates the permeation process. Typically, the drug is applied to the skin in a vehicle. The vehicle may be straightforward such as an aqueous solution, or it may be more complex, such as one with an emulsion. The physicochemical properties of the molecules neighbouring the stratum corneum will establish their partitioning into the membrane (Williams 2003:28).

The Pheriod™ system is a new manner of drug delivery aimed at overcoming the barrier function of the skin. It consists of vesicular structures the sizes of which vary from 200 - 440 nm. These vesicles, prepared from customised essential fatty acids, were found to advance the efficacy of topically administered compounds (Grobler et al., 2008:285.)

In the past 25 years, various topical and systemic drugs have been developed for the treatment of *acne vulgaris*. Acne, an ailment of the pilosebaceous units, is a familiar disease among adults and adolescents. It affects almost 80% of adolescents and young adults, and is characterised by irregular follicular differentiation, increased sebum production and inflammation (Gollnick et al., 2003:S1). Although precise mechanisms are unclear, common pathways to acne include excess sebum production, hyperkeratinisation of the hair follicle, oxidative stress and the release of inflammatory mediators.

In acne, reactive oxygen species (ROS) may be released from the damaged follicular walls, which could cause the advancement of inflammation in the pathogenesis of the disease (Arican *et al.*, 2005:380). ROS may oxidise lipids and proteins, causing oxidised products such as lipid hydroperoxidases to form (Podda *et al.*,1996:627). According to recent studies, oxidative stress is present both locally and systemically in acne patients. These patients have reduced levels of antioxidant enzymes such as super oxide dismutase and glutathione peroxidase. The studies also suggest a connection between the systemic levels of vitamin A and E and the severity of the acne (Katzman & Logan, 2007:1082).

Topical application of antioxidants to the skin is a promising approach to support the endogenous antioxidant defense and avoid oxidative injury that may lead to acne. An ideal antioxidant for topical application should have the following properties: high antioxidant activity; must penetrate the skin; be present in its active form in the skin and protect the skin against oxidative damage (Podda *et al.*, 1996:627).

Alpha-lipoic acid, and its reduced form, dihydrolipoic acid, have been described as the "universal antioxidants" because of their capacity to quench a number of free radicals in both aqueous and lipid environments, metal-chelating properties and ability to restore other antioxidants from their inactive form (Packer et al., 1995:228). Enzymes such as reduced glutathione and lipoamide dehydrogenase reduce α-lipoic acid to Dihydrolipoic acid (DHLA), which is a stronger antioxidant. DHLA may help to safeguard intracellular and extracellular vitamin C by reducing both the ascorbate free radical and dehydroascorbic acid to ascorbate. By doing so, it helps the

extracellular dehydroascorbic acid to protect cells against oxidative stress and enhances the ability to generate nitric oxide (Jones et al.,2002:83).

This study is aimed at the following:

- Formulation and stability testing of formulations containing Alpha-Lipoic Acid (ALA)
- Investigation of the possibility of a simple transdermal delivery system for ALA formulations with the aid of Pheroid™ technology.
- Experimental determination of transdermal flux of the ALA formulations.
- Experimental determination of delivery of ALA to the target site (epidermis and dermis) by means of tape stripping.
- Experimentally determining the aqueous solubility and partition coefficient (log D) of ALA.
- Developing and validating a high performance liquid chromatography (HPLC) method to quantitatively determine ALA.

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CHAPTER 2: ALPHA-LIPOIC ACID IN THE TREATMENT OF ACNE

2.1 ACNE

Acne vulgaris is an extremely common ailment which can be found in nearly all teenagers to some extent. Women in their 30's are also sometimes affected. Regardless of severity, acne frequently has a larger psychological than cutaneous effect (Webster, 2001:15). Even patients with mild to moderate acne have a higher incidence of suicidal ideation, comparable to that among patients with far more chronic and disfiguring dermatological problems. Other psychological scars include lowered self-esteem and professional expectations, social reticence, depression and anxiety. Furthermore, severe acne has been connected with decreased employability in adulthood. Therefore, it must be recognised as a serious disorder (Gollnick, 2003:1580).

2.1.1 PATHOGENESIS OF *ACNE VULGARIS*

The pathogenesis of acne is multifunctional, involving androgenic stimulation, sebaceous hyper secretion, follicular obstruction, *Propionibacterium acnes* and inflammatory mediators (Tan *et al.*, 2001:442). Genetic and hormonal factors also have a role (Layton, 2005:44). The central defect involves the formation of the comedo, a plug in the follicle that results from aberrant desquamation of the follicular wall. The cause of comedo formation is not known. Clinically, comedones are described as "open" if the pore is visible and "closed," if it is not. The black tip of an open comedo results from the oxidation of sebaceous lipid and melanin. In many patients acne remains for the most part in its first stage; in others it progresses to inflammatory lesions of varying sensitivity (Webster, 2001:15).

Grading the severity of acne as mild, moderate or severe is a useful initial assessment. Mild disease comprises open and closed comedones with sparse inflammatory lesions (Layton, 2005:44). Open comedones (blackheads) are filled with desquamated keratinous cells and serum, and have a dilated orifice. They manifest as flat or slightly raised lesions ranging from 1 – 5 mm in diameter, which can resolve spontaneously or develop into inflammatory acne lesions. As sebum accumulates following follicular blockage, a closed comedone (whitehead) appears. Closed comedones are firm, pale, 1 – 2 mm in diameter and slightly elevated, lying just beneath the skin surface (Gollnick, 2003:1581). In moderate acne, papules and pustules are more numerous (Layton, 2005:44). Pustules are raised white lesions filled with pus and are caused by the rupture

of the follicular sac into the adjacent tissue as a result of the closed comedone becoming distended. Pustules generally resolve within days with no scarring (unless traumatised) because of their superficial location. Papules (≤ 5 mm) represent a deeper dermal inflammatory reaction and appear as erythematous, raised solid lesions. As a consequence, they take longer to resolve and often do so with scarring (Gollnick, 2003:1582). Severe acne comprises extensive lesions, and may include nodules and scarring (Layton, 2005:44). Small nodules (5 – 10 mm), nodules (> 10 mm) or pseudocysts represent the most severe form of acne and are large, deep-seated abscesses that may be fluctuant when palpated (Gollnick, 2003:1582).

The sequence of events involved in acne lesion initiation, augmentation, and resolution have eluded acne researchers. It was thought that ductal keratinocytes, hyperproliferation and abnormal differentiation first give rise to the formation of a micro-comedone. Then, developments from the micro-comedone could take 1 of 2 routes: either further hyperproliferation causing accumulation of corneocytes and sebum in the follicle lumen, forming a "plug" and becoming a clinical comedone (open or closed), or inflammation caused by diffusion of soluble components from the follicle lumen into the dermis to form an inflamed lesion (papule or pustule).

Inflammation was viewed as a secondary "complication." However, the examination of both non-inflamed and inflamed lesions has led to the conclusion that these are 2 separate disorders affecting the same type of follicle, especially considering that many inflamed lesions do not present with a micro-comedone within their histological features (Holland & Jeremy, 2005:79).

2.1.1.1 THE ROLE OF SEBUM AND SEBORRHOEA

Sebum is a mixture of relatively non-polar lipids, most of which are synthesised *de novo* by the sebaceous gland for the purpose of heat insulation (Zouboulis, 2004:360). Increased sebum production and follicular epithelial cell development and abnormal desquamation play key roles in acne pathogenesis. Sebum production is controlled by androgens, mainly testosterone. Testosterone is converted to the more active 5α-dihydrotestosterone (5α-DHT) by the enzyme type I 5α-reductase. This more active androgen then stimulates increased sebum production.

The onset of acne often corresponds with increased androgen production at puberty, leading to increased sebum production. Although seborrhoea is more intense in individuals who are acne prone than in those who are free of acne, only some hyper androgenic patients present with acne virilisation. This suggests that increased sebum production may stimulate or exacerbate an

underlying abnormality, but that seborrhoea alone is not sufficient to initiate micro-comedo formation (Gollnick, 2003:1589).

2.1.1.2 ANDROGENS AND SEBUM PRODUCTION

Androgens stimulate the growth and differentiation of sebaceous glands. The exact mechanism by which this is accomplished has not been defined. It has been hypothesised that androgens play a role in follicular hyperkeratinisation in acne in addition to their effects on stimulating sebum secretion. The following clinical evidence supports an essential role for androgens in stimulating sebum production:

- The development of early acne in the prepubertal period has been associated with elevated serum levels of dehyro-epi-andosterone sulphate (DHEAS), a precursor for testosterone.
- Androgen-insensitive patients who lack functional androgen receptors do not produce sebum and do not develop acne.
- Hyperplasia or carcinomas that produce excess androgens are often associated with the development of acne.
- Systemic administration of testosterone and dehydro-epi-andosterone increases the size and secretion of sebaceous glands.
- Severe acne is often associated with elevated serum androgens (Thibuotot, 2001:144).

2.1.1.3 *PROPIONIBACTERIUM ACNES* AND INFLAMMATORY FACTORS

Propionibacterium acnes (P. acnes) is an aero-tolerant anaerobic member of the normal flora in sebaceous regions of the skin, which lives in the follicle and metabolises sebaceous triglycerides into fatty acids and glycerol (Webster, 2001:15). It is widely accepted that inflammation in *acne vulgaris* may be induced mainly by an immunologic reaction to extracellular products of *P. acnes*. However, it is by no means clear that either bacteria or bacterial products initiate follicular inflammation (Zouboulis, 2004:362).

All individuals have a significant level of *P. acnes* and some degree of follicular plugging but not all individuals have active acne. The explanation lies in the level of the immune response to the organism. Patients with excessive humeral and cellular immunity to *P. Acnes* mount a more destructive inflammatory response that produces clinical lesions. This response may represent a

true hypersensitivity to *P. acnes*, in that the organism is a beneficially commensal and of minimal infectious potential (Webster, 2001:16).

Marked overgrowth of *P. acnes*is reported in comedogenic follicles, and this proliferation can be accompanied by the generation of pro-inflammatory molecules and subsequent inflammation. Several mechanisms have been proposed as to how this bacterial overgrowth may produce inflammation. These include chemotactic-, antibody- and complement-mediated inflammatory and immune processes (Gollnick, 2003:1585).

2.1.1.4 LIPID PEROXIDATION AND ACNE

ROS produced by neutrophils are involved in the irritation and destruction of the follicular wall, responsible for the inflammatory progression of acne. In comedones the proportion of linoleic acid is markedly decreased, while palmitic acid is significantly increased, as compared to that of normal skin. Linoleic acid was found to have inhibitory effects on the action of many types of ROS released by neutrophils, such as superoxide radical anion, hydrogen peroxide and hydroxyl radical, whereas palmitic acid was only able to decrease the generation of hydrogen peroxide. Therefore, in comedo lesions, ROS overflow occurs due to a lack of inhibitors, whereas palmitic acid may be involved in the pathogenesis of acne inflammation from an oxidative tissue injury standpoint.

Among skin superficial lipids, squalene, a tri-terpenoid molecule specific to human sebum, appears to function as a quencher of singlet oxygen, protecting skin surface from lipid peroxidation; however, its oxidation generates squalene peroxidases, which exerts comedogenic effects. In both open and closed comedones a high content of polar lipids, such as squalene peroxides, was found. Based on this evidence, squalene oxidation is thought to be the link between comedogenesis and bacterial colonisation (Briganti & Picardo, 2003:665).

2.2 THE CURRENT TREATMENT OF ACNE

There is currently a range of effective treatments for *acne vulgaris,* but no recommended management outlines; however, some do exist on the national level in some countries. Early and effective intervention is extremely important. Evaluating the degree and type of scarring related to the severity and duration of acne are important before the commencement of effective therapy. The primary goals of acne treatment are, therefore, to alleviate clinical symptoms and prevent

scarring. Successful acne therapy should be based on the treatment of both the pathogenic causes and the clinical symptoms. Therapeutic goals are to:

- Reduce sebum production;
- Reverse hyperproliferation and normalise keratinisation;
- Clear existing micro-comedones and comedones;
- Reduce *P. acnes* colonisation and inflammation;
- Prevent development of new micro-comedones, subsequent comedones as well as inflammatory lesions; and
- Clear existing inflammatory acne lesions (Gollnick, 2003:S5).

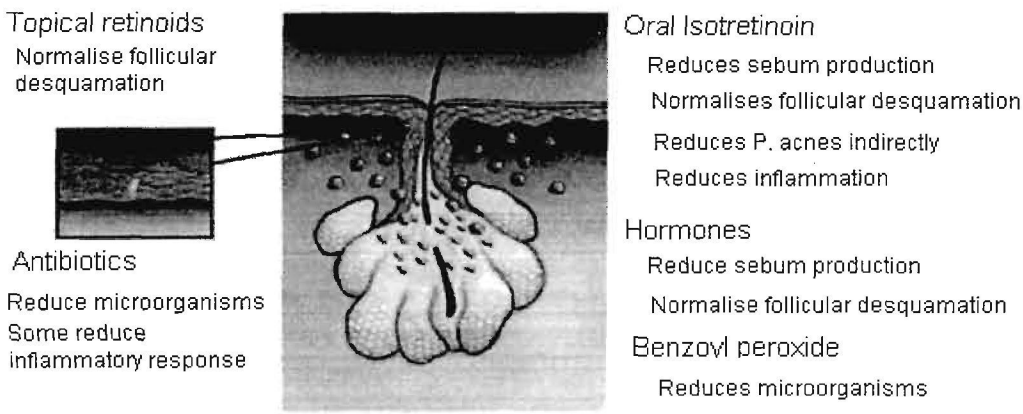

Figure 2.1 Current acne treatments (Gollnick et al., 2003:12)

2.2.1 TOPICAL RETINOIDS

Retinoids are molecular agents that act via RARs (retinoic acid receptors) to affect proliferation, differentiation and inflammation (Gollnick, 2003:S6). Topical retinoids are excellent monotherapy for all, but the most severe acne. It is prudent to add a drug that reduces inflammation by reducing *P. Acnes* populations as topical retinoid monotherapy takes several months to clear inflammatory acne (Webster, 2001:17).

Topical retinoids target the micro-comedo (the precursor of almost all other acne lesions) and should be used as first-line therapy for mild to moderate inflammatory acne in addition to

comedonal acne in most cases, excluding very severe disease states. Topical retinoids are also preferred agents for maintenance therapy. The goal is to minimise antibiotic use in acne (Gollnick, 2003:S6).

In clinical practice, all topical acne agents are regarded as palliative, not curative (Bershad, 2001:159). Topical retinoids reverse the abnormal desquamation by affecting follicular epithelial turnover and maturation of cells. In addition, some topical retinoids have an effect on inflammation by modulating the migration of inflammatory cells. Retinoids inhibit the formation of the micro-comedo by preventing the formation of both mature comedones and inflammatory lesions. Finally, retinoids alter the follicular microclimate and are likely to enhance the penetration of other compounds, including antibacterial compounds like benzoyl peroxide or topical antibiotics (Gollnick, 2003:S6).

Topical retinoids currently available for acne include tretinoin, adapalene, tazarotene and topical isotretinoin. Although these agents have differing chemical structures, they all target the micro-comedo and are comedo-suppressive in different strengths. They differ somewhat in anti-inflammatory effects and tolerability (Gollnick, 2003:S6).

The first generation retinoids, tretinoin and isotretinoin, have weak or no anti-inflammatory effects when used topically (Gollnick, 2003:1590). Tretinoin (vitamin A acid) cream is the standard to which all other anti-comedonal agents are compared. It inhibits comedo formation and eliminates comedonal acne in a few months. The only significant side effect is irritation, which usually does not require intervention (Webster, 2001:18). Molecular modification has resulted in the development of second-generation mono-aromatic compounds, etretinate and acitretin, which are synthetic analogues of the first-generation molecules. Recently, further modification has produced the poly-aromatic third-generation retinoids known as arotinoids, adapalene and tazarotene (Gollnick, 2003:1591).

Adapalene is a naphthoic acid derivative that binds to nuclear retinoid receptors and has retinoid effects. It is effective against comedonal acne and has a measure of anti-inflammatory activity (Webster, 2001:18). It has been shown to inhibit the expression of TLR2 on monocytes and macrophages, inhibiting the release of cytokines by these cells and thereby suppressing an inflammatory response.

Tazarotene is a synthetic acetylenic retinoid approved for the treatment of mild to moderate acne. Topical gel application provides direct delivery of tazarotene into the skin, where it is rapidly hydrolysed to its active metabolite, tazarotenic acid. Tazarotenic acid affects keratinocyte differentiation, cell proliferation and inflammation (Gollnick, 2003:1591).

2.2.2 TOPICAL ANTIBIOTICS

Benzoyl peroxide has demonstrated clinical efficacy in the treatment of *acne vulgaris* through both antibacterial and anti-inflammatory means (Warner & Plosker, 2002:349). Benzoyl peroxide is the gold standard in the treatment of mild to moderate acne. It has potent antibacterial and weak comedolytic activity. However, local adverse effects such as drying and irritation are common (Gollnick, 2003:1591).

Topical antibacterial agents such as tetracycline and clindamycin are good alternatives to benzoyl peroxide, as they not only reduce the population of *P. acnes* in sebaceous follicles, but also demonstrate indirect comedolytic and weak anti-inflammatory effects (Gollnick, 2003:1591). Azelaic acid is a dicarboxylic acid that has both antibacterial and comedolytic effects. Topical application of this drug is fairly effective in reducing comedones, and it is the least irritating of the preparations (Webster, 2001:18). Topical antibiotics should not be used as monotherapy due to their relatively slow onset of action, and the potential for bacterial resistance. Therapy should be discontinued if no improvement is observed within 6 - 8 weeks (Gollnick, 2003:S16).

2.2.3 HORMONAL THERAPY

Acne is often thought of as a hormonal disease because of the clear link to androgens (Webster, 2001:18). The goal of hormonal therapy is to oppose the effects of androgens on the sebaceous gland. This can be accomplished by estrogens, androgen receptor-blockers, or agents designed to decrease the endogenous production of androgens by the ovary or adrenal gland such as oral contraceptives, glucocorticoids, or gonadotrophin-releasing hormone (GnRH) agonists.

Estrogens are most commonly used to treat acne in combination with a progestin in an oral contraceptive in order to avoid the risk of endometrial cancer that is associated with unopposed estrogens (Thiboutot, 2001:149). The dose of estrogen required to suppress sebum production is greater than the dose required to suppress ovulation. Estrogens suppress the ovarian production

of androgens by suppressing gonadotrophin release. They also stimulate hepatic synthesis of sex hormone-binding globulin (SHBG) (Gollnick, 2003:S22).

Gonadotrophin releasing hormone agonists such as nafarelin, leuprolide, and buserelin inhibit ovarian androgen production by blocking the cyclic release of lutenising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. The net effect is suppression of ovarian steroidgenesis. Their use is limited by their expense and side effect profile, which includes menopausal symptoms, headache and bone loss (Thiboutot, 2001:150).

Anti-androgens, or androgen receptor blockers, include cyproterone acetate, spirinolactone and flutamide (Gollnick, 2003:S22). Cyproterone acetate is a progestational anti-androgen that blocks the androgen receptor. It is combined with ethinyl estradiol in an oral contraceptive formulation that is widely used in Europe for acne treatment. Cyproterone acetate itself has also been used to treat acne (Thiboutot, 2001:151).

Flutamide blocks the androgen receptor. It has been used at doses of 250 mg twice a day in combination with oral contraceptives for the treatment of acne or hirsutism in females. Use of flutamide in the treatment of acne is very much limited by its side effect profile. Thus it is used very seldom (Gollnick, 2003:S23).

2.2.4 SYSTEMIC ANTIBIOTICS

The oral antibacterials, erythromycin, clindamycin, co-trimoxazole, trimethoprim and the tetracyclines are widely used for the treatment of moderate to severe inflammatory acne (Gollnick, 2003:S15). Oral antibiotics are indicated for the management of moderate and severe acne, acne that is resistant to topical treatment and acne that covers large parts of the body surface. These agents act through suppressing the growth of *P. acnes* along with the inflammatory mediators synthesised and released by this pathogen. In addition, tetracyclines and erythromycin have inherent anti-inflammatory activities and decrease the production of chemotactic factors and the recruitment of neutrophils.

The efficacy of these agents, when given systemically to treat acne, depends on their ability to reach the lipid-rich environment of the pilo-sebaceous follicles where *P. acnes* proliferates (Katsambas & Papadonstantinou, 2004:412). Tetracyclines are prescribed most frequently as they are both effective and inexpensive (Gollnick, 2003:S15), but they frequently cause abdominal discomfort. Doxycycline and minocycline are more lipid-soluble than tetracycline and display better

penetration of the pilo-sebaceous follicle. They cause less gastrointestinal upset than tetracycline (Katsambas & Papakonstantinou, 2004:413).

2.2.5 SYSTEMIC RETINOIDS

Systemic retinoids, in particular isotretinoin, are the most effective therapy for moderate to severe inflammatory acne and for patients unresponsive to conventional therapy (Gollnick, 2003:S26). Isotretinoin is the only therapeutic agent that exhibits activity against all of the major etiologic factors involved in the pathogenesis of acne. It significantly reduces the excretion of sebum, formation of comedones, and colonisation of the skin with *P. acnes* and also has anti-inflammatory activity (Katsambas & Papakonstantinou, 2004:414).

The indications for the use of oral isotretinoin are as follows:

- Severe nodulocystic acne/severe acne variants;
- Inflammatory acne with scarring;
- Moderate-to-severe acne frequently relapsing; and
- Acne with severe psychological distress (Gollnick, 2003:S26).

Systemic isotretinoin therapy is associated with numerous adverse effects; the most important are teratogenicity and adverse psychiatric events. Mucocutaneous side effects are the most common, experienced by virtually all patients. Dryness of the lips (100%), skin (50%), nasal passages (30 – 50%), and eyes (20%) can result in dermatitis, chelitis, epistaxis and conjunctivitis. Before treatment, the patient should be informed of the possible adverse effects and encouraged to use moisturisers and lip balms as needed (Katsambas & Papakonstantinou, 2004:414).

2.2.6 ALPHA-LIPOIC ACID

ROS produced by neutrophils are involved in the irritation and destruction of the follicular wall, responsible for the inflammatory progress of acne (Arican *et al*, 2005:380). When the cell undergoes oxidative stress, i.e., ultraviolet radiation, ionising radiation, infection and free radicals created by metabolism, the inhibitory fraction of nuclear factor kappa-B (NFκ-B) is dissociated from the molecule. Once the inhibitory fraction is dissociated from the NFκ-B molecule, it then migrates

to the nucleus of the cell, begins transcription, and subsequent production of inflammatory mediators, including cytokines such as tumor necrosis factor alpha (TFα) and inflammatory interleukins. ALA is a powerful inhibitor of the activation of NFκ-B, and therefore, can act as an anti-inflammatory as well as an antioxidant. It would be useful to employ this substance in the treatment of chronic skin conditions (Perricone, 2003:4). Surprisingly, it has been found that ALA applied to the faces of persons with acne not only reduces erythema and acne formed scars, but also reduces pore size and the beneficial effects increase over time (Perricone, 2002:6).

The interest in ALA based skin care products has been growing significantly due to the fact that this active is less irritating than other substances such as tretinion or hydroxy acids. Therefore, it may be useful, even at low concentrations, to treat delicate areas such as those around the eyes. Additionally, ALA is an inexpensive and widely available active substance, showing a lipophilic character that might contribute to increase its loading when incorporated into lipid nanoparticles (Souto *et al.*, 2005:582).

2.3 TRANSDERMAL DRUG DELIVERY

2.3.1. STRUCTURE AND BARRIER FUNCTIONS OF THE SKIN

Skin is essentially composed of two major layers: an outer, unvascularised epithelial layer (the epidermis), and an inner layer (the dermis), which contains a rich supply of capillaries, nerves, sweat glands, sebaceous glands, and hair follicles that are supported by connective tissue. The epidermis can further be divided into several anatomical layers which represent different stages of differentiation of cells – once formed from the stem cells on the basal membrane at the interface of the epidermis and dermis – migrating towards the surface of the skin (Suhonen *et al.*, 1999:150).

The dermis is highly vascular and also includes the pilo-sebaceous units, sweat glands, dermal adipose cells, mast cells and infiltrating leucocytes. Approximately 95 % of the epidermis layer is constituted by keratinocytes, and the rest are melanocytes, Langerhans cells and Merkel cells (mechanoreceptors). The stratified epidermis, ~ 100 – 150 μm thick, is divisible into four distinct layers: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and Stratum Corneum (SC) (Menon, 2002:S4).

The excellent barrier function of the skin is accomplished almost entirely by the SC (Donnely *et al.*, 2007:195). The SC (or horny layer) is the final product of epidermal cell differentiation, and though

it is an epidermal layer, it is often viewed as a separate membrane in typical and transdermal drug delivery studies. Typically the SC comprises only 10 – 15 cell layers and is around 10 μm thick when dry, although it may swell to several times this thickness when wet. The SC serves to regulate water loss from the body whilst preventing the entry of harmful materials, including micro-organisms (Williams, 2003:9).

The SC is the major permeability barrier to external materials, and is regarded as the rate-limiting factor in the penetration of therapeutic agents through the skin due to its highly organised structure (Foldvari, 2000:418). The classic brick and mortar organisation, championed by Peter Elias, is still the most simplistic organisational description of the SC. The protein-enriched corneocytes (bricks) impart a high degree of tortuousity to the path of water or any other molecule that traverses the SC, while the hydrophobic lipids, organised into tight lamellar structures (mortar) provide a water-tight barrier property to the already tortuous route of permeation in the inter-follicular domains (Menon, 2002:S9).

The barrier nature of the SC depends critically on its unique constituents: 75 – 80% is protein, 5 – 15% is lipid with 5 – 10% unidentified on a dry weight basis (Williams, 2003:9). The SC is virtually devoid of phospholipids and is selectively enriched in ceramides, free sterols, free fatty acids, cholesterol sulphate and hydrocarbons (Elias, 1990:26).

The ceramides and neutral lipids are arranged in a bilayer format and form so-called "lipid channels." The skin's barrier function appears to depend on the specific ratio of various lipids. Studies in which non-polar and relatively polar lipids were selectively extracted with petroleum ether and acetone, respectively, indicated that the relatively polar lipids were more crucial to skin barrier integrity (Foldvari, 2000:418).

2.3.2 ROUTES OF DRUG PERMEATION ACROSS THE SKIN

Before being taken up by blood vessels in the upper dermis and prior to entering the systemic circulation; substances permeating the skin must cross the SC and the viable epidermis. There are three possible pathways leading to the capillary network: through hair follicles with associated sebaceous glands, via sweat ducts, or across continuous SC between these appendages. These three pathways are not mutually exclusive and it is likely that most molecules will pass through the SC by a combination of these routes (Williams, 2003:31). The route through which permeation

occurs is largely dependent on the penetrant's physiochemical characteristics; the most important being the relative ability to partition into each skin phase (Walters, 1989:201).

2.3.2.1 TRANSAPPENDAGEAL TRANSPORT (SHUNT ROUTE TRANSPORT)

The appendages (hair follicles, sweat ducts) essentially offer pores that bypass the barrier of the SC (Williams, 2003:31). As the fractional appendageal area available for transport is only about 0.1%, this route usually contributes negligibly to apparent steady state drug flux (Donnely *et al.*, 2007:195), but for molecules, due to their large size, for example, the shunt pathway can be significant (Sunhonen *et al.*, 1999:151).

The rapid onset of action from some topically applied medicines can only be explained by permeation bypassing the bulk of the SC. In addition to rapid drug delivery, transappendageal transport may also be important for large polar molecules and ions that would traverse poorly across the bulk of the SC (Williams, 2003:32).

2.3.2.2 TRANSCELLULAR ROUTE

The transcellular route involves transport directly across the bulk of the SC, where a solute ion sequentially crosses keratinocytes (Rastogi & Singh, 2001:2). First, there is partitioning into the keratinocyte, followed by diffusion through the hydrated keratin. In order to leave the cell, the molecule must partition into the bilayer lipids before diffusing across the lipid bilayer to the next keratinocyte. In traversing the multiple lipid bilayers the molecule must also sequentially partition into, and diffuse across the hydrophobic chains and the hydrophilic head groups of the lipids and there are estimated to be between 4 and 20 such lamellae between each keratinocyte. The rate-limiting barrier for permeation via this route remains the multiply bilayered lipids that the molecule must traverse between the keratinocytes. The use of solvents to remove lipids form the SC invariably increases drug flux for even highly hydrophilic molecules (Williams, 2003:32).

2.3.2.3 INTERCELLULAR PATHWAY

The intercellular route is widely believed to provide the principal pathway for the permeation of most drugs (Sunhonen *et al.*, 1999:151). The precise nature of the intercellular pathway is still open to debate. What is clear, however, is that the lipid bilayers provide the major limiting barrier to drug

flux. Intercellular transport is clearly through the lipid domains and transcellular permeation also requires the lipid lamellae to be crossed.

With transcellular permeation, the pathway is directly across the SC and hence the path length for permeation is usually regarded as the thickness of the SC. In contrast, the intercellular route is highly tortuous, with permeants moving through the continuous lipid domains between the keratinocytes. In this case the path length taken by the molecule is considerably greater than that of the SC thickness.

Various estimates have been proposed for the intercellular permeation distance, ranging from 150 – 500 μm; it appears likely that different path lengths are taken by permeants traversing the SC, dependent upon their physicochemical properties. As the real path length is unknown, various permeation parameters incorporating length (such as the permeability coefficient and diffusion coefficient) should always be regarded as apparent values (Williams, 2003:34). Figure 2.2 depicts the various routes of drug permeation across the skin.

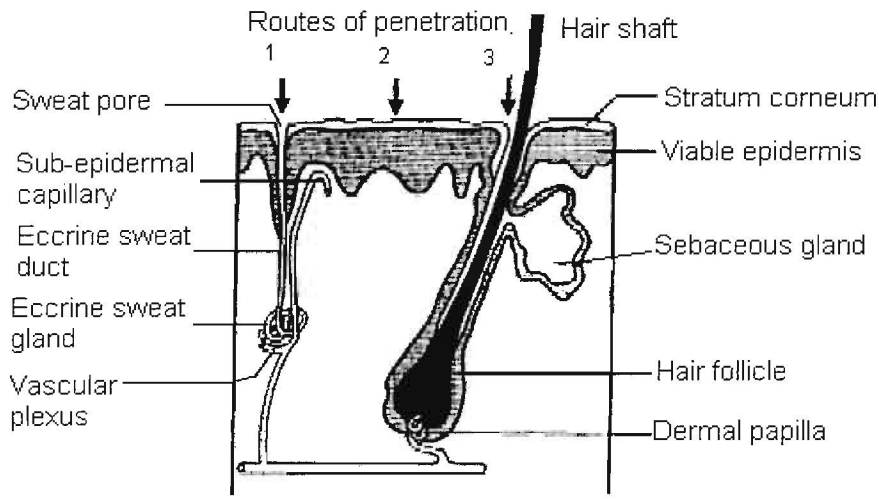

Figure 2.2 Routes of drug permeation across the skin (Barry 2001:102).

2.3.3 PHYSIOLOGICAL FACTORS AFFECTING TRANSDERMAL DRUG DELIVERY

2.3.3.1 INDIVIDUAL VARIATION

Individual variation can be as severe a problem as for other drug delivery systems, for example the absorption of hydrocortisone can show nearly a ten-fold variation between individuals. Thus dosage must be titrated to achieve a therapeutic benefit and the transdermal system does have the advantage in this respect that treatment can be stopped rapidly if too great a response is observed (Washington & Washington, 1989:188).

2.3.3.2 SKIN AGE

Skin condition and structure varies with age (Washington & Washington, 1989:188). Beyond the skin membrane, there are some age-related alterations that theoretically can affect the amounts of a topically applied drug entering the systemic circulation. Blood flow (dermal clearance of molecules traversing the tissue) tends to decrease with age and this could, *in vivo*, reduce the transdermal flux.

Whilst the ageing effects of normal skin on drug delivery are minimal, there are important morphological and hence permeability differences between normal (mature) skin and that of a neonate (pre-term infant) (Williams, 2003:14). Pre-term infants have very little barrier function, since this does not develop until 9 months after conception. In older people the SC thickens and is less hydrated, increasing its barrier function (Washington & Washington 1989:188).

2.3.3.3 BODY SITE

It is readily apparent that skin structure varies to some degree over the human body. The SC is thicker on the palms of the hands and soles of the feet than on the lips or eyelids. Though site-to-site variation in permeability is complex, there are some general trends shown in the numerous literature reports on the subject. A generalised rank order of site permeabilities is: Genitals > head and neck > trunk > arm > leg. Thus, there is a clear scientific rationale for selecting the application site based on permeability. It is valuable to put the regional variations in transdermal drug absorption into context with respect to variation found for the same site between different individuals. There is – as would be expected for a biological membrane – considerable variation in

permeation across a given body site of an individual (~ 30%) and also considerable variation between the same body site on different individuals (~40 %) (Williams, 2003:16).

2.3.3.4 OCCLUSION

Occlusion increases adsorption considerably in many cases, probably due to increased hydration of the SC, improving permeability to both polar and non-polar drugs (Washington & Washington, 1989:188).

2.3.3.5 RACE

Race appears to influence penetration to a small extent. Negroid SC has more layers and is generally less permeable, although there is no difference in actual thickness between Negroid and European SC. It is not known if the presence of melanocytes influences the penetration of drugs (Washington & Washington, 1989:189).

2.3.3.6 TEMPERATURE

The human body maintains a temperature gradient across the skin from around 37 °C inside to around 32 °C outside at the outer surface. Greatly elevating the skin temperature can induce structural alterations within the SC (Williams, 2003:18). Temperature affects drug penetration by two mechanisms. Firstly, it alters the physiology of the skin, and secondly the physicochemical diffusion rates in the device increase with temperature.

The skin temperature is strongly influenced by its surroundings, and may be 20 °C cooler or several degrees hotter. In disease the body temperature may vary. Temperature-induced variations in the diffusion coefficient may alter the absorption rate by up to a factor of two over this temperature range. Temperature also influences blood flow in the surface vasculature and so might be expected to influence adsorption through this route (Washington & Washington, 1989:189).

2.3.3.7 DISEASE

The skin is the part of the body which comes into direct contact with the environment and hence it is usually the first part of the body to sustain damage or be exposed to irritant substances. Inflammation occurs in response to a number of factors e.g. mechanical, chemical, thermal stimuli, infections or imbalance in the auto-regulation processes. All these processes can reduce barrier action and lead to increased permeability of the skin. Any damaged or diseased area of the skin is

likely to display compromised barrier properties and consequently, higher drug absorption (Washington & Washington, 1989:189).

2.3.4 PHYSIOCHEMICAL FACTORS INFLUENCING PERCUTANEOUS ABSORPTION

2.3.4.1 PARTITTION COEFFICIENT

In order to cross the SC, a permeant must first partition into the membrane. The partition coefficient of a permeant is usually the governing factor in dictating which pathway it will follow through the skin (Williams, 2003:35). When the drug reaches the viable tissue it encounters a phase change. It has to transfer from the predominantly lipophilic intercellular channels of the SC into the living cells of the epidermis, which will be largely aqueous in nature and essentially buffered at pH 7.4 (Potgieter, 2002:14).

For molecules with intermediate partition coefficients, showing some solubility in both oil and water phases, the intercellular route probably predominates. This would typically encompass most molecules with a log $P_{(Octanol/water)}$ of 1 to 3. For more highly lipophilic molecules (log $P > 3$), the intercellular route will be almost exclusively the pathway used to traverse the SC. However, for these molecules a further consideration is the ability to partition out of the SC into the essentially aqueous viable epidermal tissues.

For more hydrophilic molecules (log $P < 1$) the transcellular route becomes increasingly important, yet there are still lipid bilayers to cross between the keratinocytes. For highly hydrophilic (and charged) molecules, the appendageal pathway may also become significant, as is shown by the localisation around the pilosebaceous units of topically applied dyes (Williams, 2003:36).

2.3.4.2 MOLECULAR SIZE

It is well documented that the molecular size of a substance directly affects its diffusion across simple or complex membranes. The diffusion of molecules through liquids is inversely proportional to the molecular weight of the molecules or to the square or cube root thereof. This phenomenon may also be expected in the case of diffusion across the skin (Monene, 2003:34).

Most conventional therapeutic agents (small organic molecules) that are selected as candidates for transdermal delivery tend to lie within a relatively narrow range of molecular weights (100 –

500 Da). Within such a narrow range, the influence of molecular weight on drug flux appears to be relatively minor if compared to the influence of, for example, changes in partition coefficients. When selecting much larger molecules as therapeutic agents, for example, peptides or proteins, the molecular weight dependency on transdermal flux is much more apparent (Williams, 2003:37).

For compounds ranging in molecular weight from 18 to < 750, Potts and Guy derived an equation for predicting the permeability through human skin (Potgieter, 2002:14).

$\log k_p\,(cm.sec^{-1}) = -6.3 + 0.71 \log K_{oct} - 0.0061 MW$

Where:

$\log K_p$ = permeability coefficient

$\log K_{oct}$ = octanol/water partition coefficient

MW = molecular weight.

2.3.4.3 SOLUBILITY/MELTING POINT

The melting point of substances reflects their relative hydrophobia associated with a low level of crystalline interactions. Drug crystallinity, or melting point, influences permeability and was found to be inversely proportional to lipophilicity (log K_{oct}). The melting point of a substance is often considered to be indicative of the maximum flux attainable through the skin. The lower the melting point, the greater is the drugs ability to permeate the skin (Monene, 2003:30).

The steady state flux of a drug across any membrane is proportional to the concentration gradient between the two faces of the membrane. If sink conditions occur on one side of the membrane and an infinite dose of drug is applied to the other, the concentration gradient will be proportional to the solubility of the drug in the lipid phase of the membrane (Potgieter, 2002:18).

From the discussion of the intercellular permeation pathway, it is evident that lipophilic molecules tend to permeate through the skin faster than more hydrophilic molecules. Thus, solubility within the intercellular lipids (usually described by the partition coefficient) can be correlated with the permeability coefficient for a homologous series of compounds. However, whilst lipophilicity is generally a desired feature of transdermal candidates, it is also necessary for the molecule to

exhibit some aqueous solubility since topical medicaments are generally applied from an aqueous formulation (Williams, 2003:37).

2.3.4.4 IONISATION

According to the pH-partition hypothesis, which was developed through the gastrointestinal tract, only the unionised form of a drug can permeate through the lipid barrier in significant amounts. However, with the complex structure of human skin, this model cannot be rigidly applied (Williams 2003:38).

An ionisable drug will be present in both charged and uncharged form depending on its pKa and the pH of the environment (Smith, 1990:27). The unionised moiety of a drug is more lipid soluble and may dissolve more rapidly in the lipid material of the skin, thereby facilitating transport by passive diffusion (Abdou, 1989:438). The ionised moiety, on the other hand, is usually less lipid soluble, limiting transdermal permeation.

The solubility of ionised species is considerably higher than for unionised species. It may therefore be possible that, given a set pH or permeant concentration, the amounts of drug permeating, will be dominated by the diffusion of the ionised moieties (Potgieter, 2002:24).

Permeation across human skin can occur via several pathways, none of which is mutually exclusive, and all of which probably operate for most molecules traversing the skin. Some appendages offer an essentially aqueous pathway through the SC, albeit one of limited cross-sectional area.

The transcellular route can be viewed as one of intermediate properties, whereas the intercellular pathway is essentially a lipophilic route. Thus, it is likely that charged permeants (ionised drugs) can cross the membrane (by the shunt route), but that the amounts of these permeants may be somewhat less than if the species were unionised and were to pass largely via the lipoidal intercellular route (Williams, 2003:38). Table 2.1 depicts the formulation concentrations for passive ransdermal delivery and the ideal limits.

Table 2.1 Formulation considerations for passive transdermal delivery and ideal limits (Naik *et al.*, 2000:319):

Aqueous solubility	> 1 mg ml^{-1}
Lipophilicity	10 < $K_{o/w}$ < 1000
Molecular weight	< 500 Da
Melting point	< 200°C
pH of saturated aqueous solution	pH 5-9
Dose deliverable	< 10 mg day^{-1}

2.3.5 MATHEMATICS OF SKIN PERMEATION

Percutaneous absorption has been analised by a variety of mathematical models in the literature. Among the mathematical models used in the analysis of percutaneous absorption, diffusion-partitioning models based on Fick's law of diffusion are widely applied. If the model parameters can be determined from separate experiments, the diffusion-partitioning model can predict the rate of skin penetration under various modes of transdermal treatment (Tojo, 1997:113).

Drug absorption across human skin is passive and hence, can be described in physical terms. The following description of the mathematical treatments applied to transdermal drug delivery studies is intended to provide an overview. Only two situations will be considered:

1. Where the drug is applied as an infinite dose – it does not deplete over the time of application (e.g. as with a transdermal patch).

2. Where a small finite dose is applied and so pseudo-steady state permeation would not be encountered (e.g. with a cream for a local action) (Williams, 2003:41).

Molecules move in response to a thermodynamic force arising from a concentration gradient (Williams, 2003:41). Fick's first law of diffusion states that the rate of transfer of a diffusing substance through a unit area of a section is proportional to the concentration gradient measured

normal to the section (Crank, 1975:139). The amount of material passing through a unit area per unit time is termed the flux (J). Where:

$$J = -D\frac{\partial c}{\partial x} \qquad \textbf{(Equation 2.1)}$$

J is the flux of the permeant (Williams, 2003:41), C is the concentration of the diffusing substance, x is the space coordinate measured normal to the section, and D is the diffusion coefficient. The negative sign arises because diffusion occurs in the direction opposite to that of increasing concentration (Crank, 1975:139).

The homogenous membrane model for describing the drug transport across the skin has been widely applied since diffusion across the SC is a rate-limiting step for most drugs. If the skin contains no drug molecules prior to the application of the delivery device, drug movement in the skin can be described by Fick's second law of diffusion:

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial^2 x} \qquad \textbf{(Equation 2.2)}$$

where D is the diffusion coefficient in the SC, C is the concentration, t is the time and x is the distance from the surface of the skin (Tojo, 1997:113). Thus, 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, 2003:42).

The skin membrane is highly complex and hence precise mathematical solutions to permeation data are not possible. For example, different skin membranes are often used (SC alone or dermal membranes) and multiple pathways for permeation may operate at one time (appendageal, intercellular and transcellular). Thus, the following solutions to Fick's second law must be treated as useful approximations that allow comparisons and standardisation of permeation data.

Most *in vitro* experimental designs aim to mimic as closely as possible the *in vivo* situation. The most common *in vitro* design is one where a membrane (usually the epidermis) separates two compartments. One compartment contains the permeant in a vehicle, possibly a simple aqueous or buffer solution (termed the donor solution), and the other compartment contains a receptor (or receiver) solution that provides sink conditions (i.e. essentially zero concentration). After sufficient time, steady-state permeation across the membrane is achieved when the concentration gradient of

the permeant across the membrane is constant. Under these conditions, the following equation applies:

$$\frac{dM}{dt} = \frac{DC_o}{h}$$ **(Equation 2.3)**

where M is the cumulative mass of permeant that passes through per unit area of the membrane in time t. C_0 is the concentration of the permeant in the first layer of the membrane (at the skin surface, in contact with the donor solution) and h is the membrane thickness. It is very difficult to measure C_0, the concentration of permeant in the first layer of the membrane.

The concentration of the permeant in the vehicle (donor solution) bathing the skin membrane (C_v) is usually known or can be determined relatively easily. Since C_0 and C_v are simply related by:

$$C_0 = PC_v$$ **(Equation 2.4)**

where P is the partition coefficient of the permeant between the membrane and vehicle. Substitution of Equation 2.4 into Equation 2.3 gives:

$$\frac{dm}{dt} = \frac{DPC_V}{h}$$ **(Equation 2.5).**

This is the most applied equation in examining transdermal drug delivery data.

The permeability coefficient of a permeant through a membrane can be defined by (Williams, 2003:43):

$$kp = \frac{PD}{h}$$ **(Equation 2.6).**

Neither the SC nor the whole skin is a unique inert membrane. Therefore, the drug concentrations in the formulation are not the same as at the skin surface, but are related to the vehicle membrane distribution coefficient K_m. When the difference between the concentration at the upper membrane surface and its lower surface is ΔC, and the thickness of the skin is h, the equation can be stated as follows (Goosen, 1998:24):

$$J = \frac{k_m D\Delta C}{h} = k_p \Delta C$$ **(Equation 2.7).**

J	=	flux of drug
K_m	=	vehicle membrane distribution coefficient
D	=	diffusion coefficient
ΔC	=	concentration difference between the upper and lower surfaces of the skin
h	=	thickness of skin
K_p	=	permeability coefficient

2.3.6 PENETRATION ENHANCERS

The use of agents to enhance the penetration of drugs through the skin has been widely studied, although phenomena associated with the modification of the barrier function and the molecular nature of the incorporating molecules remain unclear. An understanding of these interactions, however, is fundamental to the design of topical delivery vehicles and their modes of interaction with the skin (Ward & Tallon, 1990:55).

The SC is a highly efficient and effective barrier, with the multiple bilayered lipids providing the principal pathway for permeation through the membrane (Wlliams, 2003:83). The increase of thermodynamic activity of the active ingredient in the vehicle is one approach to enhance dermal and transdermal delivery without influencing the physicochemical characteristics of the SC (Otto, 2008:9). For maximum penetration rate, the drug should be at its highest thermodynamic activity (Barry, 2001:102).

However, by changing the properties of the SC, the cutaneous and percutaneous absorption can also be enhanced. Physical enhancement methods actively affect the barrier properties or circumvent the SC and include iontophoresis, electroporation, sonophoresis, magnetophoresism, microneedles, skin perforation and needleless injection (Otto, 2008:9). The specific mechanism of penetration enhancement can fall into one of three categories:

1) disruption of the highly ordered structure of intercellular lipid channels,

2) interaction with corneocyte intracellular protein components, and

3) enhanced partitioning of the drug in the presence or absence of the enhancer compound, which can be described by the 'lipid protein partitioning (LPP) theory' (Foldvari, 2003:419).

Chemical penetration modifiers affect the skin barrier properties by diffusing into the SC and altering the solubility properties of the skin for the permeant and/or disrupting the lipid packing of the SC. The former results in the change of the partition coefficient *K* between skin and vehicle and the latter influences the diffusion coefficient, *D* (Otto, 2008:9).

2.3.6.1 WATER

Hydration of the SC increases the penetration rate of most (but not all) substances (Barry, 2001:105). Though the mechanisms of action are unclear, the effects of increasing SC hydration on drug delivery are evident (Williams, 2003:84). Water, due to its polar nature, is likely to interact with the polar head groups of the bilayer, thereby upsetting the packing at the polar plane (Sunhonen *et al.*, 1999:153).

The increase in water content in the SC generally results in an increase in transdermal delivery of both hydrophilic and lipophilic permeants (Otto, 2008:10). Moisturising factors, occlusive films, hydrophobic ointments and transdermal patches all enhance drug bioavailability into skin (Barry, 2001:106). However, one should be careful with a generalisation as it has also been reported that occlusion does not necessarily enhance transdermal delivery of hydrophilic compounds (Otto, 2008:10).

2.3.6.2 ALCOHOLS, FATTY ALCOHOLS AND GLYCOLS

Ethanol is commonly used in many transdermal formulations and is often the solvent of choice for use in patches. It is also used as a co-solvent with water for ensuring sink conditions during *in vitro* permeation experiments (Williams, 2003:94). Ethanol has been suggested to induce modifications at the polar head group region of the bilayers. Disruption of the polar head region may result in an increase of the interfacial area of the lipids between limited regions of interdigiated and the predominant non-interdigiated lipids in the same lamella, which may play a role in the observed enhanced penetration (Sunhonen *et al.*, 1999:153).

Fatty alcohols (also called alkanols) are usually applied to skin in a co-solvent (often propylene glycol) at between 1 and 10%. When comparing activities for saturated fatty alcohols, ranging from octanol to myristyl alcohol, a parabolic relationship was found with a maximum effect for decanol. Enhancement activity also showed a general increase, when up to two unsaturated bonds were added to the alcohols, but activity fell when three double bonds were introduced (Sunhonen *et al.*, 1999:153).

Propylene glycol has been used as a 'stand alone' penetration enhancer, but is more widely used as a vehicle for the application of accelerants (Williams, 2003:95). Propylene glycol (PG) alone is able to enhance the permeation of lipophilic drugs, probably because of a solvent drag effect (Foldvari, 2000:421). As a vehicle, propylene glycol works synergistically with many enhancers, including Azone, oleic acid, fatty alcohols and terpenes. Propylene glycol permeates well through the human SC and its mechanisms of action are probably similar to those suggested for ethanol (Williams, 2003:95).

2.3.6.3 SULPHOXIDES

Dimethylsulphoxide (DMSO) is one of the earliest and most widely studied penetration-enhancing materials. It is a powerful aprotic solvent which hydrogen bonds with itself rather than with water. It is colourless, odourless and hygroscopic, and is often used in many areas of pharmaceutical sciences as a 'universal solvent' (Williams, 2003:87).

Alterations in SC protein structures have been frequently attributed to penetration enhancers such as DMSO. They have been suggested to replace water molecules, bound to polar protein side chains, resulting in conformational alterations of the keratinised proteins from α-helix to β-sheet (Sunhonen *et al.*, 1999:157).

2.3.6.4 PYRROLIDONES

The pyrrolidones and derivatives have been investigated as potential penetration enhancers in human skin. These compounds apparently have greater enhancement effects with hydrophilic permeants than with lipophilic materials, although this might be attributed to the greater enhancement potential for the poorer hydrophilic permeants. N-methyl-2-pyrrolidone (NMP) and 2-pyrrolidone (2-P) are the most widely studied enhancers of this group.

The pyrrolidones partition well into human SC, where they alter the solvent nature of the tissue. Thus, their prime mechanism of action appears to be the generation of a permeant reservoir within the tissue. This drug reservoir formation offers potential for sustained release of the permeant from the SC over extended time periods (Willams, 2003:91).

2.3.6.5 FATTY ACIDS

Among the fatty acids, oleic acid has been investigated extensively as a skin penetration enhancer for numerous compounds. The magnitude of enhancement is affected by several factors, including

fatty acid chain length, the presence of double bonds and the solvent or vehicle in which the fatty acid is dissolved (Foldvari, 2000:420). From thermal analyses, it is apparent that oleic acid interacts with the lipid domains within the SC – as would be expected for a long-chain fatty acid.

Spectroscopic investigations have used perdeuterated oleic acid, in both human SC and in model lipid mixtures. These studies show that oleic acid exists in separate phases (or 'pools') within the olbilayer lipids. The formation of pools provides permeability defects within the bilayers lipids, thus, facilitating permeation of hydrophilic permeants via these defects. Lipophilic molecules are less hindered by the lipid domains within the SC, and hence their permeation is not enhanced to as great an extent as for the hydrophilic molecules (Williams, 2003:93).

2.3.6.6 TERPENES

Terpenes are naturally occurring constituents of plant volatile oils. These molecules can be classified as hydrocarbons, alcohols, ketones or oxides, and have been shown to enhance the penetration of several groups of drugs. The more lipophilic oxygen-containing terpenes, in particular, have been found to increase the penetration of various drugs (Foldvari, 2000:420).

One mechanism by which these agents operate is to modify the solvent nature of the SC in order to improve drug partitioning into the tissue. Terpenes also modify drug diffusivity through the membrane. A reduction in the lag time for permeation is usually found. Small angle X-ray diffraction studies have indicated that d-limonene and 1,8-cineole disrupt SC bilayers lipids, whereas nerolidol (a long-chain sesquiterpene) reinforces the bilayers, possibly by orientating alongside the SC lipids (Williams, 2003:100).

2.3.6.7 SURFACTANTS

Surfactants are of amphiphilic nature consisting of a hydrophobic 'tail' and a hydrophilic 'head'. They are used in formulations as emulsifiers, wetting agents and solubilisers, and are classified into cationic (e.g. cetyltrimethyl ammonium bromide and benzalkonium chloride), anionic (e.g. sodium dodecyl sulphate and fatty acid salts), non-ionic (e.g. alkyl polyethylene oxide, poloxamers and fatty alcohols) and zwitterionic surfactants (e.g. dodecyl betaine) (Otto, 2008:11). Their usual role is to aid in drug solubilisation and to attribute water washability to the vehicle for cosmetic appeal.

Surfactants interact extensively with the skin, usually by penetrating into the skin and disrupting lipids or proteins. One frequently used non-ionic surfactant, sodium lauryl sulphate (SLS), has

been shown to accumulate in the skin after topical treatment. Recent studies have demonstrated that SLS has a detrimental effect on epidermal lipid processing and consequently, the formation of the lipid barrier domain. Cationic and anionic surfactants have also been used to enhance drug permeation through the skin. These substances, however, tend to be even more damaging to the skin than non-ionic surfactants and are unlikely to become accepted for use in pharmaceutical preparations (Foldvari, 2000:421).

2.3.6.8 AZONE

Azone (1-dodecylazacycloheptan-2-one or laurocapram) was specifically designed as a chemical penetration enhancer. Its chemical structure can be considered as a hybrid between a cyclic amide and an alkylsulphoxide, but it is missing the aprotic sulphoxide group that gives rise to the disadvantages listed for DMSO. Azone is a colourless, odourless liquid (melting point - 7 °C) with a smooth, oily but non-greasy feel. It is a highly lipophilic material with a log $P_{octanol/water}$ around 6.2 and it is soluble in, and compatible with, most organic solvents including alcohols. The chemical has little pharmacological activity, although some evidence exists for an antiviral effect and it has very low toxicity. Thus, Azone appears to possess many of the desirable properties for the 'ideal' penetration enhancer (Williams, 2003:89).

Extensive studies have also been conducted on the mechanism of action of Azone and its various derivatives. Azone inserts itself into the intercellular lipid layer of the skin and through hydrogen bonding to an adjacent ceramide head group, creates a 'channel' on the other side where hydrogen bonding to the other ceramide molecule is not possible. The length of the alkyl chain of the Azone molecule also influences its location within the lipids. This disordering effect is responsible for enhancing drug permeation (Foldvari, 2000:421).

3.6.2.9 PHOSPHOLIPIDS

There is no compelling evidence to suggest that phospholipids interact directly with SC lipid packing, though this may be expected when considering their physicochemical properties and structures. However, it is apparent that phospholipids can occlude the skin surface and thus can increase tissue hydration which can increase drug permeation.

When applied to the SC as vesicles, phospholipids can fuse with SC lipids. This collapse of structure liberates permeant into the vehicle in which the drug may be poorly soluble, hence thermodynamic activity could be raised, in this way facilitating permeation (Williams, 2003:102).

2.3.7 DRUG DELIVERY VEHICLES

The most desirable method to improve the performance of an active in a cosmetic formulation is an appropriate delivery system. Many innovative cosmetic technologies control the delivery of active ingredients, and they can be divided into three broad types: vesicular (liposomes and niosomes), molecular (cyclodextrines), and particulate (micro-capsules and matrix particles). All of these delivery systems achieve a balance between the physicochemical requisites for stability of active and inactive constituents, preservation against microbial spoilage and presentation of the active molecules to the skin in a system that will allow appropriate release of the active to SC or viable skin layers (Morganti *et al.*, 2001:317).

2.3.7.1 LIPOSOMES

Liposomes are microscopic vesicles consisting of amphipathic lipids arranged in one or more concentric bilayers. These thermodynamically stable, lamellar structures form spontaneously when lipid is brought into contact with an aqueous phase. Unlike micelles, emulsions and micro-emulsions; liposomes have an entrapped, discontinuous aqueous phase separated by 4 nm thick, bilayered lamellae from the continuous aqueous phase (Uster, 1990:327). The lipid molecules are usually phospholipids, with or without cholesterol, and the lipids may be arranged in one or more bilayers (Williams, 2003:124).

Table 2.2: Liposome nomenclature (Uster, 1990:328).

Acronym	Vesicle diameter (μm)	Number of lamellae	Captured volume (ml/g)
MLV (Multilammellar vesicles)	> 0.1	≥ 5	≥ 0.6
SUV (small unilamellar vesicles)	0.03 < 0.06	1	0.6 – 1.9
LUV (large, unilamellar vesicle)	> 0.06	1	≥ 1.9
OLV (Oligolamelllar vesicles)	> 0.06	1 < ≤ 5	≥ 0.06

2.3.7.2 NIOSOMES

Niosomes are non-ionic surfactant vesicles prepared primarily from non-ionic surfactants. As with conventional liposomes prepared from phospholipids, the properties of niosomes can be modified by incorporating other excipients, such as cholesterol, into the membrane and they can possess one or more lipid bilayers encapsulating an aqueous core. Niosomes offer potential benefits over liposomes prepared from phospholipids. The components tend to have less inherent toxicity and would generally be less irritating to the skin. Both liposomes and niosomes are versatile in their compositions, sizes and entrapment efficiencies. From the comparisons available, it appears that drug delivery from niosomes may be less effective than from liposomes, though the increased fluxes reported from liposomes when compared to niosomes may simply result from using an optimised liposome system in comparison with a sub-optimal niosomal formulation (Williams, 2003:129).

2.3.7.3 ETHOSOMES

Ethosomes are comprised of phospholipids as with traditional liposomal formulations, but also incorporate high levels of an alcohol – usually ethanol. The use of ethanol, typically at 30% in the formulation, creates a 'soft' vesicle that can be modified in terms of size and lamellarity. They have also been shown to be capable of delivering compounds to the deeper skin layers or to the systemic circulation and they can be designed to improve transdermal delivery of lipophilic or hydrophilic molecules (Williams, 2003:134).

2.3.7.4 EMULSIONS AND MICRO-EMULSIONS OR NANO EMULSIONS

An emulsion is a system consisting of two immiscible liquid phases, one of which is dispersed through the other. The particles of the dispersed phase are usually between 0.1 – 10 µm in diameter. Since most drugs are hydrophobic and water-insoluble, oil in water (o/w) emulsions are used as vehicles to deliver such drugs. Nano-emulsions, also referred to as submicron emulsions, with droplet diameters in the size range of 0.02 – 0.20 µm and with a narrow size distribution, can solubilise water-insoluble drugs within the hydrocarbon core (Grobler *et al.*, 2008:285).

Nanotopes™ are a new carrier system for cosmetic actives constructed by ultra small spherical particles with a size of < 30 nm and therefore smaller than the inter-corneocyte pores. In contrast to phospholipid membrane systems, the monolayer membrane of the so-called Nanotopes™ comprises both phospholipids and a specific co-surfactant in a defined ratio. The co-surfactant acts as a membrane stabiliser and is inserted with its large hydrophilic conelike head group between the rather cylindrically shaped phospholipid molecules (Morganti *et al.*, 2001:317).

2.3.7.5 PHEROID™

Although several delivery technologies are available, most of them are hampered to some extent by stability problems, high cost to market and limited field of application, low solubility and bioavailability. Pheroid™ technology, based on what was previously called Emzaloid™ technology, is able to enhance the absorption and/or efficacy of various categories of active ingredients and other compounds and has been shown to result in marked improvements in the control of size, charge and the hydrophilic-lipophilic characteristics of therapies, when compared to other systems

(Grobler *et al.*, 2008:284). This formulation is used in several preparations for the treatment of many skin disorders such as psoriasis, eczema, and dermatitis and contains ethyl esters of the essential fatty acids, linoleic acid and linolenic acid as well as oleic acid, emulsified in water saturated with nitrous oxide (N_2O) (Saunders *et al.*, 1999:100).

The addition of a dispersed gas phase to the respective oil and water phases adds another dimension to the basic Pheroid™. The association of N_2O with the dispersed phase has been shown to have at least three functions:

- Contributing to the miscibility of the fatty acids in the dispersal medium;
- Contributing to the self-assembly process of the Pheroid™, as determined by zeta-potential and particle size analysis; and
- Contributing to the stability of the formed Pheroid™, as shown by accelerated and formal stability studies, as well as by its stability at high and low pH (Grobler *et al.*, 2008:284).

Molecular modelling indicates that there is some interaction between the fatty acids and the nitrous oxide, resulting in stable vesicular Pheroid™ structures. The nitrous oxide essential fatty acid (NOEFA) matrix thus provides a functional model for the transport of hydrophobic and hydrophilic drugs. It was noted in controlled experiments on various formulations that if either the N_2O or the essential fatty acids were absent from the formulations, the efficacy and stability of the formulation were dramatically decreased (Grobler *et al.*, 2008:284).

The design of the Pheroid™ allows for manipulation of both its structural and functional features. Surface charge of the Pheroid™ can be adapted by the degree of hydrogenation of the fatty acids. Mean particle size can be reproducibly manipulated by changing the composition and ratio of the fatty acids and by modification of the fatty acids is an inherent feature of the Pheroid™ and not an outer coating of the structure, as is the case in stealth liposomes or pegylation of drugs or delivery structures. Modifications also serve to negate direct uptake of the Pheroid™-entrapped compounds by phagocytes and increase the longevity. Various investigations into different Pheroid™ formulations have shown that the structural and functional characteristics of Pheroid™ can be manipulated by (Grobler *et al.*, 2008:284):

- Changing the fatty acid composition or concentrations;
- The addition of non-fatty acids or phospholipids such as cholesterol;

- The addition of cryo-protectants;
- The addition of charge-inducing agents;
- Changing the hydration medium (ionic strength, pH);
- Changing the method of preparation (subtle changes may have dramatic results);
- Changing the character and the concentration of the active compound; or
- The addition of sunscreen formulations (Grobler *et al.*, 2008:284).

The Pheroid™ is sterically stabilised by electro-chemical interaction and not by cholesterol, as is the case in most lipid-based delivery systems. Cholesterol results in rigid vesicular structure with an inability to extravastate, i.e., to deform in order to cross densely packed cohesive capillary walls, without fracturing, whereas the electro-chemical stabilisation of the Pheroid™ allows a very elastic vesicular structure. Pheroid™ have been shown to cross capillary walls. The dense SC offers a similar challenge and it's thought that the fluidity of the Pheroid™ membrane contributes to efficient dermal and transdermal delivery. The uptake of Pheroid™ by cells may be influenced by the Pheroid™ formulation and by the mechanism of uptake by the cells. The permeation of the Pheroid™ formulation is determined by one or more of the following factors:

- Size of the Pheroid™;
- Morphology of the Pheroid™;
- Molecular geometry of the fatty acids themselves;
- Concentration and ratios of the various fatty acids;
- Hydration medium (ionic strength, etc.);
- pH of the preparation'
- Presence of charge-changing molecules;
- Presence of molecules that influence the electrostatic milieu;
- Character and concentration of the active drug; and
- State of the Pheroid™ (i.e., either gel state or fluid state or in between) Grobler *et al.*, 2008:285).

The Pheroid™ is probably one of the most effective, versatile and inexpensive delivery systems in commercial use. All components used in the manufacturing of Pheroid™ are pharmaceutically safe and the system is based on the naturally occurring molecules of the body. The stability of the Pheroid™ delivery system has been proved for Pheroid™-based commercialised products. Nevertheless, although various studies support the use of Pheroid™ technology in cosmetic and pharmaceutical applications, the elucidation of all critical parameters is necessary if the technology is to be used to its full potential (Grobler *et al.*, 2008:289).

2.4 TRANSDERMAL DELIVERY OF ALPHA-LIPOIC ACID

2.4.1 TREATMENT OF ACNE WITH ALPHA-LIPOIC ACID

Acne vulgaris should not only be treated according to the symptoms of pustule, papule and comedone formation, but scar formation should be minimised and atrophic acne formed scars left after resolution of the active phase, should be treated. Scars are the result of the formation of granulation tissue as well as matrix formation following inflammation. ALA and/or its derivatives provide surprising benefits when used with currently employed acne medications as adjunct ingredients, because skin irritant side effects are minimised or eliminated.

Because of its solubility, ALA is sometimes referred to as a universal antioxidant. It acts as a free radical scavenger and neutraliser and prevents the cross-linking of cell membranes that is often seen in acne in its post-inflammatory phases. By the same token, ALA modulates free radicals and other oxidative species, and by doing so it affects gene expression, including expression of NFκ-B, nitric oxide synthetase and other mediators at all stages of pro-inflammation and inflammation. The alteration of lipid peroxidation, protein cross-linking, growth factor stimulation and membrane permeability may explain the effects of ALA on the symptoms of acne (Perricone, 2002:6).

ALA is fat-soluble. Therefore, preparations containing ALA can be applied neatly to skin areas subject to damage or already damaged. Because of its fatty nature, ALA physically contributes to the lubrication and soothing of affected skin areas (Perricone, 1998:3). The topical application of ALA provides a simple, non-invasive, non-toxic, over-the-counter topical method for treating all phases of acne. ALA compositions decrease pore size, minimising sebum accumulation and keratinous debris that cause both whiteheads and blackheads observed in acne. ALA minimises scar formation and provides marked losses of scar borders and decreases in scar depth where

scars have already formed. Topically applied ALA also seems to fill in scar tissue, making it more equal to adjacent normal skin (Perricone, 2002:2).

2.4.2 PROBLEMS ASSOCIATED WITH THE ABSORPTION OF ALPHA-LIPOIC ACID

2.4.2.1 PHYSICOCHEMICAL PROPERTIES OF ALPHA-LIPOIC ACID

Table 2.3 The physicochemical properties of ALA according to Merck & Co., Inc., 1999.

CAS Registry number	[62-46-4]
CAS name(s):	1,2-Dithiolane-3-pentanoic acid; 1-2-dithiolane-3-valeric acid
Molecular weight	206.33
Melting point	46 – 48 °C
pKa	5.4

2.4.2.2 POSSIBLE SOLUTIONS TO THE PROBLEM OF TRANSDERMAL DELIVERY OF ALPHA-LIPOIC ACID

Due to its very low aqueous solubility, ALA will have difficulty permeating the skin. This problem can be overcome by placing ALA in a carrier, particularly one in which it is soluble or in which it can be effectively solubilised (e.g. as an emulsion or micro-emulsion) (Perricone, 2002:6).

Another possible solution is to entrap ALA into the Pheroid™ delivery system. The efficacy of entrapment of a compound can be expressed as the percentage of the initial amount of compound added to the formulation that is entrapped (Grobler *et al.*, 2008:285).

The entrapment efficiency of Pheroid™-based products is generally determined by confocal laser scanning microscopy (CLSM), and both the Pheroid™ and the active compound are visualised through fluorescence labelling. An entrapment efficiency of more than 90% has been aimed at for all products in development. Due to the pliable system design of the Pheroid™, the number of colloidal particles per volume can be increased or decreased to suit the required concentration of active compound. The interior entrapment volumes of the Pheroid™ can be determined by the

general volume formula (πr^3), where r is the radius of the Pheroid™. The theoretical entrapment volume for increasing sizes of vesicular Pheroid™ can be calculated as follows:

Number of Pheroid™ % = (Total number of active molecules ÷ Average number of active molecules per Pheroid™) x 200 **(Equation 2.8)**

In this formula the term active molecule can also mean chemical compounds, biological compounds or structures such as peptides, crystals or the smallest unit within which the compound may occur within the formulation. Multiplication of the ratio with 200 is done as the number of Pheroid™ should preferably be double that of the molecules to ensure complete entrapment in equilibrium. The simplest Pheroid™ formulation contains an average of 150 Pheroid™/µl or 150 000 Pheroid™/ml of formulation. This number can be increased or decreased as required (Grobler *et al.*, 2008:285).

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

FORMULATION, *IN VITRO* RELEASE AND TRANSDERMAL DIFFUSION OF ALPHA-LIPOIC ACID

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Abstract

The pathogenesis of acne is multifunctional. A possible approach to acne treatment is to reduce reactive oxygen species that lead to the irritation and destruction of the follicular wall as well as the inflammatory progression of acne. Topical application of antioxidants to the skin is a promising approach to support the endogenous antioxidant defence and avoid oxidative injury that may lead to acne. The cosmeceutical active alpha-lipoic acid was employed in this study due to its anti-oxidant and anti-inflammatory effects. The aim of the study was to investigate *in vitro* transdermal diffusion of alpha-lipoic acid with the aid of the novel Pheroid™ drug delivery system. Four 1% alpha-lipoic acid formulations for topical application were investigated by means of vertical Franz cell diffusion studies. These studies were conducted over a period of 12 h, using female abdominal skin. Phosphate buffered solution (pH 7.4) was used as the receptor phase. Each formulation (1 ml) was applied to the donor phase. *In vitro* penetration of alpha-lipoic acid was assayed by HPLC. The Pheroid™ proved beneficial for transdermal diffusion of alpha-lipoic acid. Stability tests were conducted on the formulations. HPLC proved alpha-lipoic acid to be the most stable in the Pheroid™ cream formulation.

Keywords: Alpha-lipoic acid, Acne, Transdermal diffusion, Pheroid™, Formulation

1. Introduction

Acne vulgaris is a disease of the pilosebaceous units and is driven by a variety of pathogenic factors. While the exact mechanisms are not fully elucidated, the most common pathways to acne include excess sebum production, hyperkeratinisation of the hair follicle, oxidative stress and the release of inflammatory mediators. The actual disease process in acne is not life-threatening, however, acne is not merely a cosmetic problem as the condition itself can bring about significant psychological effects (Katzman & Logan, 2007). Psychological scars caused by acne include lowered self-esteem and professional expectations, social reticence, depression and anxiety. Furthermore, severe acne has been connected with decreased employability in adulthood. Therefore, it must be recognised as a serious disorder (Gollnick, 2003).

In acne, reactive oxygen species (ROS) may be released from damaged follicular walls. ROS are toxic molecules that play a critical role in inflammatory skin diseases. *P. acnes* causes the release of some chemotactic factors leading to neutrophil accumulation, which leads to damage of the follicular epithelia after the release of some inflammatory factors such as lysosome enzymes as a result of phagocytosis. ROS are released from the active neutrophils in the inflammatory tissue (Arican et al., 2005). Oxidative stress is present both locally and systemically in patients with acne (Katzman & Logan, 2007). Therefore, both dermal and transdermal drug delivery of alpha-lipoic acid will contribute to the reduction of ROS that may lead to acne. Destruction of the follicular wall is mostly responsible for the release of ROS which may lead to acne (Arican et al., 2005). The follicular wall is located in the dermis which is thus the main target of delivery for alpha-lipoic acid.

The cosmeceutical active, alpha-lipoic acid was employed during this study due to its anti-oxidant and anti-inflammatory effects. The term "cosmeceutical" is attributed to Dr. Albert Kligman and can be regarded as a hybrid category of products lying on the border between drugs and cosmetics. Cosmeceuticals exert a pharmaceutical therapeutic benefit but not necessarily a biologic therapeutic benefit. The difference between a drug and a cosmeceutical, is that the former is

defined by having a biological effect on living tissue (Choi and Berson, 2006). Alpha-lipoic acid is unique among antioxidant molecules, because it retains protective functions in both its reduced and oxidised forms (Navari-Izzo et al., 2002). Its small molecular weight in combination with its solubility characteristics suggest the possibility of being absorbed by the skin (Beitner, 2003). It is soluble in both water and fat, which suggests that it connects the activity of antioxidants in the membranes with that of antioxidants in the cytoplasm, strengthening the antioxidant network of the cells (Navari-Izzo et al., 2002).

Penetration of compounds into or through the skin has been widely investigated and the factors that play a role in the absorption of these molecules by the skin are well understood. Conventional topical vehicles, such as ointments, creams or gels, predominantly exert their effect by releasing the drug onto the surface, and the drug molecules then diffuse through the skin layers (Foldvari, 2000).

The patented Pheroid™ delivery system was employed as a carrier system for the selected cosmeceutical active in order to determine whether it would enhance its penetration to the skin. Pheroid™ is a colloidal system that contains unique stable lipid-based submicron- and micron-sized structures, called Pheroid™, which are equally distributed in a dispersion medium (Grobler et al., 2008). In this study the *in vitro* transdermal delivery of formulations containing alpha-lipoic acid, entrapped in the Pheroid™ delivery system was investigated.

2. Materials and methods

2.1 Materials

The cosmeceutical active alpha-lipoic acid was obtained from Sigma. Sodium hydroxide and dihydrogen potassium phosphate, used for the preparation of phosphate buffered solution (PBS) were supplied by Merck. Reagents used during the high pressure liquid chromatography (HPLC) method included: acetonitrile, dried methanol, orthophosphoric acid and iso-octane obtained from Merck and Saarchem. Water used during this study was purified by a Milli-Q® Academic purification system (Millipore, Milford, USA). Reagents used during the manufacturing of large batches of alpha-lipoic acid creams and gels included: vitamin F and *d-l*-alpha tocopherol (which were both obtained from Chempure), cetyl alcohol, liquid paraffin, span 60, tween 80, methylparaben and butylated hydroxytoluene (BHT) (which were obtained from Merck), butylated hydroxyanisole (BHA) which was obtained form Sigma, xantam gum (which was obtained from DB Fine chemicals) and nitrous oxide water.

2.2 Methods

2.2.1 Stability testing

The purpose of stability testing is to provide evidence on how the quality of a drug substance or product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light; and to establish a re-test period for the drug substance or a shelf-life for the drug product as well as recommend storage conditions (International conference on harmonisation, 2003).

The stability program comprised the storage of all products at temperatures and humidities of 25 °C/55% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH, over a period of three months. Test intervals were at onset of the stability program (initial), at one month, two months and three months. All stability tests were done using calibrated and/or validated apparatus, where appropriate.

The following tests were conducted on Pheroid™ cream, Pheroid™ gel, cream and gel over a period of three months: assay, pH, weight variation, viscosity, physical appearance and confocal laser scanning microscopy (CLSM).

2.2.1.1 Assay

All alpha-lipoic acid formulation assays were conducted on HPLC. The HPLC analysis of alpha-lipoic acid was performed by using an Agilent 1100 Series HPLC. The machine is designed with a G1311A quaternary pump, G1315A diode array detector, G1313A auto sampler injection mechanism, G1322A vacuum degasser, solvent module and HP Chemstation Software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25 °C. A high performance silica based, reversed phase Phenomenex Luna C18 (2) column, (150 x 4.6 mm) with a 5 µm particle size was used. To maintain the condition of the column, it was rinsed each time before storage with HPLC grade water at a flow rate of 1.0 ml/min for 20 min, then with 70% acetonitrile for a further 20 min and finally with isopropyl alcohol for the last 20 min. The mobile phase consisted of 50 volumes of methanol and 50 volumes of distilled water containing octan-sulphonic acid. The flow rate was set at 1 ml/min and the injection volume 20 µl.

2.2.1.2 pH

A Mettler Toledo Seveneasy (Switzerland) pH meter, which was calibrated with buffer solutions of pH 4.01, 7.00 and 10.01 prior to measurements, was used to measure the pH of the formulations.

2.2.1.3 Weight variation

Weight variation from months 1 to 3 was determined on a Mettler Toledo (Switzerland) balance. Formulations were weighed in their closed, semi-permeable plastic containers at month 0, 1, 2 and 3. The weight of the empty container (with lid) was then subtracted from the weight to determine the weight of the formulation.

2.2.1.4 Viscosity

The viscosity was determined with a Brookfield Model DV –II+ (United States of America) viscometer. Viscosities of the four different formulations were measured. Spindle S 96 was used, but the speed was adjusted according to the assumed viscosity of each formulation.

2.2.1.5 Physical appearance

Physical assessment of each formulation was carried out once a month at month 0, 1, 2 and 3. The physical appearance of each product was examined and compared to the initial results. Changes in colour and texture were noted.

2.2.1.6 Confocal laser scanning microscopy

The purpose of CLSM is to determine the size distribution of the Pheroid™ microsponges. Phospholipid components of the Pheroid™ were stained with phenoxazine dye nile red (Haugland, 2005). A sample was placed on a glass slide and covered with a glass cover-slip and sealed with an adhesive to prevent the Pheroid™ from drying out. The CLSM used to capture the images was a Nikon PCM 2000 CLSM, with a DXM 1200 digital camera for real-time imaging and ApoPlanar oil immersion objective with numerical aperture (NA) of 1.4, and was equipped with a red helium/neon laser and green argon laser.

2.3 Experimental aqueous solubility determination

The aqueous solubility of alpha-lipoic acid was obtained by preparing PBS at pH 7.4. Super-saturated solutions of alpha-lipoic acid in PBS were placed in a pre-heated water bath of 32 °C and stirred for 24 h. Hereafter the samples were filtered and diluted by factor 100 before being analysed by means of HPLC. The experiment was conducted in triplicate.

2.4 Experimental octanol-buffer partition coefficient (log D) determination

n-Octanol (200 ml) was pre-saturated with 200 ml PBS (pH 7.4) 24 h prior to experimental procedures. Hereafter the two layers were separated and stored in different containers. 2.4 mg

(0.8 mg/ml) of alpha-lipoic acid, dissolved in pre-saturated octanol (3 ml), was shaken in a water bath for 10 min. Pre-saturated buffer (3 ml) was added to the solution above and shaken for 45 min. Octanol-buffer mixtures were centrifuged at 4000 rpm for 30 min at 32 °C. The PBS phase was diluted ten times and analysed by means of HPLC. Experiments were done in triplicate.

2.5 Skin preparation for diffusion studies

Excised human skin from female patients, who had undergone abdominal plastic surgery, was used (Leveque et al., 2004). The skin was frozen at -20 °C within 24 h after removal. At this temperature the skin is stable with regard to the penetration of drugs, as well as the thickness of the stratum corneum over a time period of 3 and 6 months, respectively (Leveque et al., 2004). Full-thickness skin (containing the overlying dermis, the viable epidermis and the outermost stratum corneum) (Williams, 2003) was used during this study. The innermost subcutaneous fat layer (hypodermis) (Williams, 2003) was carefully separated from the skin with a scalpel to avoid damage to the skin. Damaged or ruptured skin may lead to incorrect results. The skin was punched into circles with a diameter of approximately 15 mm and placed onto Whatman® filter paper with the stratum corneum side facing upwards and left to air-dry. The skin circles on the filter paper were then covered with aluminium foil and kept frozen at -20 °C until used. Frozen skin samples were thawed at room temperature (25 °C) prior to the diffusion study and thoroughly examined for any defects before being placed on the Franz cells. Ethical approval for the procurement and exploitation of the skin was provided by the Research Ethics Committee of the North-West University under reference number 04D08. The identities of the skin donors were confidential and informed consent was obtained beforehand.

2.6 Franz cell diffusion method

Vertical Franz cells with 2 ml receptor compartments and 1.0751 cm^2 effective diffusion areas were used for the permeation studies. These Franz cells consist of a donor (top) and a receptor (bottom) compartment where the drug solution and the receptor fluid (PBS, pH 7.4) were placed,

respectively. A small magnetic stirring bar was placed in each receptor compartment in order to maintain stirring throughout the experiment. Full-thickness skin was thawed and mounted between the receptor and donor compartments, with the stratum corneum facing upwards. Dow Corning® high vacuum grease was applied to each Franz Cell in order to prevent any leakage. The receptor compartments were filled with PBS (pH 7.4). Special care was taken to ensure that no air bubbles were trapped beneath the skin. The donor compartments were filled with 1 ml of the alpha-lipoic acid formulation under investigation and subsequently covered with Parafilm® to prevent evaporation for the duration of the experiment. A horseshoe clamp was used to secure the donor and receptor compartments. In order to control the temperature, the receptor compartments were placed directly in a 37 °C water bath to attain a skin temperature of 32 °C (Cleary, 1993). Only the receptor compartments (equipped with stirring magnets) were submerged in the water. The cells were stirred by using a magnetic stirrer plate at a speed of 750 rpm. Ten Franz cells were used for each experiment. The entire receptor compartment was emptied and replaced with fresh 37 °C PBS (pH 7.4) after 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 4.00, 6.00, 8.00, 10.00 and 12.00 h. The entire receptor volumes were withdrawn to mimic sink conditions as they occur in the human body. The withdrawn samples were directly assayed by HPLC to determine the drug concentration which had permeated through the stratum corneum.

2.7 Tape stripping

At 12 h, after the removal of the donor and receptor phases, the diffusion cells were carefully dismantled and the skin was pinned to a piece of Parafilm® which was stapled to a solid surface. Skin was dabbed dry with tissue paper and pieces of tape were cut to a length that covered the diffusional area; but did not overlap with the areas outside of the diffusion cell imprints. Exposed diffusional area (≈1.075 cm^2) was visibly marked by the groove from the diffusion cells (≈11.7 mm diameter). The first tape strip was discarded and the following 15 tape strips were placed in a vial containing 5 ml PBS. Complete removal of the stratum corneum was indicated by the glistening of

the viable epidermal layer after 15 strips (Pellet, 1997). Excess skin was trimmed away from the flange imprints of the diffusion cells and the remaining skin (dermis) was cut into pieces to enlarge the surface area. It was then placed in 2 ml of PBS (pH 7.4). Samples were filtered and assayed.

2.8 Statistical analysis of diffusion studies and tape stripping

For statistical analysis, the median (statistically calculated 50 percentile or centre of a given set of data) of the flux values were examined. The median flux is a more accurate method for determining flux, if a huge difference in the experimental average flux values occurs (Gerber et al., 2008). A Wilcoxon two-sample test (Steyn et.al., 1998) was performed to evaluate the differences in median flux values using the NPAR1WAY procedure of SAS (SAS Institute, Inc., 2005). A Wilcoxon two-sample test was also performed to evaluate the differences in median flux between the formulations that diffused into the epidermis and dermis, respectively. A p-value less than 0.05 would indicate a statistical significance between the values.

3. Results and discussion

3.1 Stability testing

3.1.1 Assay

Significant change for a drug product is defined as a 5% change in assay from its initial value (International conference on harmonisation, 2003). From the stability test results, the amount of alpha-lipoic acid in the Pheroid™ cream, Pheroid™ gel, cream and gel appeared unstable at elevated temperatures. The change in assay for alpha-lipoic acid, methylparaben, BHA, BHT and *d-l*-alpha tocopherol revealed that significant change took place between month 0 and month 3. Alpha-lipoic acid proved to be the most stable when formulated in the Pheroid™ cream. A possible explanation for the increase in the amount of alpha-lipoic acid in Pheroid™ cream after one month was that some of the water might have evaporated form the formulation, thus increasing the concentration of the active. Further investigation is necessary in order to improve the stability of the formulations as all the formulations proved to be unstable over time.

3.1.2 pH

Significant change is observed in a product when the product fails to meet the acceptance criterion for pH (International conference on harmonisation, 2003). Alpha-lipoic acid is a weak acid with a p_{Ka} value of 5.4. The pH of formulations should be maintained at a pH between 5 and 9 in order to obtain ideal transdermal delivery (Naik et al, 2000). The pH of the Pheroid™ cream, Pheroid™ gel, cream and gel was found to be stable and favourable with regard to alpha-lipoic acid, but was not favourable for ideal transdermal delivery.

3.1.3 Weight variation

According to the US Pharmacopoeia (2009) weight variation is determined by weighing 10 containers individually. The contents of the containers should be carefully removed and accurately weighed. The empty containers should then be weighed and the weight of the contents subtracted to render gross weight. Weight variation could not be determined according to the USP standards,

because the size of the manufactured batch was insufficient. The weight of only one container of each formulation at each temperature was measured and noted.

3.1.4 Viscosity

The viscosity of the Pheroid™ cream varied significantly from months 0 to 3, proving that significant change had taken place. The viscosity values of Pheroid™ gel fluctuated marginally from months 0 to 2 indicating that the spreadability remained stable for 2 months. Month 3 differed largely from month 2, indicating significant change. The viscosity values for cream over the three-month period fluctuated only slightly. This fluctuation could have been regarded as insignificant and thus proves that the cream was stable for the three-month period. The aforementioned was also depicted for the gel formulation.

3.1.5 Physical appearance

No significant change in appearance was observed at 25 °C/55% RH for the Pheroid™ cream from months 0 to 3. The Pheroid™ cream at 30 °C/60% RH depicted slight change in colour after 3 months and at 40 °C/75% RH a clear change in colour could be seen after 2 months. As with the Pheroid™ cream, no significant change in colour was observed for the Pheroid™ gel at 25 °C/55% RH over the three-month period. No significant change was seen at 30°C/60% RH either. Pheroid™ gel at 40 °C/75% RH changed to a yellow colour after 3 months. No significant change in the physical appearance in cream was seen over the three-month period at all temperatures. The appearance of the gel at 25 °C/55% RH and 30 °C/60% RH remained the same for the three-month period. At 40 °C/75% RH the colour changed significantly after 3 months. It appears that the aesthetics of the formulations without Pheroid™ stayed acceptable for a longer time period than that of the formulations containing Pheroid™.

3.1.6 *Confocal laser scanning microscopy*

Visual assessment of the Pheroid™ sponges on microscopic level was done with CLSM. No crystals or other impurities were observed in any of the formulations at any of the three

temperatures. No change in the microscopic consistency of the Pheroid™ cream or cream was observed over the three-month period at any of the three temperatures. The size of oil droplets in both the Pheroid™ gel and gel seemed to increase after one month at 40 °C/75 %RH.

3.2 Experimental aqueous solubility and log D determinations

The solubility of alpha-lipoic acid was found to be 8.602 mg/ml in PBS (pH 7.4) at 32 °C, which is above the value of 1 mg/ml required for ideal transdermal delivery (Naik et al., 2000). To enable a compound to traverse the stratum corneum, it needs to have an octanol-water partition coefficient (log P) in the region of 1 to 3 (Williams, 2003). The log D value determined for alpha-lipoic acid was -0.78 which is a value unfavourable for transdermal permeation. The aqueous solubility proved favourable for penetration through the skin whereas the log D value predicted that the permeation may not be optimal.

3.3 Franz cell diffusion study and tape stripping data

Figure 1 Box-plots and red lines of the flux values of alpha-lipoic acid in the Pheroid™ cream and Pheroid™ gel formulations after application to the skin to illustrate median and average flux, respectively.

3.3.1 Pheroid™ cream diffusion

As seen in figure 1, the cumulative concentration of alpha-lipoic acid that permeated through the epidermis was plotted against time. Average flux was calculated from the slope of the straight line. The profile of the Pheroid™ cream chart exhibited a biphasic flux pattern. A clear steady state flux from 0 to 2 h was observed after which the cumulative concentration of alpha-lipoic acid remained the same from 3 to 6 h, indicating that the entire amount of alpha-lipoic acid in Pheroid™ cream had diffused through the skin. The average and median flux values from 0 to 2 h were 58.01 ± 6.63 $\mu g/cm^2.h$ and 57.831 $\mu g/cm^2.h$, respectively. The average and median flux values were found to be

very similar. Either one of the values could be used to indicate flux. If there had been a significant difference between these two values, the median flux value would have been used to indicate flux.

3.3.2 Pheroid™ gel diffusion

The cumulative concentration of alpha-lipoic acid that permeated through the epidermis was plotted against time. Average flux was obtained by the slope of the straight line. Pheroid™ gel exhibited a linear flux pattern from 4 to 12 h. The average and median flux of Pheroid™ gel from 4 to 12 h were 22.18 ± 3.33 μg/cm^2.h and 20.299 μg/cm^2.h, respectively. Only a slight difference between the average and median flux values was found, indicating that either one could be used as the flux value.

3.3.3 Cream diffusion

The average flux for alpha-lipoic acid in the cream could not be calculated as none of the active diffused into the receptor phase of the Franz cells. The alpha-lipoic acid in cream was detected in the epidermal layer during tape stripping.

3.3.4 Gel diffusion

The average flux for alpha-lipoic acid in gel could not be calculated, as none of the active diffused into the receptor phase of the Franz cells. The alpha-lipoic acid in the gel was detected in the dermis during tape stripping.

3.4 Tape stripping data

The concentration of alpha-lipoic acid in Pheroid™ cream and cream found in the epidermis was 569.10 and 764.93 μg/ml, respectively; both the formulations revealed 0 μg/ml in the dermis. The concentration of alpha-lipoic acid in Pheroid™ gel and gel found in the dermis was 23.62 and 61.06 μg/ml, respectively; both the formulations depicted 0 μg/ml in the epidermis. Alpha-lipoic acid in both the cream formulations favoured the epidermis whereas in the gel formulations it diffused into the dermis.

3.5 Statistical analysis of diffusion studies and tape stripping

A Wilcoxon two-sample test was performed to evaluate the differences in median flux values for two groups: Pheroid™ gel and gel after 12 h using the NPAR1WAY procedure of SAS (SAS Institute, Inc., 2005). A p-value less than 0.05 would indicate a statistical significance between the medians of the two groups on a 5% level of significance. A p-value of 0.0002 was obtained, indicating a highly statistically significant result.

A p-value of < 0.0001 was obtained to evaluate the differences in median flux between the formulations that diffused into the dermis. The same test was performed on the formulations that remained in the epidermis after diffusion. A p-value of < 0.0001 was obtained, rendering a statistically significant difference between the two medians .

A correlation value of -0.857 between flux and the concentration of alpha-lipoic acid in the dermis was obtained after a CORR procedure of SAS (SAS Institute, Inc., 2005). This means that the concentration of alpha-lipoic acid in the dermis increased as the flux decreased. A p-value of < 0.0001 was obtained which indicated that the correlation was statistically significant. A correlation value of -0.824 between the concentration of alpha-lipoic acid in the epidermis and alpha-lipoic acid in the dermis was further obtained. This means that the concentration of alpha-lipoic acid in the epidermis decreased as the concentration of alpha-lipoic acid in the dermis increased. A p-value of < 0.0001 was obtained rendering a statistically significant correlation.

4. Conclusion

Alpha-lipoic acid was detected in the receptor compartments of the formulations containing Pheroid™ and was absent in the receptor compartments of the formulations not containing Pheroid™. This proved that the Pheroid™ was favourable for penetration of alpha-lipoic acid through full-thickness skin. According to Katzman & Logan (2007), the ROS that may lead to acne are present in both the dermis and the systemic circulation. The target areas for transdermal and topical delivery of alpha-lipoic acid were thus reached during the diffusion studies. There are various explanations for the improved transdermal diffusion of formulations containing Pheroid™. The cetyl alcohol used in the cream formulations as a thickener may work synergistically with Pheroid™ sponges to facilitate improved penetration of the SC. The Pheroid™ gel formulations did not contain cetyl alcohol, and therefore, may have exhibited lower average flux values. Another possible way of enhancing transdermal flux for formulations is to alter the pH of the formulations. It has been found that formulations with a pH value of between 5 and 9 exhibit improved transdermal drug delivery (Naik et al., 2000). The increased systemic delivery of formulations containing Pheroid™ may be due to the interaction of alpha-lipoic acid with nitrous oxide (NO) present in the Pheroid™ sponges, which was proved to provide a functional model for the transport of both hydrophobic and hydrophilic drugs (Grobler et al., 2008). Another possible reason for improved systemic drug delivery of the Pheroid™ is the fact that it contains a large amount of surface-active ingredients, which may act as penetration enhancers.

Tape stripping showed significant differences between the epidermis and dermis. The amount of alpha-lipoic acid in the dermis was higher in the Pheroid™ gel and the gel formulations, whereas the cream formulations were more effective in delivering alpha-lipoic acid to the epidermis. This may be due to the reaction of the more hydrophobic cream formulations with the lipids of the SC and the diffusion of the more hydrophilic gel formulations through the SC into the dermis.

When comparing the average flux with both dermal and transdermal drug delivery, alpha-lipoic acid will contribute to the reduction of ROS that may lead to acne. Both dermal and transdermal drug delivery of alpha-lipoic acid will contribute to the reduction of ROS that may lead to acne. Median flux values of Pheroid cream™ and Pheroid™ gel revealed that the flux values differed only slightly, due to small variation within the flux values. This indicates that either of the values can be used as the true flux value.

None of the four formulations complied with all of the stability tests over the three-month period. Further investigations are necessary to ascertain the exact cause of the instability of the formulations in order to improve the results of stability tests. Micro-organisms might be a possible cause of the degradation of the formulations, especially at high temperatures. A possible solution for the instability is to incorporate different preservatives into the formulations, preferably preservatives with broad antibacterial spectra. The oxidation of *d-l*-alpha tocopherol is a possible explanation for the changes in colour. A replacement for *d-l*-alpha tocopherol will possibly improve the aesthetics of the formulations over the three-month period.

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FIGURE LEGENDS

Figure 1 Box-plots and red lines of the flux values of alpha-lipoic acid in the Pheroid™ cream and Pheroid™ gel formulations after application to the skin to illustrate median and average flux, respectively.

FIGURES

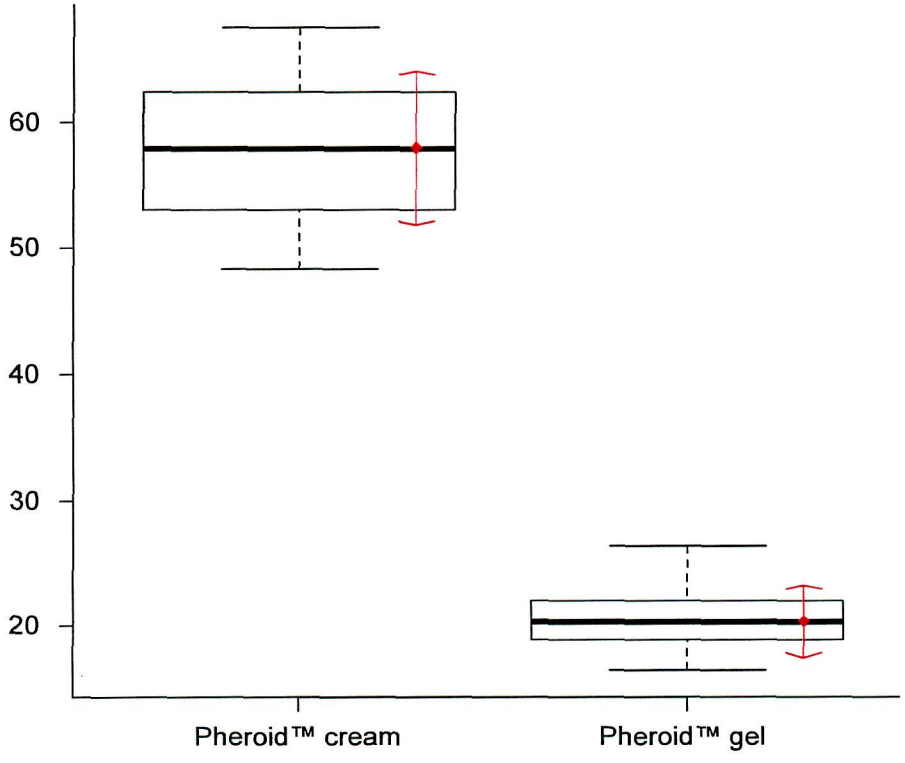

Figure 1 Box-plots and red lines of the flux values of alpha-lipoic acid in the Pheroid™ cream and Pheroid™ gel formulations after application to the skin to illustrate median and average flux, respectively

CHAPTER 4: FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

The main objective of this study was to investigate the possibility of improving the transdermal delivery of formulations containing alpha-lipoic acid by using an innovative delivery system, namely Pheroid™ technology. The interest in alpha-lipoic acid based skin care products has been growing significantly due to the fact that this active is less irritating than other acne treatments such as tretinoin or hydroxy acids. Therefore, it might be useful, even at low concentrations, to treat delicate areas such as those around the eyes. Additionally, alpha-lipoic acid is an inexpensive and widely available active substance, showing a lipophilic character that might contribute to increase its loading when incorporated into lipid nanoparticles (Souto *et al*, 2005:581).

Acne vulgaris is an extremely common disease. It can be found in nearly all teenagers to some degree as well as women in their thirties. Regardless of severity, acne often has a larger psychological effect than cutaneous effect. Studies have shown that people with severe acne as teens are less employable as adults and that self-esteem is low (Webster, 2001:15). The pathogenesis of acne is complex, with strong evidence supporting the involvement of sebaceous hyperplasia, follicular hyperkeratinisation, bacterial hypercolonisation, as well as immune reactions and inflammation (Gollnick, 2003:1579).

The physicochemical properties of compounds greatly influence their penetration through the skin (Williams, 2003:35). Actives with balanced log P values in the range of 1 to 3 will partition reasonably well between the hydrophilic and lipophilic domains in the skin (Hadgraft, 2004:292). The aqueous solubility and log D values of alpha-lipoic acid were experimentally determined. The log D value of alpha-lipoic acid was determined to be -0.78, whereas the solubility of alpha-lipoic acid was found to be 8.60 mg/ml in PBS (pH 7.4) at 32 °C. According to Hadgraft (2004:292) the log D value proved to be unfavourable for permeation through the skin. The aqueous solubility, on the other hand, was favourable for transdermal diffusion.

The experimental determination of transdermal flux of the alpha-lipoic acid formulations revealed that Pheroid™ improved the transdermal delivery of the active ingredient. Average flux of Pheroid™ cream from 0 to 2 hours was 58.01 ± 6.63 $\mu g/cm^2.h$. The average flux of Pheroid™ gel from 4 to 12 hours was 22.18 ± 3.33 $\mu g/cm^2.h$. The average flux for alpha-lipoic acid in the cream and gel formulations could not be calculated, as none of the active diffused into the receptor phase of the Franz cells. Cream remained in the epidermis whereas the gel was found in the dermis.

Tape stripping data established that alpha-lipoic acid in both the cream formulations favoured the epidermis whereas in the gel formulations the active diffused into the dermis. The concentrations of alpha-lipoic acid in Pheroid™ cream and cream that remained in the epidermis after application to the skin, were 569.10 µg/ml and 764.93 µg/ml, respectively. Concentrations of alpha-lipoic acid in Pheroid gel and gel that diffused into the dermis were 23.62 µg/ml and 61.06 µg/ml respectively.

A further aim of the study was to conduct stability tests on the formulations. The stability program comprised the storage of all products at temperatures and humidities of 25 °C/55% RH, 30 °C/60% RH and 40 °C/75% RH, over a period of three months. Test intervals were at onset of the stability program (initial), at one month, two months and three months. All stability tests were done using calibrated and/or validated apparatus, where appropriate.

The following tests were conducted on Pheroid™ cream, Pheroid™ gel, cream and gel over a period of three months: Assay, pH, weight variation, viscosity, physical appearance and confocal laser scanning microscopy. Assay results revealed that the concentration of methylparaben decreased significantly over the 3 month period. This indicated that it was insufficient to use methylparaben as the only preservative. Preservation may be improved by combining methylparaben with another preservative. BHA and *d-l*-alpha tocopherol failed to comply with the stability test standards and is thus not usable for anti-oxidant purposes. Alpha-lipoic acid was found to be unstable in all four of the formulations over the three month period.

According to Naik *et al* (200:319), none of the pH values of the formulations were ideal for transdermal delivery (pH 5 - 9). The pH values were however ideal for the transdermal permeation of alpha-lipoic acid (p_{Ka} 5.4) as it is predominantly unionised at the pH values obtained.

The small changes in the viscosity over the 3 month period in formulations without Pheroid™ compared to the large differences in viscosity in formulations containing Pheroid™, revealed that the formulations without Pheroid™ were more stable. This can be seen when the % RSD values are compared to each other.

Colour changes were clear in the Pheroid™ cream, Pheroid™ gel and gel formulations over the three-month period, especially at 40°C/75%RH. No apparent change in colour was observed in the cream formulation. The changes in colour may be due to oxidation.

Confocal scanning laser microscopy revealed that the formulations were homogenous. At 40 °C/75 % RH there was a clear increase in the oil droplet size of the Pheroid™ gel and gel

formulations. The Pheroid™ cream and cream formulations remained the same throughout the 3 month period.

In future it may be advisable to render further investigation into the following aspects:

- The stability of the formulations can be improved. This may be done by using different preservatives and anti-oxidants.
- Clinical trials can be performed to test the efficacy of formulations containing the Pheroid™ technology compared to formulations without Pheroid™ for the treatment of acne.

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ANNEXURE A: ANALYSIS OF ALPHA-LIPOIC ACID

A.1 THE HPLC SYSTEM

Analytical instrument: The HPLC analysis of Alpha-lipoic acid was performed by using an Agilent® (Agilent Technologies, Palo Alto, (A)) 1100 Series HPLC (High Performance Liquid Chromatograph). The instrument consisted of a G1311A quaternary pump, G1315A diode array detector, G1313A autosampler injection mechanism, G1322A vacuum degasser, solvent module and HP Chemstation Software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25 °C.

Column: A high performance silica based, reversed phase Phenomenex® Luna C18 (2) column, (150 x 4.6 mm) with a 5 µm particle size was used (Phenomenex, Torrance, CA). To maintain the condition of the column, it was rinsed each time before storage with HPLC grade water at a flow rate of 1.0 ml/min for 20 minutes, then with 70% CH_3CN for a further 20 minutes and finally with $(CH_3)_2CHOH$ for the last 20 minutes.

Mobile phase: The mobile phase consisted of 50 volumes of acetonitrile and 50 volumes of distilled water containing 0.1% ortho-phosphoric acid.

Flow rate: 1 ml/min

Injection volume: 50 µl

Retention time: The analyte elutes at approximately 5.5 minutes.

Detection: Diode array detector at 210 nm

A.2 PREPARATION OF THE STANDARD SOLUTION

Weigh approximately 10 mg of alpha-lipoic acid accurately and dissolve in 100 ml HPLC water. Subsequently, transfer 5 ml of the above mentioned solution into a 50 ml volumetric flask. Make up to volume with HPLC grade water. The concentration range includes standard solutions of 100, 50, 10, 5, 1, 0.5 and 0.1µg/ml. Standards are transferred to the autosampler vials for analysis.

A.3 VALIDATION OF THE HPLC METHOD

A.3.1 LINEARITY

The linearity of an analytical method is its capacity (within a given range) to elicit test results which are directly proportional to the amount (concentration) of analyte in the sample. The linearity of alpha-lipoic acid was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml) of the standards, prepared as described above.

The regression value (r^2) of 0.999 obtained for alpha lipoic acid indicated a high degree of linearity and therefore, demonstrates good stability of the analysis system. Figure A.1 depicts the linear regression curve of peak area of alpha-lipoic acid versus concentration

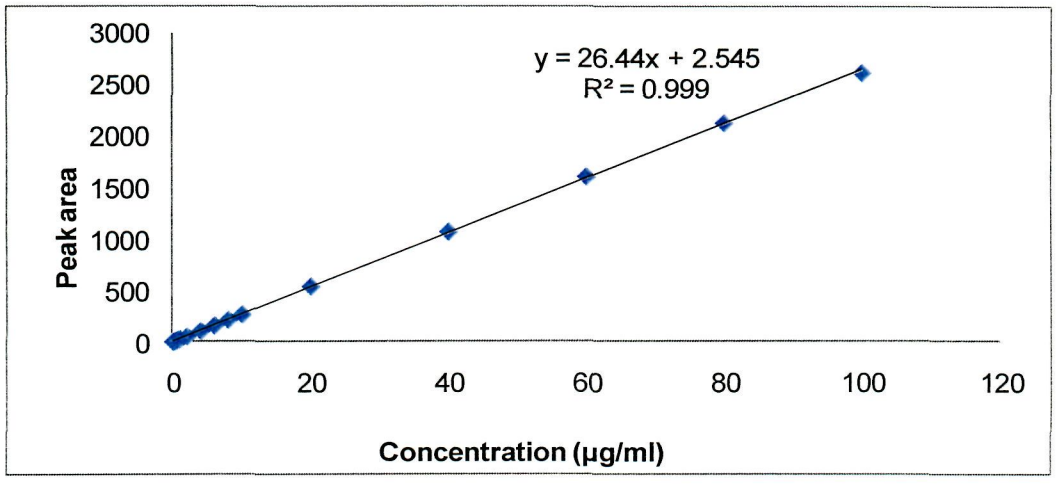

Figure A.1 Alpha-lipoic acid linear regression curve of peak area versus concentration

A.3.2 ACCURACY

The accuracy of an analytical procedure represents the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Concentrations of 1 µg/ml, 10 µg/ml and 100 µg/ml were prepared from a standard solution and injected in duplicate into the chromatograph. Recovery proved to be between 98 % and 102 %.

Table A.1 Accuracy parameters of alpha-lipoic acid

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
1.0	28.77	28.94	28.86	0.99	99.47
1.0	29.04	28.91	28.98	1.00	99.93
1.0	29.67	29.22	29.45	1.02	101.70
10.0	262.41	261.33	261.87	9.80	98.05
10.0	265.63	266.69	266.16	9.97	99.7
10.0	266.56	265.18	265.87	9.96	99.6
100.0	2602.55	2598.32	2600.44	98.22	98.2
100.0	2698.74	2672.31	2685.53	101.44	101.4
100.0	2625.74	2691.72	2658.73	100.43	100.4

Mean	99.83
SD	1.18
%RSD	1.18

Conc. refers to concentration (µg/ml)

AUC refers to the area under the curve

SD refers to standard deviation

%RSD refers to relative standard deviation

A.3.3 PRECISION

A.3.3.1 INTRA-DAY PRECISION (REPEATABILITY)

Concentrations of 100, 10 and 1 µg/ml was prepared from a standard solution and injected in triplicate into the chromatograph on the same day (n = 3). The percentage recovery was found to

be between 98 and 102 % with an SD of 1.21. Repeatability complied with the pharmaceutical standard that allows a SD of 2.

Table A.2 Intra-day precision parameters of alpha-lipoic acid

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
102.34	2197.09	2198.02	2197.56	104.21	101.83
99.80	2089.29	2085.60	2087.44	98.99	99.19
120.25	2508.39	2569.34	2538.87	120.40	100.12
9.56	199.76	199.97	199.87	9.48	99.14
9.42	194.04	194.68	196.36	9.31	98.85
9.31	194.39	194.64	194.52	9.22	99.08
1.03	22.15	22.08	22.12	1.05	101.82
0.89	18.86	18.01	18.44	0.87	98.23
1.12	23.73	23.61	23.67	1.12	100.22

Mean	99.83
SD	1.21
%RSD	1.21

A.3.3.2 INTER-DAY PRECISION

A concentration of 10 µg/ml was prepared from a standard solution and injected in triplicate into the chromatograph (n = 3). The % recovery was found to be between 85 % and 94 % with an SD of 2.74. Inter-day repeatability complied with the pharmaceutical standard that allows a SD of 5.

Table A.3 Inter-day precision parameters of alpha-lipoic acid

Conc (µg/ml)	Day 1	Day 2	Day 3	Between days
	91.65	86.34	88.14	
	92.81	86.24	88.87	
	93.99	85.85	89.59	
Mean	92.82	86.14	88.87	89.28
SD	0.96	0.21	0.59	2.74
RSD %	1.03	0.25	0.67	3.07

A.4 RUGGEDNESS

A.4.1 SAMPLE STABILITY

A concentration of 10 µg/ml was prepared from a standard solution. The sample vial was loaded into the autosampler tray and analysed at hourly intervals for 12 hours. The results complied with pharmaceutical standards.

Table A.4 Sample stability parameters of alpha-lipoic acid

Time (hours)	Peak Area	%
0	255.06	100.00
1	240.27	94.20
2	264.56	103.73
3	257.51	100.96
4	254.14	99.64
5	258.63	101.40
6	255.27	100.08
7	252.22	98.89
8	248.61	97.47
9	248.07	97.26
10	248.98	97.62
11	253.79	99.50
12	264.74	103.58

Mean	253.99	99.58
SD	6.47	2.54
RSD%	2.55	2.55

A.4.2 SYSTEM REPEATABILITY

A concentration of 1.0 µg/ml was prepared from a standard solution and injected for six consecutive times into the chromatograph. The variation in the response (% RSD) proved to be excellent with a value of 2.29 for peak area and 0.28 for retention time.

Table A.5 System repeatability parameters of alpha-lipoic acid

	Peak area	Retention times (minutes)
	27.32	4.53
	27.27	4.52
	26.83	4.52
	25.76	4.53
	26.60	4.54
	25.88	4.56
Mean	26.61	4.53
SD	0.61	0.01
RSD %	2.29	0.28

ANNEXURE B: METHODS FOR PREPARATION OF PHEROID™

B.1 INTRODUCTION

The Pheroid™ delivery system is a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures, called Pheroids, uniformly distributed in a dispersion medium that may be adapted to the indication. Pheroids are typically formulated to have a diameter of between 200 nm and 2 µm. Parameters such as required capacity (i.e. the amount and size of the active compound to be entrapped), the rate of delivery and the administration route are taken into account when deciding on the type and diameter of the Pheroids (Grobler *et al.*, 2008:285).

Pheroids consist primarily of ethylated and pegylated polyunsaturated fatty acids, including the omega-3 and omega-6 fatty acids but excluding arachidonic acid. The fatty acids are in the *cis*-formation and therefore compatible with the orientation of the fatty acids in man. These fatty acids can be formulated with various compounds for novel and innovative dosage forms. Colloidal dosage forms commonly used include liposomes, emulsions and micro-emulsions, polymeric microspheres and macromolecular microspheres. In the design of Pheroid™, one or more features of each of these dosage forms have been incorporated (Grobler *et al.*, 2008:285).

As in the case with liposomes, Pheroids generally contain a lipid bilayer, but it contains no phospholipids or cholesterol. In contrast to liposomes, Pheroids are formed by a self-assembly process similar to that of low-energy emulsions and micro-emulsions and no lyophilization or hydration of the lipid components is necessary (Grobler *et al.*, 2008:288).

The Pheroids contain one unique component, namely nitrous oxide (N_2O), which is found distributed in association with the dispersed phase throughout the continuous phase. The association of N_2O with the dispersed phase has been shown to have at least three functions:

1. Contributing to the miscibility of the fatty acids in the dispersal medium.
2. Contributing to the self-assembly process of the Pheroids.
3. Contributing to the stability of the formed Pheroids, as shown by accelerated and formal stability studies (Grobler et al., 2008:289).

B.2 ALPHA-LIPOIC ACID FORMULATIONS CONTAINING PHEROID™

B.2.1 FORMULA OF THE PHEROID™ GEL

The formula of the Pheroid™ gel is given in Table B.1

Table B.1 Formula of the Pheroid™ gel

INGREDIENTS	% m/m	ACTIVITY
Vitamin F	2.8	Anti-oxidant
d-l-alpha-tocopherol	0.2	Anti-oxidant
Liquid paraffin	10	Co-solvent
Span 60	0.5	Thickener
Tween 80	4.5	Co-solvent
Methylparaben	0.4	Preservative
Alpha-lipoic acid	1	Active ingredient
BHA	0.02	Anti-oxidant
BHT	0.2	Anti-oxidant
Xantam gum	2	Thickener
Pheroid™ components	1.5	Active ingredient
$N_2O.H_2O$	To 100%	Solvent

Preparation of the formulation:

- Weigh off $N_2O.H_2O$ and heat to 80°C
- Weigh off and slowly add xantam gum to heated $N_2O.H_2O$ while homogenising at a speed of 13 500 rpm.
- Weigh off and add together alpha-lipoic acid and liquid paraffin. Stir until dissolved.
- Weigh off and heat together: vitamin F, span 60, tween 80, methylparaben, BHA and BHT. Heat to 80°C.
- Add the alpha-lipoic acid and liquid paraffin mixture to the mixture above and cool down to 55°C.
- Weigh off *d-l*-alpha-tocopherol and add to the mixture mentioned above.
- Add the remaining ingredients while homogenising at 13 500 rpm until a temperature of 40°C is reached.

- Slowly stir to room temperature.
- Transfer the formulation into 25 ml, white, pre-sterilised, non-transparent plastic containers.

Discussion

Trial batches were formulated and examined over a period of one week to ensure that the formulations were fit to be manufactured in bulk for stability testing purposes. This white, homogenous gel appeared to be stable and was incorporated into the stability study.

B.2.2 FORMULA OF THE PHEROID™ CREAM

The formula of the Pheroid™ cream is given in table B.2

Table B.2 Formula of the Pheroid™ cream

INGREDIENTS	% m/m	ACTIVITY
Vitamin F	2.8	Anti-oxidant
d-l-alpha-tocopherol	0.2	Anti-oxidant
Liquid paraffin	10	Co-solvent
Span 60	0.5	Thickener
Tween 80	4.5	Co-solvent
Methylparaben	0.5	Preservative
Alpha-lipoic acid	1	Active ingredient
Cetyl alcohol	7	Thickener
BHA	0.02	Anti-oxidant
BHT	0.2	Anti-oxidant
Pheroid™ components	1.5	Active ingredient
$N_2O.H_2O$	To 100%	Solvent

Preparation of the formulation:

- Weigh $N_2O.H_2O$ off and heat to 80°C.
- Weigh off together alpha-lipoic acid and liquid paraffin. Stir until dissolved.
- Weigh off and heat together: vitamin F, cetyl alcohol, span 60, tween 80, methylparaben, BHA, and BHT. Heat to 80°C.

- Add the alpha-lipoic acid mixture to the mixture mentioned above. Cool down to 55°C.
- Weigh off *d-l*-alpha tocopherol and add to the mixture mentioned above.
- Add the mixture to $N_2O.H_2O$ while homogenising at 13 500 rpm until a temperature of 50°C is reached.
- Continue to homogenise to 40°C at 8 000 rpm.
- Stir slowly to room temperature.
- Transfer the formulation into 25 ml, white, pre-sterilised, non-transparent plastic containers.

Discussion

Trial batches were formulated and examined over a period of one week to ensure that the formulations were suitable for bulk manufacturing. This white, homogenous cream appeared to be stable and was incorporated into the stability study.

B.3 ALPHA-LIPOIC ACID FORMULATIONS WITHOUT PHEROID

B.3.1 FORMULA OF THE ALPHA-LIPOIC ACID GEL

The formula of the alpha-lipoic acid gel is given in Table B.3

Table B.3 Formula of the alpha-lipoic acid gel

INGREDIENTS	% m/m	ACTIVITY
Vitamin F	2.8	Anti-oxidant
d-l-alpha-tocopherol	0.2	Anti-oxidant
Liquid paraffin	10	Co-solvent
Span 60	0.5	Thickener
Tween 80	4.5	Co-solvent
Methylparaben	0.5	Preservative
Alpha-lipoic acid	1	Active ingredient
Xantam gum	2	Thickener
BHA	0.02	Anti-oxidant
BHT	0.2	Anti-oxidant
H_2O	To 100%	Solvent

Preparation of the formulation:

- Weigh off alpha-lipoic acid and liquid paraffin and mix together.
- Weigh off the following ingredients and heat to 80°C: vitamin F, *d-l*-alpha tocopherol, span 60, tween 80, methylparaben, BHA and BHT.
- Add the alpha-lipoic acid mixture to the mixture mentioned above.
- Weigh off the water and xantam gum and mix together.
- Add the alpha-lipoic acid mixture to the xantam gum mixture and homogenise at 13 500 rpm to room temperature.
- Transfer the formulation into 25 ml, white, pre-sterilised, non-transparent plastic containers.

Discussion

Trial batches were formulated and examined over a period of one week to ensure that the formulations were fit to be manufactured in bulk for stability testing purposes. This white, homogenous gel appeared to be stable and was incorporated into the stability study.

B.3.2 FORMULA OF THE ALPHA-LIPOIC ACID CREAM

The formula of the alpha-lipoic acid cream is given in Table B.4

Table B.4 Formula of the alpha-lipoic acid cream

INGREDIENTS	% m/m	ACTIVITY
Vitamin F	2.8	Anti-oxidant
d-l-alpha-tocopherol	0.2	Anti-oxidant
Liquid paraffin	10	Co-solvent
Span 60	0.5	Thickener
Tween 80	4.5	Co-solvent
Methylparaben	0.5	Preservative
Alpha-lipoic acid	1	Active ingredient
Cetyl alcohol	7	Thickener
BHA	0.02	Anti-oxidant
BHT	0.2	Anti-oxidant
H_2O	To 100%	Solvent

Preparation of the formulation:

- Weigh off alpha-lipoic acid and liquid paraffin and mix together.
- Weigh off the following ingredients and heat to 80°C: vitamin F, *d-l-alpha tocopherol*, span 60, tween 80, methylparaben, BHA and BHT.
- Add the alpha-lipoic acid mixture to the mixture mentioned above.
- Add the alpha-lipoic acid mixture to the water and homogenise at 13 500 rpm to room temperature.
- Transfer the formulation into 25 ml, white, pre-sterilised, non-transparent plastic containers.

Discussion

Trial batches were formulated and examined over a period of one week to ensure that the formulations were fit to be manufactured in bulk for stability testing purposes. This white, homogenous cream appeared to be stable and was incorporated into the stability study.

B.3 CONCLUSION

All the formulations appeared homogenous after one week and were therefore used for stability tests.

ANNEXURE C: METHODS FOR STABILITY TESTING

C.1 INTRODUCTION

The purpose of stability testing is to provide evidence on how the quality of a drug substance or product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, and to establish a re-test period for the drug substance or a shelf life for the drug product and recommend storage conditions (ICH Q1A (R2) 2003:1).

Stability testing evaluates a product's ability to maintain its original aesthetic, physical and chemical characteristics under controlled conditions designed to accelerate ageing. Such testing provides an early indication of problems that may occur in formulations. This process assists the formulator to acknowledge if the formulation is stable, meaning it will not change significantly over time (Ganz, 2006:33).

Stability studies should include testing of those attributes of the drug substance that are susceptible to change during storage and are likely to influence quality, safety and/or efficacy. The testing should cover, as appropriate, the physical, chemical, biological and microbiological attributes (ICH Q1A (R2) 2003:3).

A drug substance should be evaluated under storage conditions that test its thermal stability and its sensitivity to moisture (ICH Q1A (R2), 2003:3). Elevated temperature storage is critical, since the rate of chemical reactions roughly double for every 10 °C increase in temperature. A potential drawback is that at high temperatures one may be forcing reactions to occur that would not have happened at all at lower temperatures (Ganz, 2006:35).

The stability program comprised the storage of all products at temperatures and humidities of 25 °C/55 % RH, 30 °C/60 % RH and 40 °C/75 % RH, over a period of 3 months. Test intervals were at onset of the stability program (initial), at one month, two months and three months.

In general, "significant change" for a drug product is defined as:

- A 5 % change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
- Any degradation product's exceeding its acceptance criterion;

- Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., colour, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions; and, as appropriate for the dosage form;
- Failure to meet the acceptance for pH; or
- Failure to meet the acceptance criteria for dissolution for 12 dosage units (ICH Q1A (R2), 2003:9).

C.2 STABILITY TESTS DONE

All tests were done using calibrated and/or validated apparatus, where appropriate. The stability tests done on the four formulations are given in table C.1.

Table C.1 Stability tests conducted on the four formulations

Test	Pheroid™ cream	Pheroid™ gel	Alpha-lipoic acid cream	Alpha-lipoic acid gel
Assay	√	√	√	√
pH	√	√	√	√
Viscosity	√	√	√	√
Weight Variation	√	√	√	√
Physical appearance	√	√	√	√
Confocal laser scanning microscopy (CLSM)	√	√	√	√

C.2.1 Assay

All Alpha-lipoic acid formulation assays were done on HPLC. Validation of the HPLC method for Alpha-lipoic acid is shown in Annexure E. Methanol was used as a solvent for all four formulations in order to get good recovery of all the components that need to be analysed. The components

analysed by HPLC are the following: alpha-lipoic acid, methylparaben, BHA, BHT and *d-l*-alpha tocopherol.

Standard preparation of Alpha-lipoic acid formulations

The following amounts of ingredients were accurately weighed and transferred into a 50 ml volumetric flask:

- Alpha-lipoic acid 20 mg
- BHA 0.4 mg
- BHT 4 mg
- Methylparaben 10 mg
- *d-l*-alpha tocopherol 4 mg

The volumetric flask was filled to 50 ml with methanol and sonicated for 10 minutes. The standard was then transferred to HPLC sample vials and analysed.

Sample preparation of Alpha-lipoic acid formulations

2 g of each formulation was accurately weighed and transferred into a 50 ml volumetric flask, using a clean syringe with a rubber tube attached to the tip for each sample. Methanol was added and the samples were shaken by hand and then sonicated for 10 minutes thereafter. The samples were then filtered through 0.45 µm filters after which they were transferred into HPLC vials and analysed.

HPLC parameters:

Analytical instrument: The HPLC analysis of Alpha-lipoic acid was performed by using an Agilent® (Agilent Technologies, Palo Alto, (A)) 1100 Series HPLC (High Performance Liquid Chromatograph). The instrument consisted of a G1311A quaternary pump, G1315A diode array detector, G1313A autosampler injection mechanism, G1322A vacuum degasser, solvent module and HP Chemstation Software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25°C.

Column:	A high performance silica based, reversed phase Phenomenex® Luna C18 (2) column, (150 x 4.6 mm) with a 5 µm particle size was used (Phenomenex, Torrance, CA).To maintain the condition of the column, it was rinsed each time before storage with HPLC grade water at a flow rate of 1.0 ml/min for 20 minutes, then with 70 % CH_3CN for a further 20 minutes and finally with $(CH_3)_2CHOH$ for the last 20 minutes.
Mobile phase:	The mobile phase consisted of 50 volumes of methanol and 50 volumes of distilled water containing 0.1 % octan-sulphonic acid.
Flow rate:	1 ml/min
Injection volume:	20 µl
Retention time:	*d-l*-alpha tocopherol, which had the highest affinity for the stationary phase, eluted at 23 minutes. The total run time was set at 25 minutes.
Detection:	Diode array detector at 220 nm

C.2.2 Results and discussion

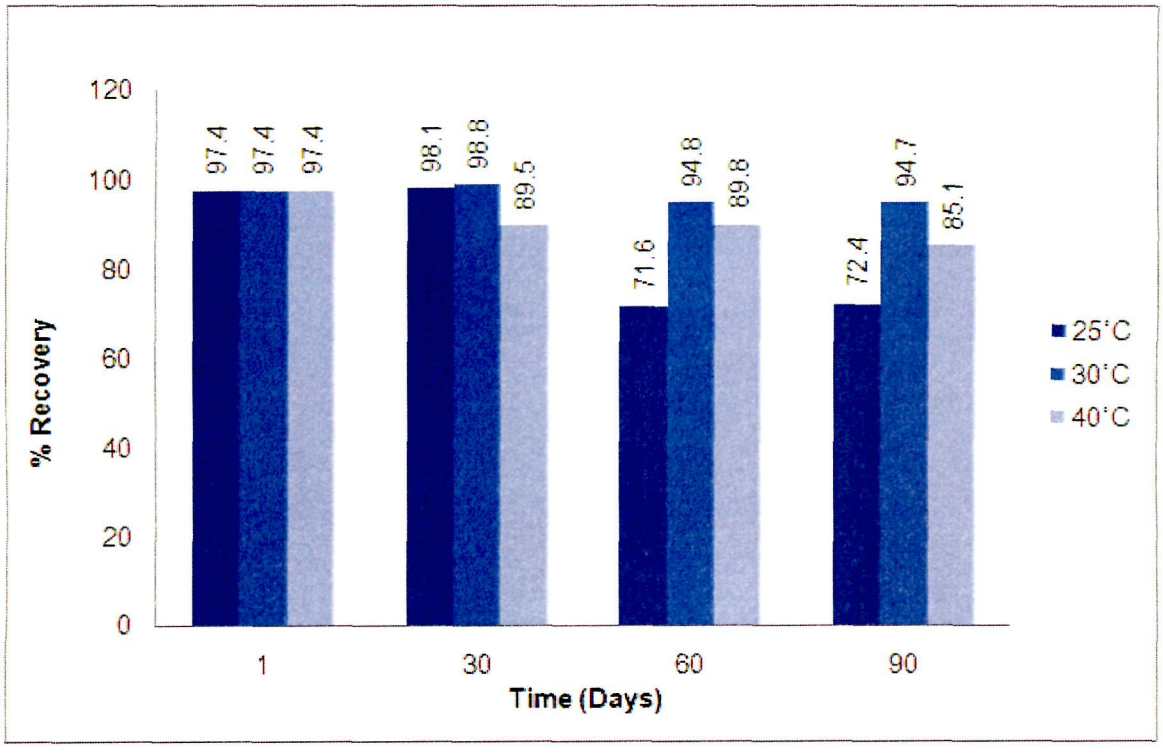

Figure C.1 (a) Accelerated stability test results for methylparaben in Pheroid™ cream

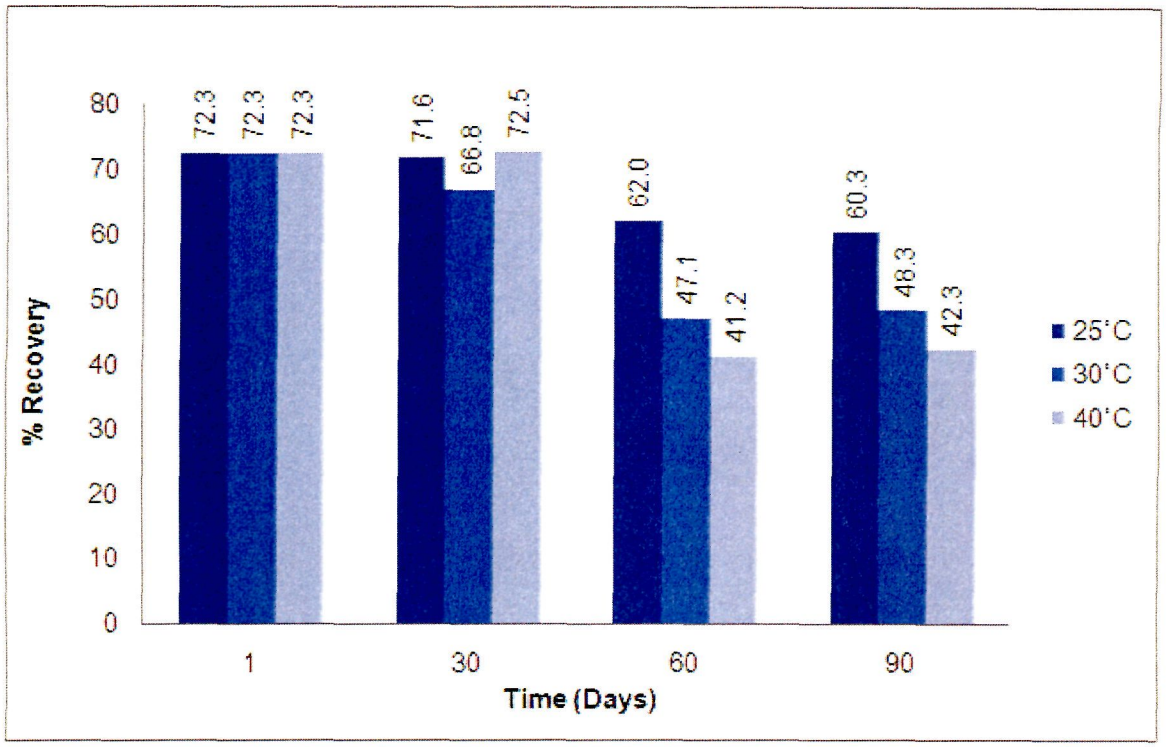

Figure C.1(b) Accelerated stability test results for methylparaben in Pheroid™ gel

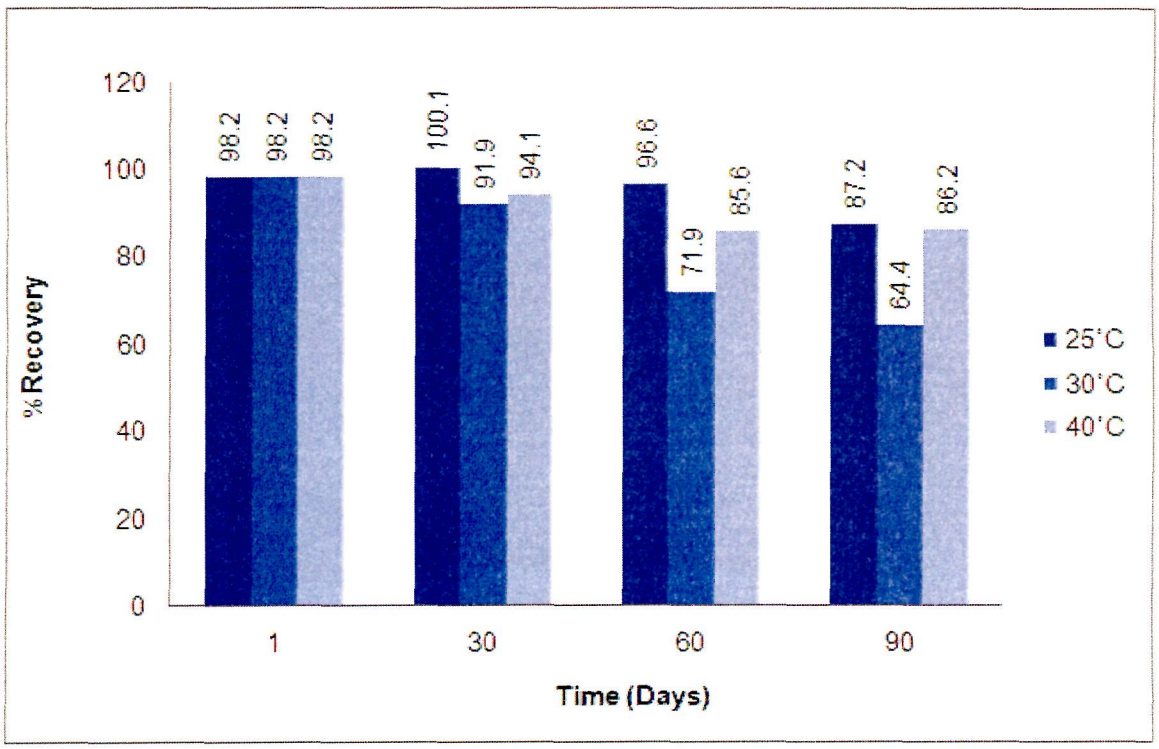

Figure C.1 (c) Accelerated stability test results for methylparaben in cream

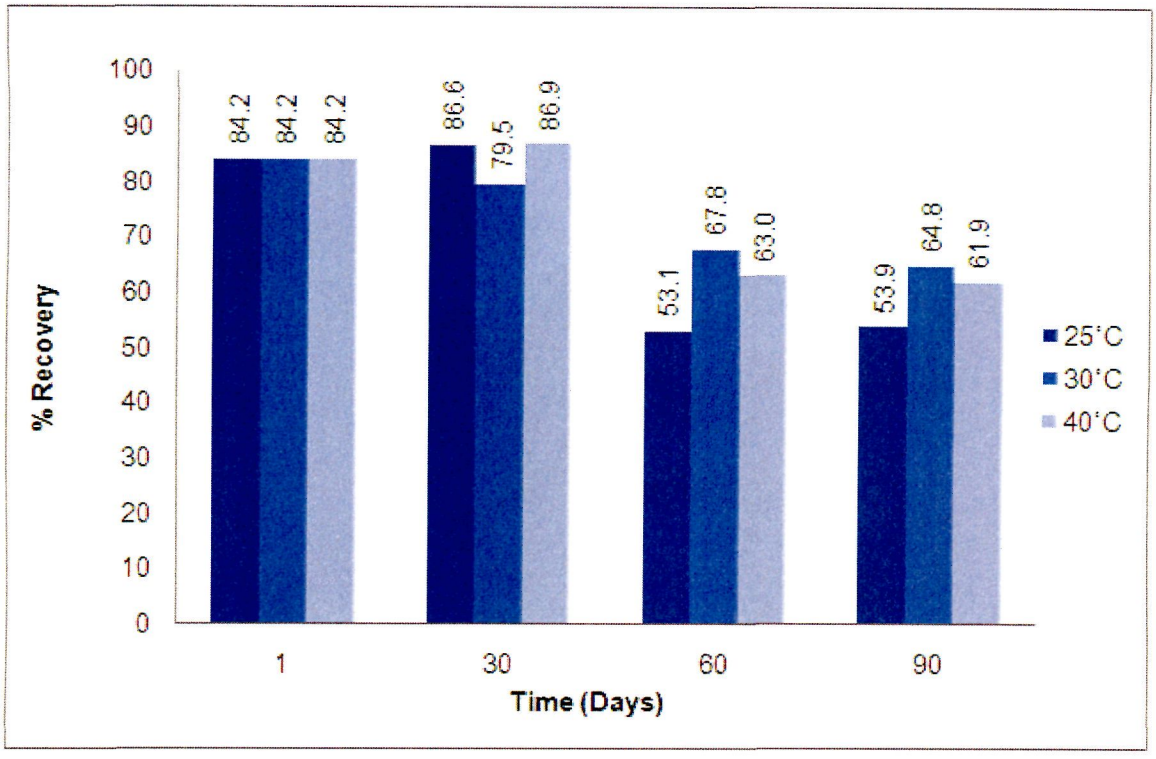

Figure C.1 (d) Accelerated stability test results for methylparaben in gel

Discussion

A significant change in the amount of methylparaben in the Pheroid™ cream was observed after one month at 40 °C/75 % RH. After two months there was also a significant decline in the amount of methylparaben in Pheroid™ cream at 25 °C/60 % RH. No significant change was observed at 30 °C/60 % RH. Methylparaben in Pheroid™ gel showed a significant decline after one month at 30 °C/60 % RH. After two months the formulations which were maintained at 25 °C/60 % RH and 40 °C/75 % RH also exhibited significant change.

After one month, methylparaben in cream showed a significant decline at 30 °C/60 % RH. After two months, the assay of methylparaben at 40 °C/75 % RH revealed significant change. After three months, significant change was observed at all temperatures.The amount of methylparaben in gel showed significant change at all three temperatures after two months.

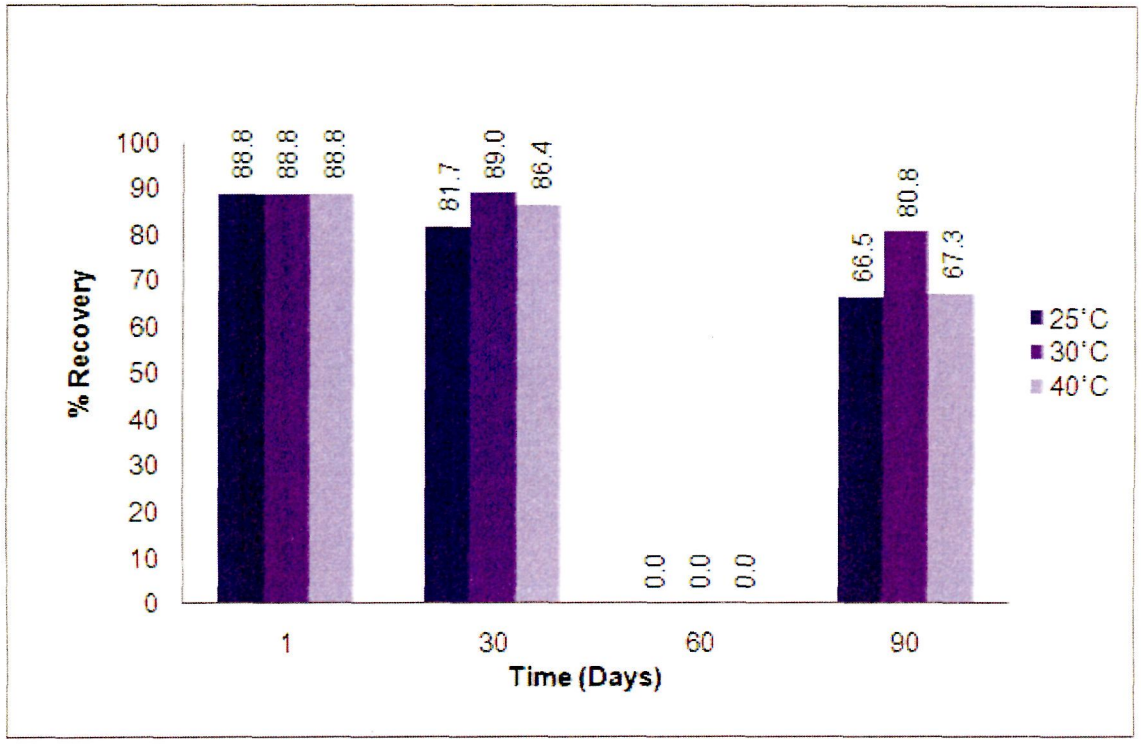

Figure C.2 (a) Accelerated stability test results for BHA in Pheroid™ cream

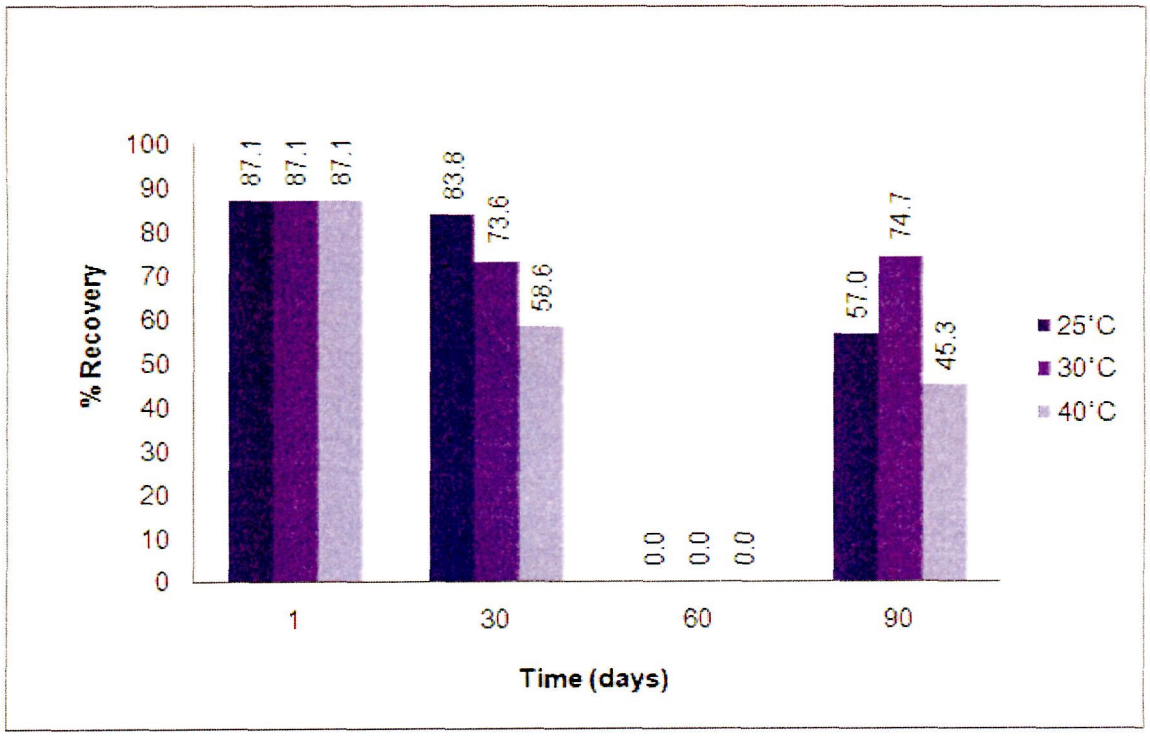

Figure C.2 (b) Accelerated stability test results for BHA in Pheroid™ gel

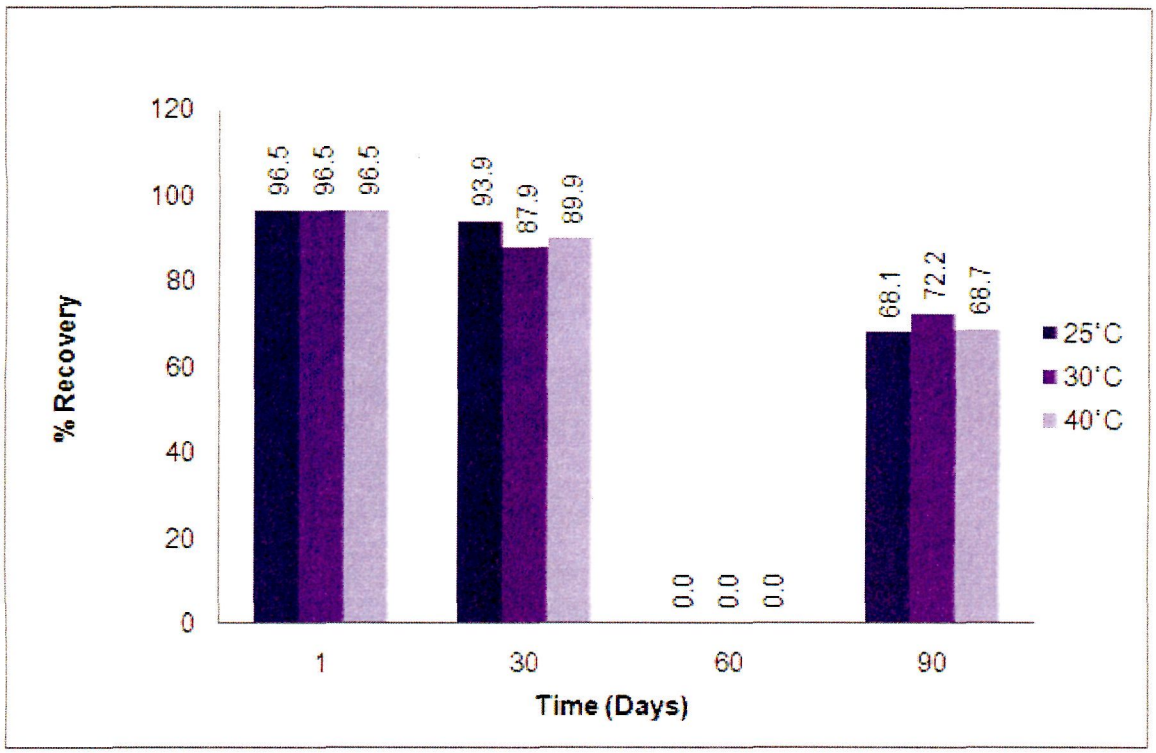

Figure C.2 (c) Accelerated stability test results for BHA in cream

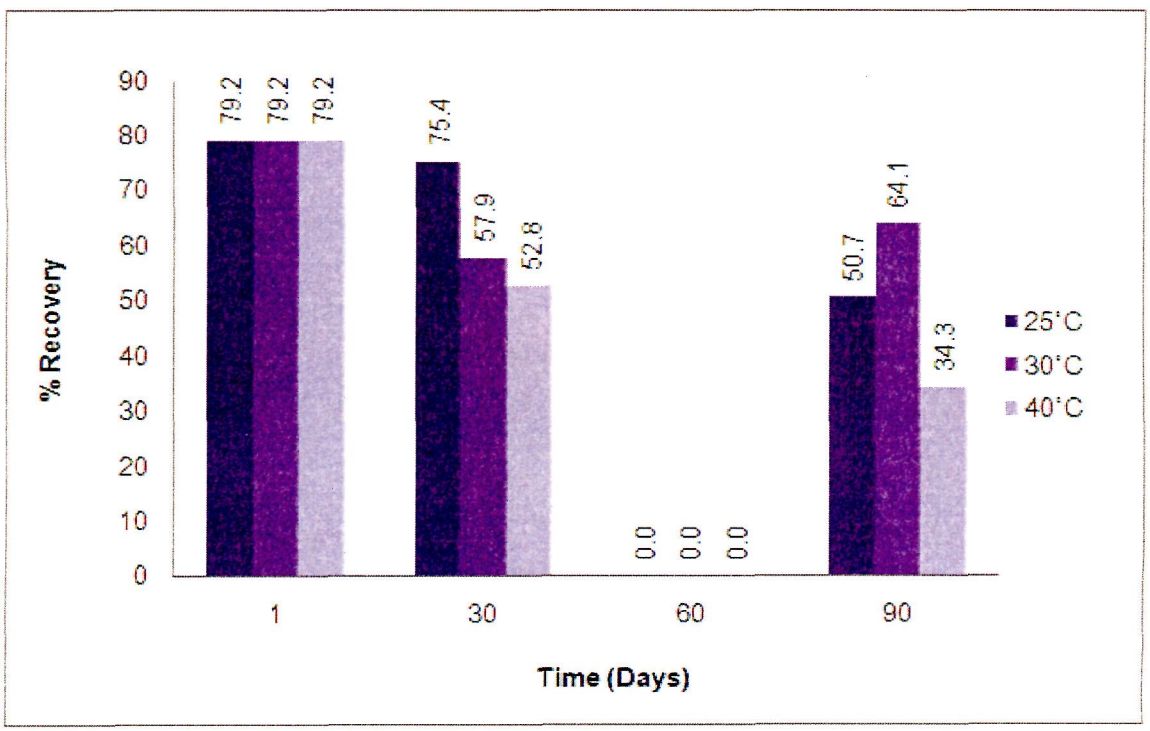

Figure C.2 (d) Accelerated stability test results for BHA in gel

Discussion

Due to an analytical flaw, the amount of BHA in each formulation could not be determined at month 2. A significant decline in the amount of BHA is observed in the Pheroid™ cream at 25 °C/60 % RH after one month. After three months, the amount of BHA in the Pheroid™ cream was not only significantly lower at 25 °C/60 % RH but also at 30 °C/60 % RH and 40 °C/75 % RH.

The amount of BHA in Pheroid™ gel, cream and gel exhibited significant change after one month at 30 °C/60 % RH and 40 °C/75 % RH. After three months, the amounts of BHA in Pheroid™ gel, cream and gel were significantly lower at all 3 temperatures and relative humidities.

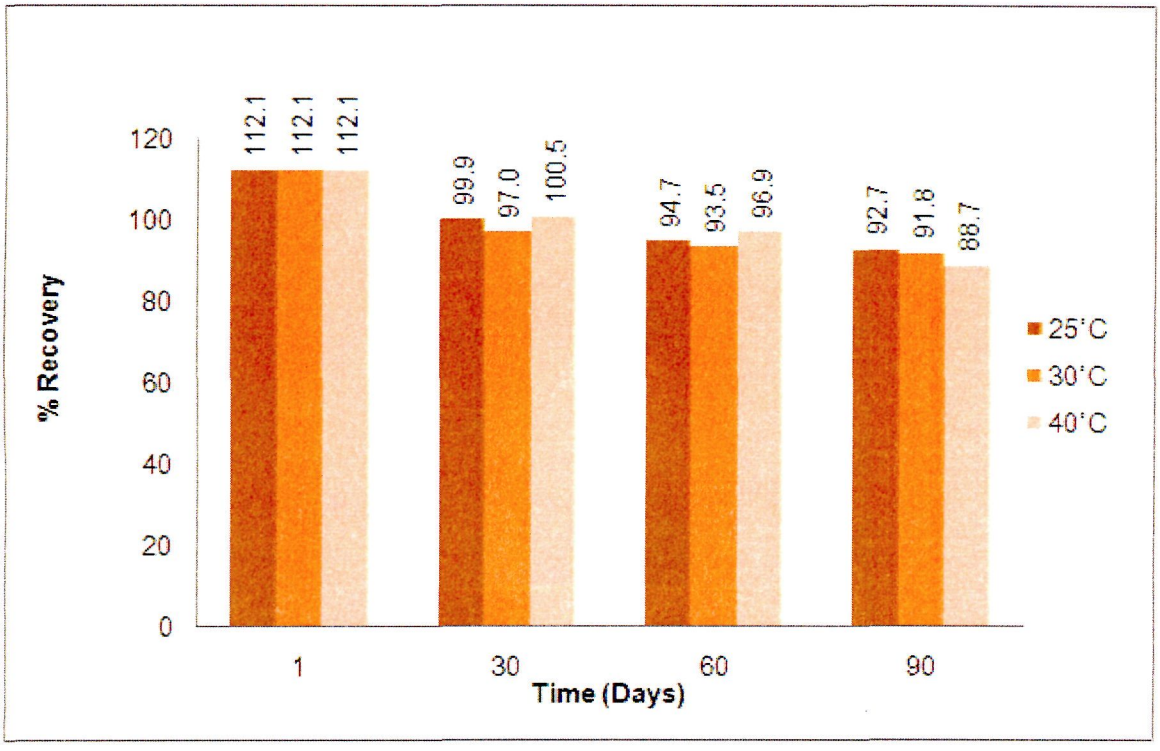

Figure C.3 (a) Accelerated stability test results for BHT in Pheroid™ cream

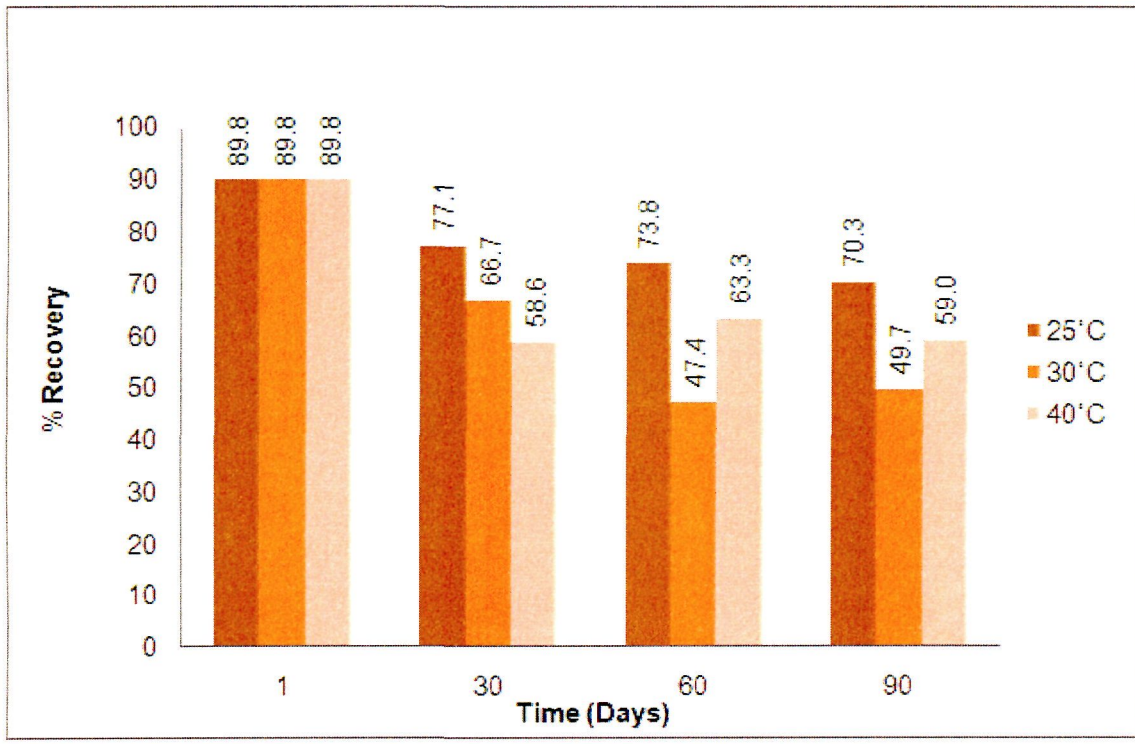

Figure C.3 (b) Accelerated stability test results for BHT in Pheroid™ gel

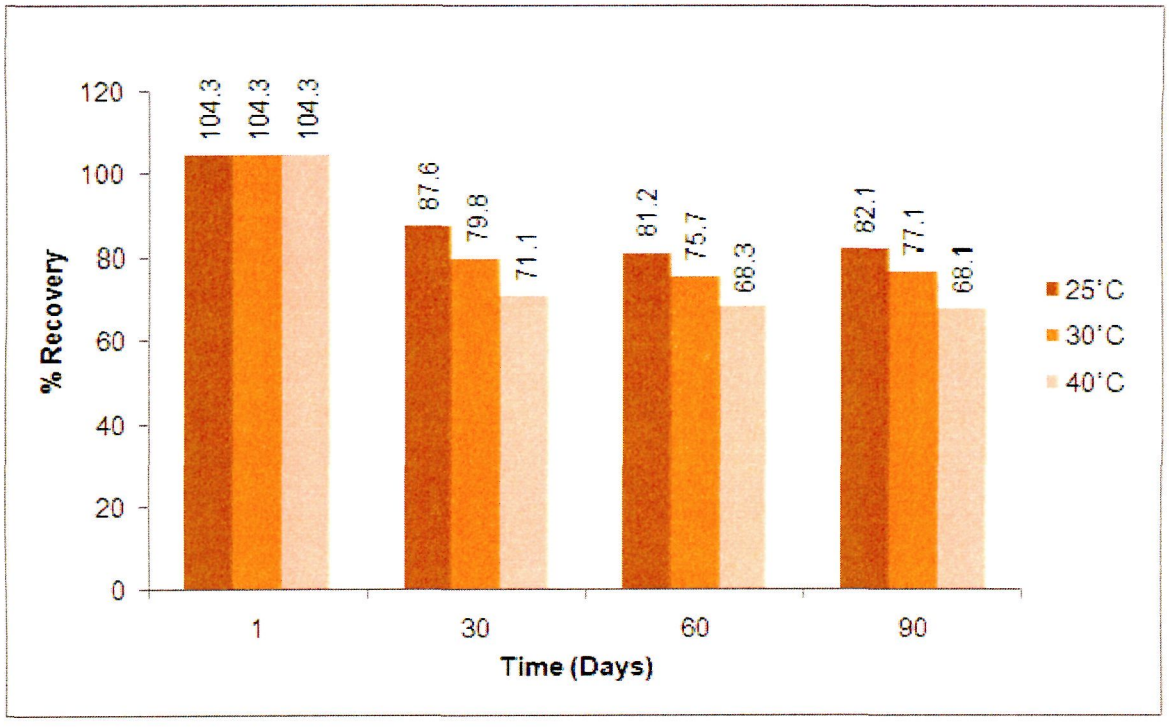

Figure C.3 (c) Stability test results for BHT in cream

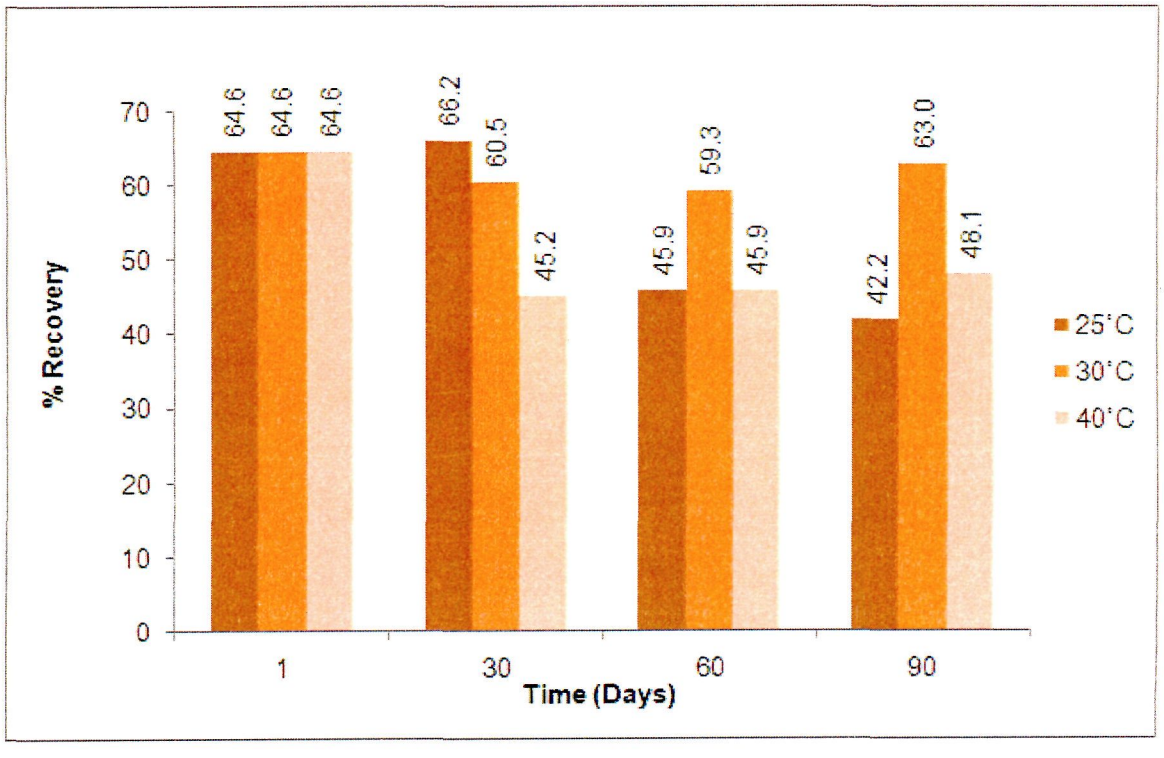

Figure C.3 (d) Stability test results for BHT in gel

Discussion

The amount of BHT in Pheroid™ cream, Pheroid™ gel™ and cream showed a significant decline after one month at all temperatures and relative humidities. The amount of BHT in gel at 40 °C/75 % RH was significantly lower after one month. After two months BHT at 30 °C/60 % RH and 25 °C/60 % RH has also significantly decreased.

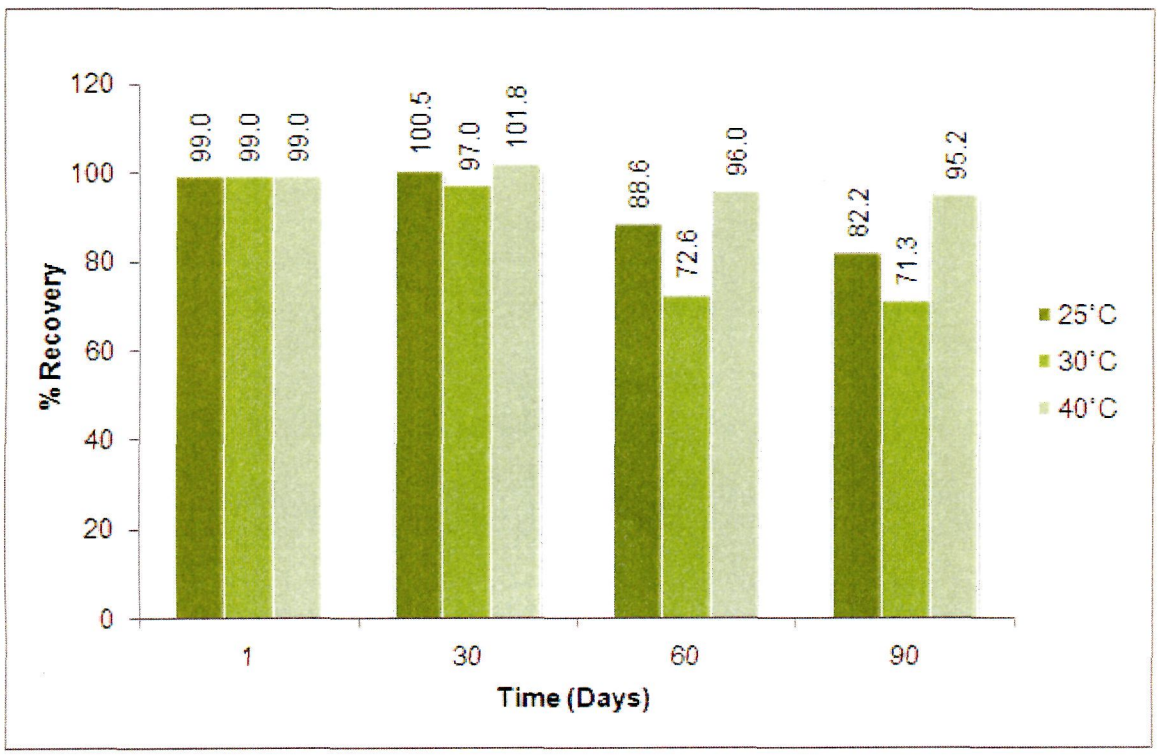

Figure C.4(a) Accelerated stability test results of alpha-lipoic acid in Pheroid™ cream

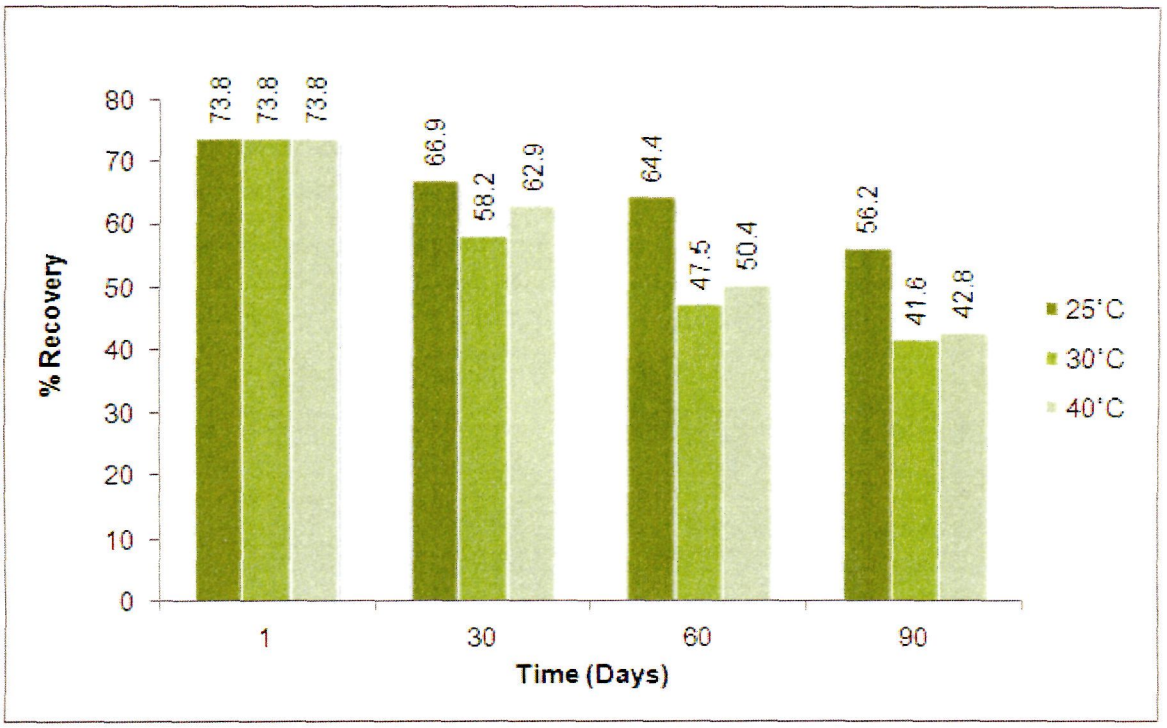

Figure C.4 (b) Accelerated stability test results for alpha-lipoic acid in Pheroid™ gel

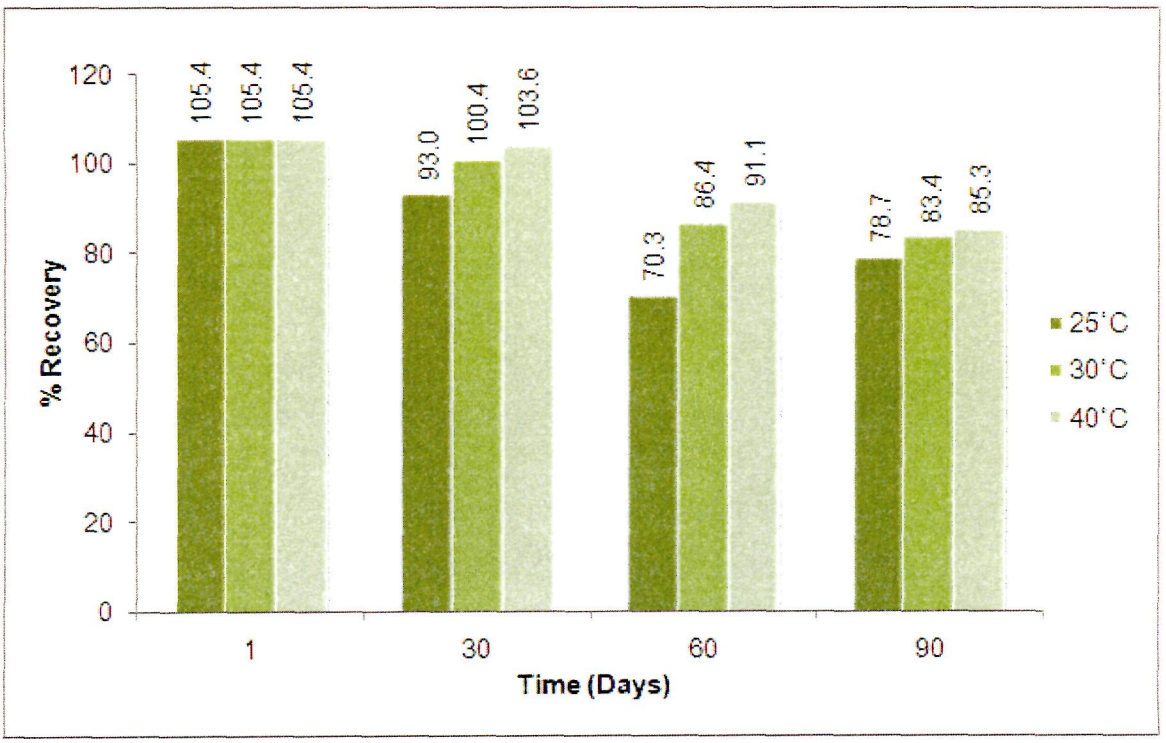

Figure C.4 (c) Accelerated stability test results for alpha-lipoic acid in cream

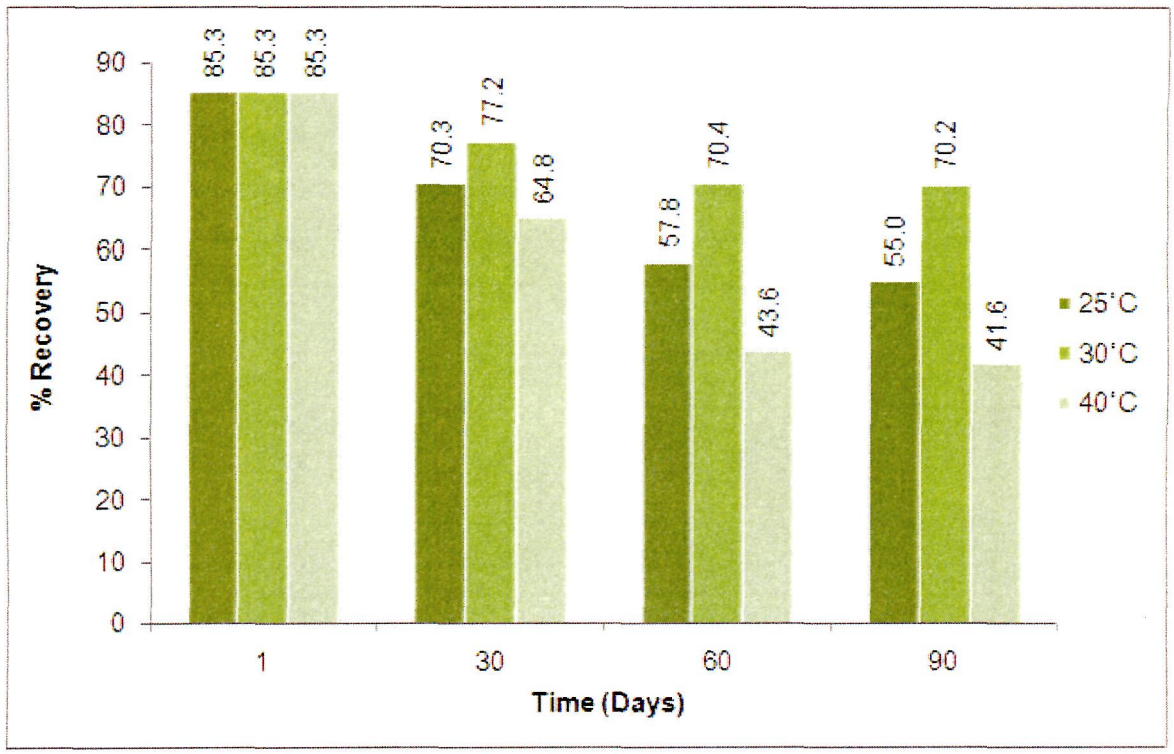

Figure C.4 (d) Accelerated stability test results for alpha-lipoic acid in gel

Discussion

A significant decrease in the amount of alpha-lipoic acid was observed in Pheroid™ cream after two months at 25 °C/60 % RH and 30 °C/60 % RH. No significant decrease in the amount of alpha-lipoic acid was observed over the entire three-month period at 40 °C/75 % RH in Pheroid™ cream.

A significant decline in the amount of alpha-lipoic acid in Pheroid™ gel was noticeable after one month at 25°C/60 % RH, 30 °C/60 % RH and 40 °C/75 % RH.

After one month, the amount of alpha-lipoic acid in cream at 25 °C/60 % RH was significantly less. The same occurs at RH, 30 °C/60 % RH and 40 °C/75 % RH after 60 days.

Alpha-lipoic acid in gel was significantly lower after one month at 25 °C/60 % RH, 30 °C/60 % RH and 40 °C/75 % RH.

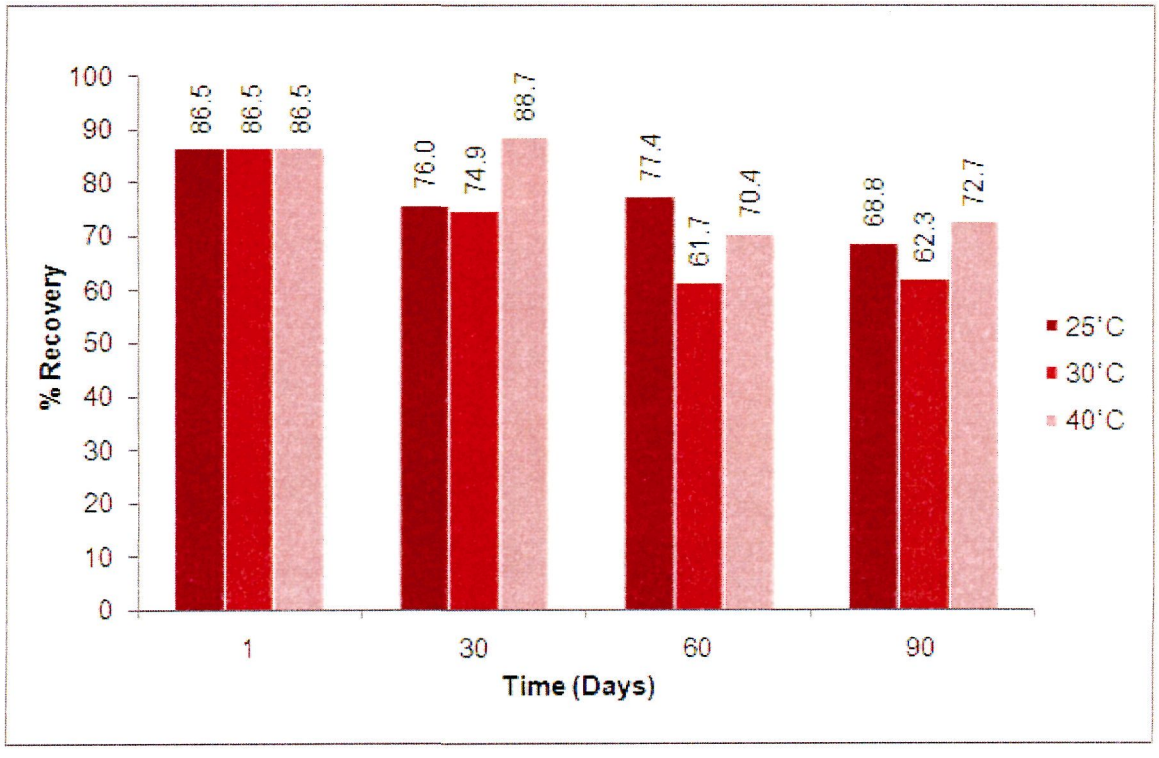

Figure C.5 (a) Accelerated stability test results for *d-l*-alpha tocopherol in Pheroid™ cream

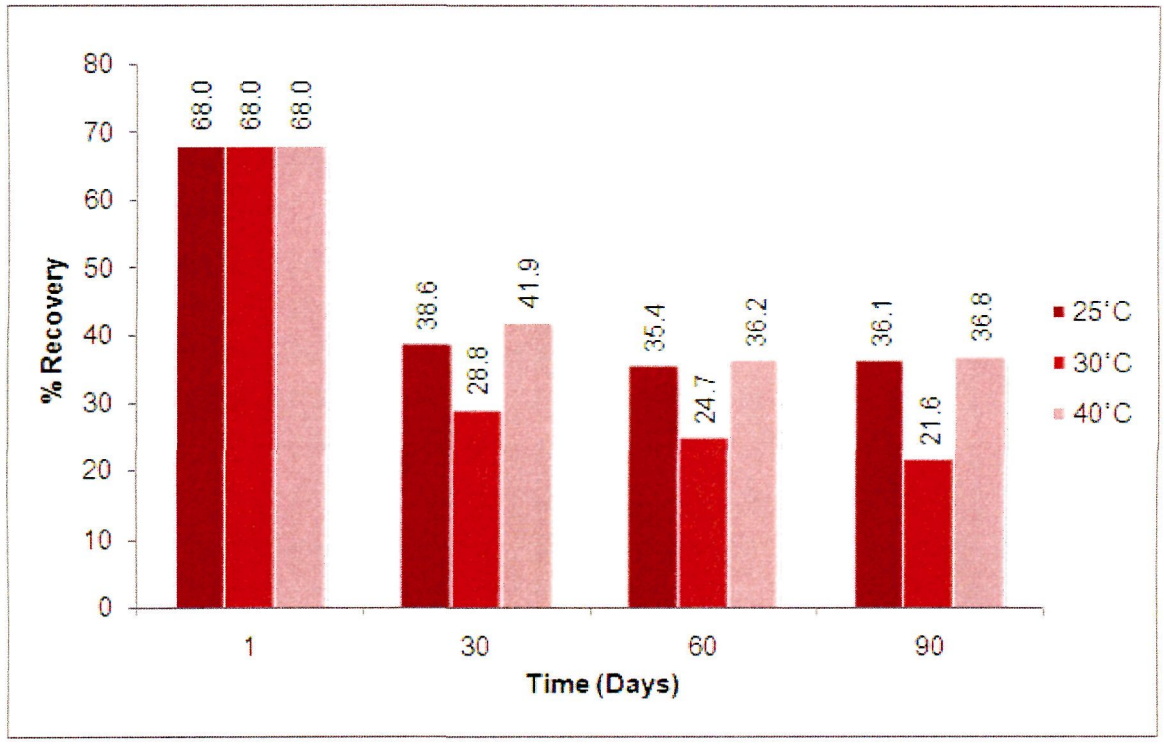

Figure C.5 (b) Accelerated stability test results for *d-l*-alpha tocopherol in Pheroid™ gel

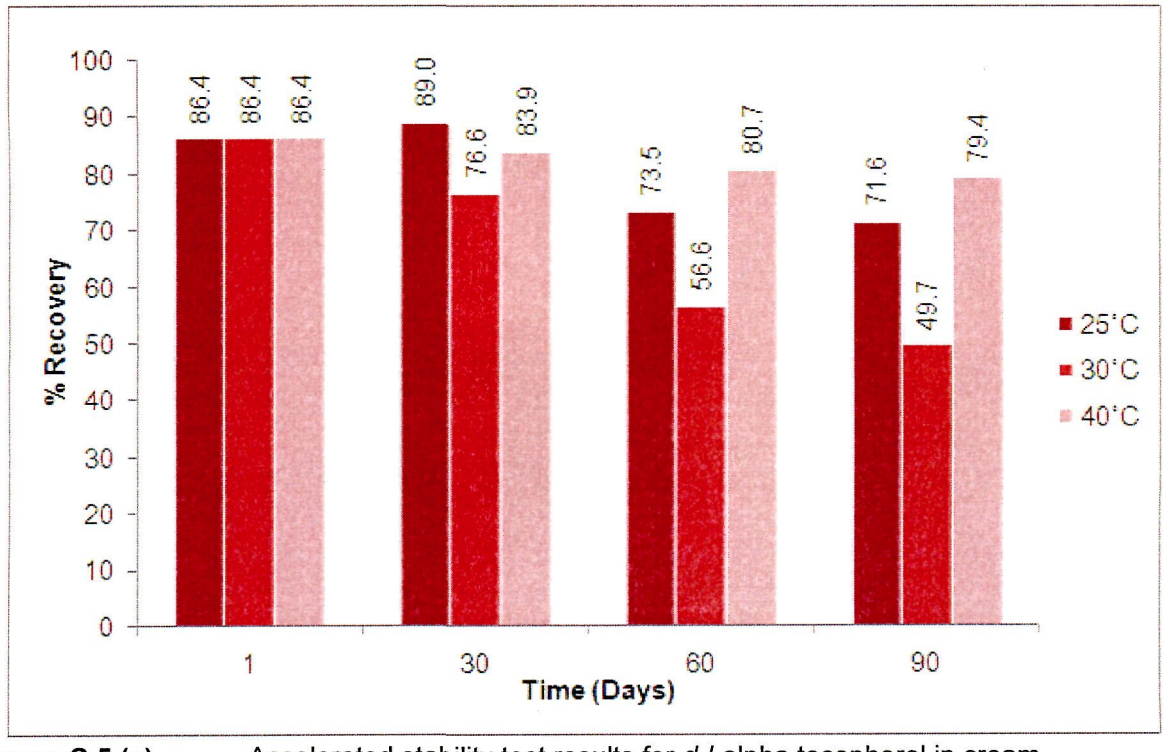

Figure C.5 (c) Accelerated stability test results for *d-l*-alpha tocopherol in cream

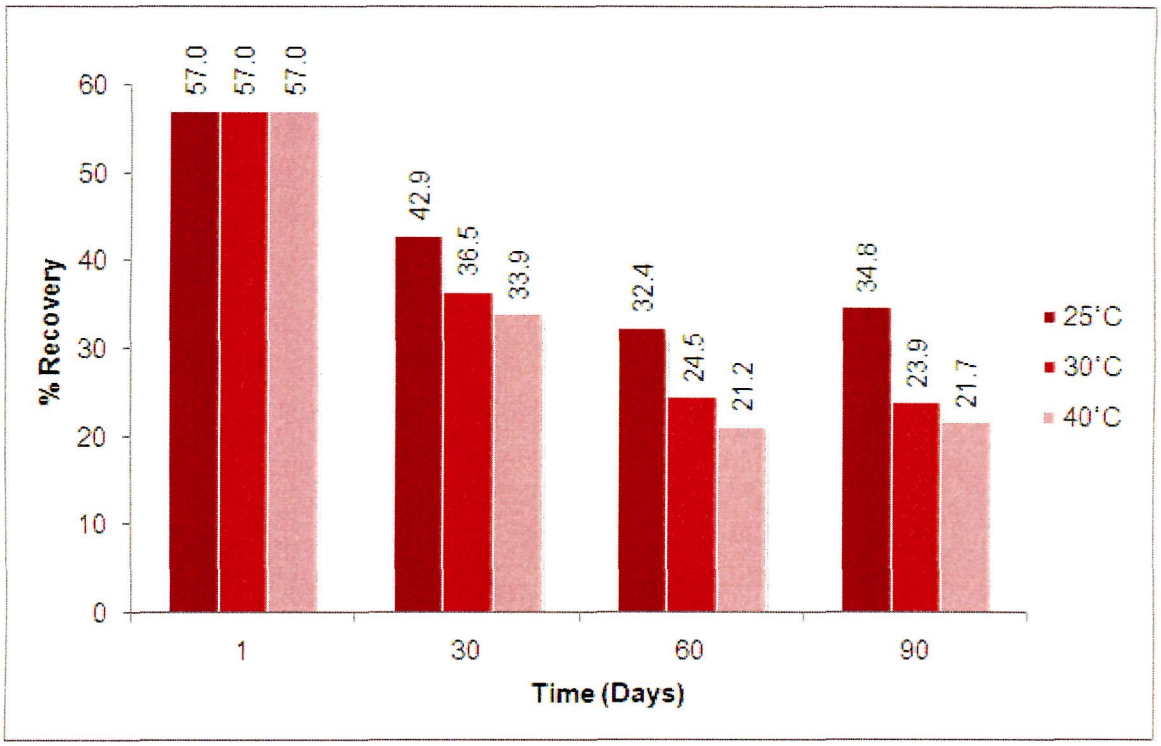

Figure C.5 (d) Accelerated stability test results for *d-l*-alpha tocopherol in gel

Discussion

A significant decline in the amount of *d-l*-alpha tocopherol in Pheroid™ cream was observed at 25 °C/60 % RH and 30 °C/60 % RH after one month. At 40 °C/75 % RH, a significant decline was only observed after two months.

Pheroid™ gel showed a significant decline in the amount of *d-l*-alpha tocopherol at 25 °C/60 % RH, 30 °C/60 % RH and 40 °C/75 % RH after one month.

In the lotion, there was a significant decrease in the amount of *d-l*-alpha tocopherol at RH, 30°C/60% RH after one month. At 25 °C/60 % RH and 40 °C/75 % RH a significant decline was only noticeable after two months.

At 25 °C/60 % RH, 30 °C/60 % RH and 40 °C/75 % RH, there was a significant decline in the amount of *d-l*-alpha tocopherol in gel.

C.2.2 pH

pH is defined as the value given by a suitable, properly standarised, potentiometric instrument (pH meter), capable of reproducing pH values to 0.02 pH units, using an indicator electrode, sensitive to hydrogen-ion activity, the glass electrode, and a suitable reference electrode. (USP 32(2009):313). A Mettler Toledo Seveneasy (Mettler Toledo, Switzerland) pH meter, which was calibrated with buffer solutions of pH 4.01, pH 7.00 and pH 10.01 immediately prior to measurements, was used to measure the pH of the formulations.

C.2.3 Viscosity

Viscosity is a property of liquids that is closely related to the resistance to flow. The United States Pharmacopoeia (USP 32) (2009:387) defines viscosity as the force required to move one plane surface continuously past another under specific steady state conditions, when the space between is filled by the liquid in question. Viscosity is also defined as the sheer stress divided by the sheer rate of shear strain. The basic unit is the *poise*; however, viscosities commonly encountered represent fractions of the poise, so that the *centipoise* (1 poise = 100 centipoise) proves to be the more convenient unit.

The viscosity was determined on a Brookfield Model DV –II+ (Brookfield, United States of America) viscometer. The viscosities of the four different formulations were measured. Spindle S 96 was used but the speed was adjusted according to each formulation's assumed viscosity. The viscosity parameters are listed in table C.2

Table C.2 Viscosity parameters

Formulation	Spindle	Rpm	Temperature	Time (minutes)
Pheroid™ lotion	S 96	1.5	25°C	5
Pheroid™ gel	S 96	1.5 - 3.0	25°C	5
Lotion	S 96	0.6	25°C	5
Gel	S 96	1.5 - 3.0	25°C	5

C.2.4 Weight variation

The weight variation from month 1 to 3 was determined on a Mettler toledo (Mettler Toledo, Switzerland) AB 204-S balance. Formulations were weighed in their closed, semi-permeable plastic containers at month 0, 1, 2 and 3. The weight of the empty container (with lid) was then subtracted from the weight to determine the weight of the formulation.

C.2.5 Discussion

(a) Pheroid™ cream

Table C.3 revealed the decline in the pH value at all three temperatures and humidites and indicated that hydrolysis took place during the three month stability test period. Since Alpha-lipoic acid is a weak acid with a pKa of 5.4, it was found to be predominantly unionised at the pH values obtained during the stability tests. The unionised state of alpha-lipoic acid proved to be favourable for transdermal diffusion (Naik *et al.*, 2000:319). The decrease in viscosity revealed that the formulation showed less resistance to flow and suggested that a change in the composition of the Pheroid™ cream took place from month 0 to month 3.

(b) Pheroid™ gel

As with the Pheroid™ cream, the pH values obtained during the stability tests on the Pheriod™ gel are values at which alpha-lipoic acid was mainly in the unionised state, which was favourable for transdermal delivery (Naik *et al.*, 2000:319). The decrease in viscosity shows that the gel also may have undergone hydrolysis from month 0 to month 3. The results for Pheroid™ gel can be seen in table C.4.

(c) Cream

A decline in the pH indicates that hydrolysis took place. The decrease in viscosity for the cream is much smaller than for Pheroid™ cream and, indicating that the degree of hydrolysis may be smaller. The results for cream are shown in table C.5.

(d) Gel

The results for gel can be seen in Table C.6. The pH of gel, as with the other 3 formulations, has declined over the three month period. The degree of decrease in pH also does not influence the state of ionisation of alpha-lipoic acid.

C.2.6 Results

Table C.3 Results for Pheroid™ cream.

Stability test	Mass (g)	pH	Viscosity (cP)
Storage condition	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH
Month 0	25.80 25.80 25.80	4.03 4.03 4.03	887814.59
Month 1	26.06 25.32 24.96	3.91 3.98 4.00	590297.43
Month 2	26.00 25.27 24.93	3.86 3.87 3.93	229124.5
Month 3	25.93 25.23 24.88	3.58 3.89 3.71	226846.7
SD	0.10 0.23 0.38	0.16 0.07 0.13	276339.36
%RSD	0.37 0.91 1.51	4.29 1.66 3.20	57.15

Table C.4 Results for Pheroid™ gel.

Stability test	Mass (g)	pH	Viscosity (cP)
Storage condition	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH
Month 0	25.22 25.22 25.22	4.45 4.45 4.45	85163.73
Month 1	25.19 24.61 24.66	4.43 4.53 4.21	85949.40
Month 2	25.16 23.49 24.36	4.36 4.29 4.11	89940.49
Month 3	25.13 22.69 24.20	4.20 4.14 3.92	59080.14
SD	0.03 0.98 0.39	0.10 0.15 0.19	12232.22
%RSD	0.13 4.08 1.58	2.25 3.45 4.58	15.28

Table C.5 Results for cream.

Stability test	Mass (g)	pH	Viscosity (cP)
Storage condition	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH
Month 0	25.26 25.26 25.26	3.92 3.92 3.92	389836.17
Month 1	25.17 24.69 25.79	3.99 4.04 3.93	364841.51
Month 2	25.16 24.15 25.75	3.98 3.96 3.86	317774.94
Month 3	25.10 24.01 25.68	3.96 3.99 3.96	343424.70
SD 0	0.06 0.49 0.21	0.03 0.04 0.04	26579.4
%RSD	0.23 2.01 0.83	0.68 1.10 0.93	7.51

Table C.6 Results for gel.

Stability test	Mass (g)	pH	Viscosity (cP)
Storage condition	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH 30°C/60% RH 40°C/75% RH	25°C/60% RH
Month 0	26.35 26.35 26.35	4.44 4.44 4.44	52267.07
Month 1	24.74 25.84 23.89	4.38 4.40 4.26	50583.96
Month 2	23.91 25.81 23.68	4.47 4.21 4.16	48587.34
Month 3	22.86 25.77 24.06	4.34 4.20 3.96	45736.21
SD	1.28 0.24 1.08	0.05 0.11 0.17	2432.10
%RSD	5.22 0.91 4.41	1.15 2.52 4.12	4.93

C.2.7 Physical appearance

A physical assessment of each formulation was carried out once a month at month 0, 1, 2 and 3. The physical appearance of each product was examined and compared to the *initial results*. Changes in colour and texture were noted. Colour charts were used to describe the change in colour from month 0 to month 3.

Discussion

Over the three month period, the changes in the appearance of Pheroid™ cream were observed in both colour and texture, especially at the higher temperatures. A possible explanation for the change is the oxidation of the Pheroid™ components and anti-oxidants. The changes in Pheroid™ cream are discussed in Table C.7.

As depicted in Table C.8, Pheroid™ gel mainly exhibited changes in colour. At month 3 changes in textures were also noted.

Only a slight change in colour and no change in texture was observed in cream during the three-month period. Table C.9 explains the changes in cream.

In the Pheroid™ gel, a significant colour change occured at high temperatures. No major changes in texture were observed.

Table C.7 Changes in Physical appearance of Pheroid™ cream.

Storage Condition	25 °C/60 % RH	30 °C/60 % RH	40 °C/75 % RH
Month 0	White Smooth, non-fluent, no air bubbles, applies easily	White Smooth, non-fluent, no air bubbles, applies easily	White Smooth, non-fluent, no air bubbles, applies easily
Month 1	White Smooth, non-fluent, no air bubbles, applies easily	Off-white Spongy, air bubbles present, applies easily.	Light yellow Spongy with air bubbles, applies easily.
Month 2	Light yellow No change	Off-white No change	Yellow Spongy, oily, applies easily
Month 3	Light yellow More fluent than previous months, air bubbles present, applies easily.	Light yellow More fluent than previous months, spongy, oily, applies easily.	Yellow Spongy, fluent, oily, applies easily

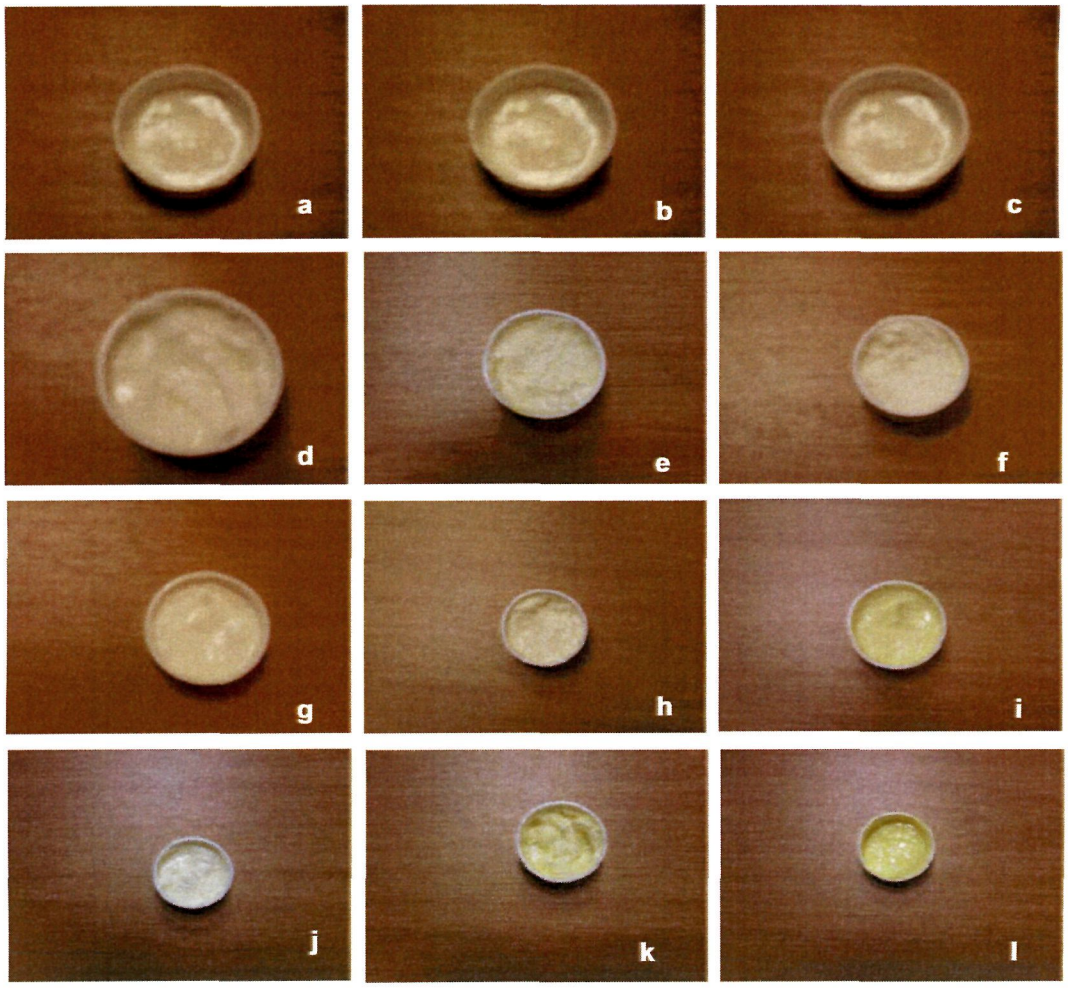

Figure C.6 Physical appearance of Pheroid™ cream at **(a)** Month 0 (25 °C/60 % RH), **(b)** Month 0 (30 °C/60 % RH), **(c)** Month 0 (40 °C/75 % RH), **(d)** Month 1 (25 °C/60 % RH), **(e)** Month 1 (30 °C/60 % RH), **(f)** Month 1 (40 °C/75 % RH), **(g)** Month 2 (25 °C/60 % RH), **(h)** Month 2 (30 °C/60 % RH), **(i)** Month 2 (40 °C/75 % RH), **(j)** Month 3 (25 °C/60 % RH), **(k)** Month 3 (30 °C/60 % RH) and **(l)** Month 3 (40 °C/75 % RH).

Table C.8 Changes in Physical appearance of Pheroid™ gel.

Storage Condition	25 °C/60 % RH	30 °C/60 % RH	40 °C/75 % RH
Month 0	White Smooth, non-fluent, no air bubbles, applies easily	White Smooth, non-fluent, no air bubbles, applies easily	White Smooth, non-fluent, no air bubbles, applies easily
Month 1	White No change	White No change	White No change
Month 2	White No change	Off-white No change	Off-white No change
Month 3	White More fluent than previous months, no air bubbles present, applies easily.	Light yellow More fluent than previous months, smooth, applies easily.	Off-white More fluent than previous months, smooth, applies easily.

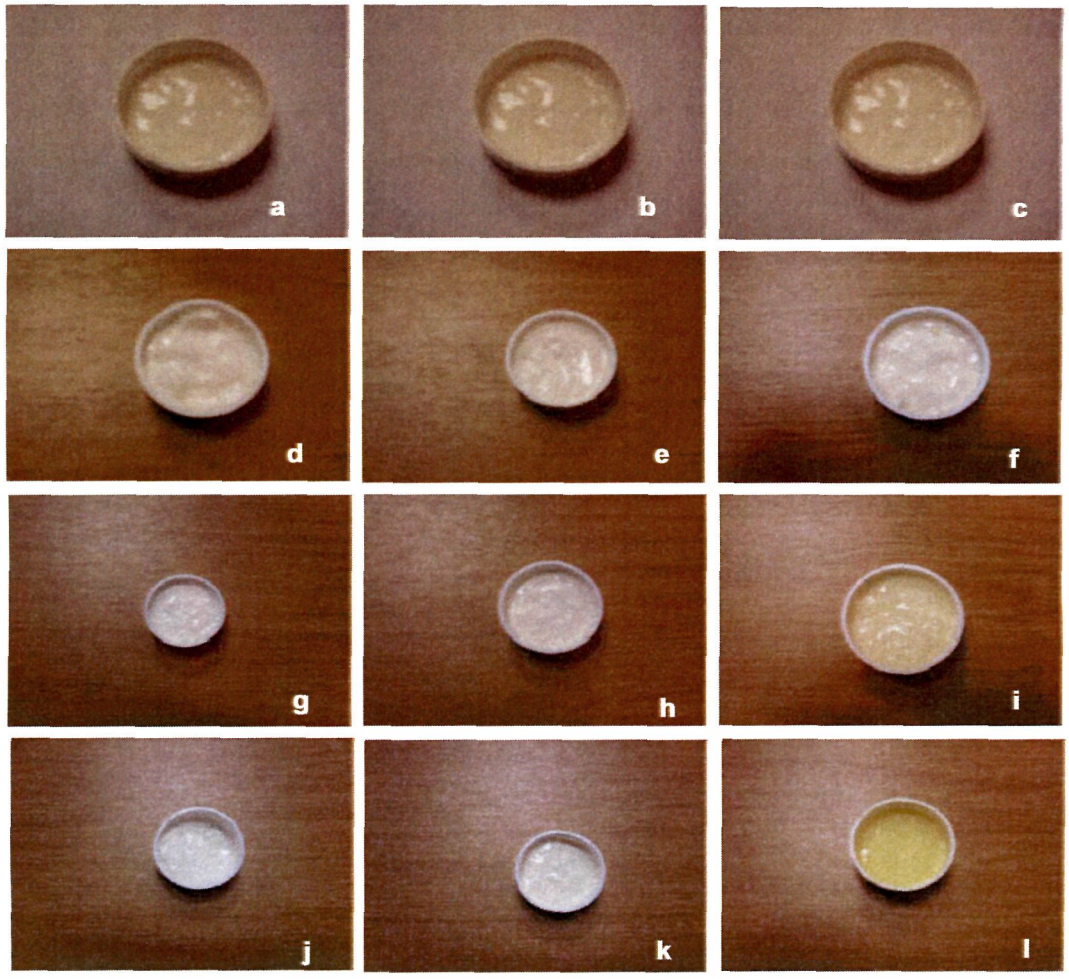

Figure C.7 Physical appearance of Pheroid™ gel at **(a)** Month 0 (25 °C/60 % RH), **(b)** Month 0 (30 °C/60 % RH), **(c)** Month 0 (40 °C/75 % RH), **(d)** Month 1 (25 °C/60 % RH), **(e)** Month 1 (30 °C/60% RH), **(f)** Month 1 (40 °C/75 % RH), **(g)** Month 2 (25 °C/60 % RH), **(h)** Month 2 (30 °C/60 % RH), **(i)** Month 2 (40 °C/75 % RH), **(j)** Month 3 (25 °C/60 % RH), **(k)** Month 3 (30 °C/60 % RH) and **(l)** Month 3 (40 °C/75 % RH).

Table C.9 Changes in physical appearance of cream.

Storage Condition	25°C/60% RH	30°C/60% RH	40°C/75% RH
Month 0	White Smooth, non-fluent, no air bubbles, applies easily	White Smooth, non-fluent, no air bubbles, applies easily	Light yellow Smooth, non-fluent, no air bubbles, applies easily
Month 1	Light yellow No change	Light yellow No change	Light yellow No change
Month 2	White No change	Light yellow No change	Light yellow No change
Month 3	White No change	Light yellow No change	Light yellow No change

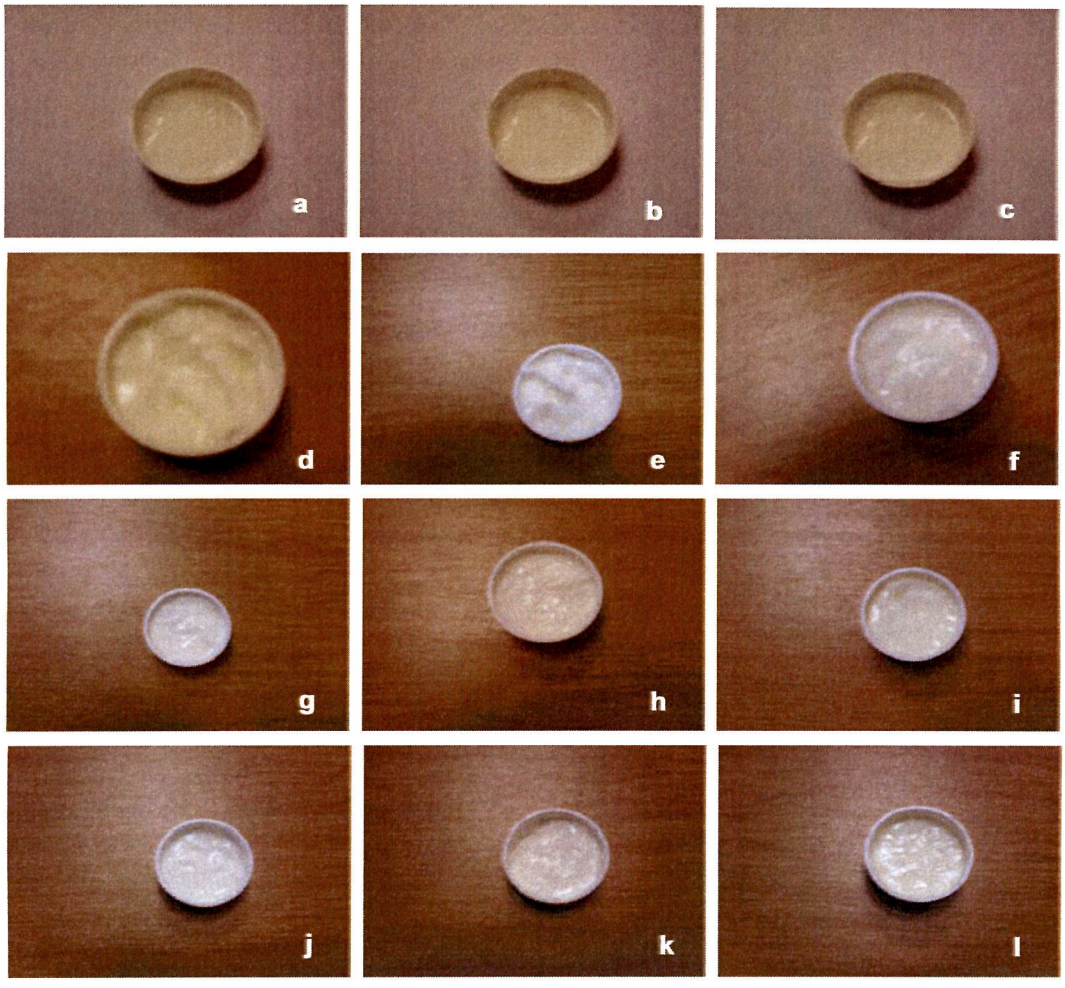

Figure C.8 Physical appearance of cream at **(a)** Month 0 (25°C/60% RH), **(b)** Month 0 (30°C/60% RH), **(c)** Month 0 (40°C/75% RH), **(d)** Month 1 (25°C/60% RH), **(e)** Month 1 (30°C/60% RH), **(f)** Month 1 (40°C/75% RH), **(g)** Month 2 (25°C/60% RH), **(h)** Month 2 (30°C/60% RH), **(i)** Month 2 (40°C/75% RH), **(j)** Month 3 (25°C/60% RH), **(k)** Month 3 (30°C/60% RH) and **(l)** Month 3 (40°C/75% RH).

Table C.10 Changes in physical appearance of gel.

Storage Condition	25°C/60% RH	30°C/60% RH	40°C/75% RH
Property	Colour Texture	Colour Texture	Colour Texture
Month 0	White Smooth, non-fluent, no air bubbles, applies easily	White Smooth, non-fluent, no air bubbles, applies easily	Light yellow Smooth, non-fluent, no air bubbles, applies easily
Month 1	Off-white No change	White No change	Light yellow No change
Month 2	Off-white No change	Light yellow No change	Bright yellow No change
Month 3	Off-white No change	Light yellow No change	Bright yellow No change

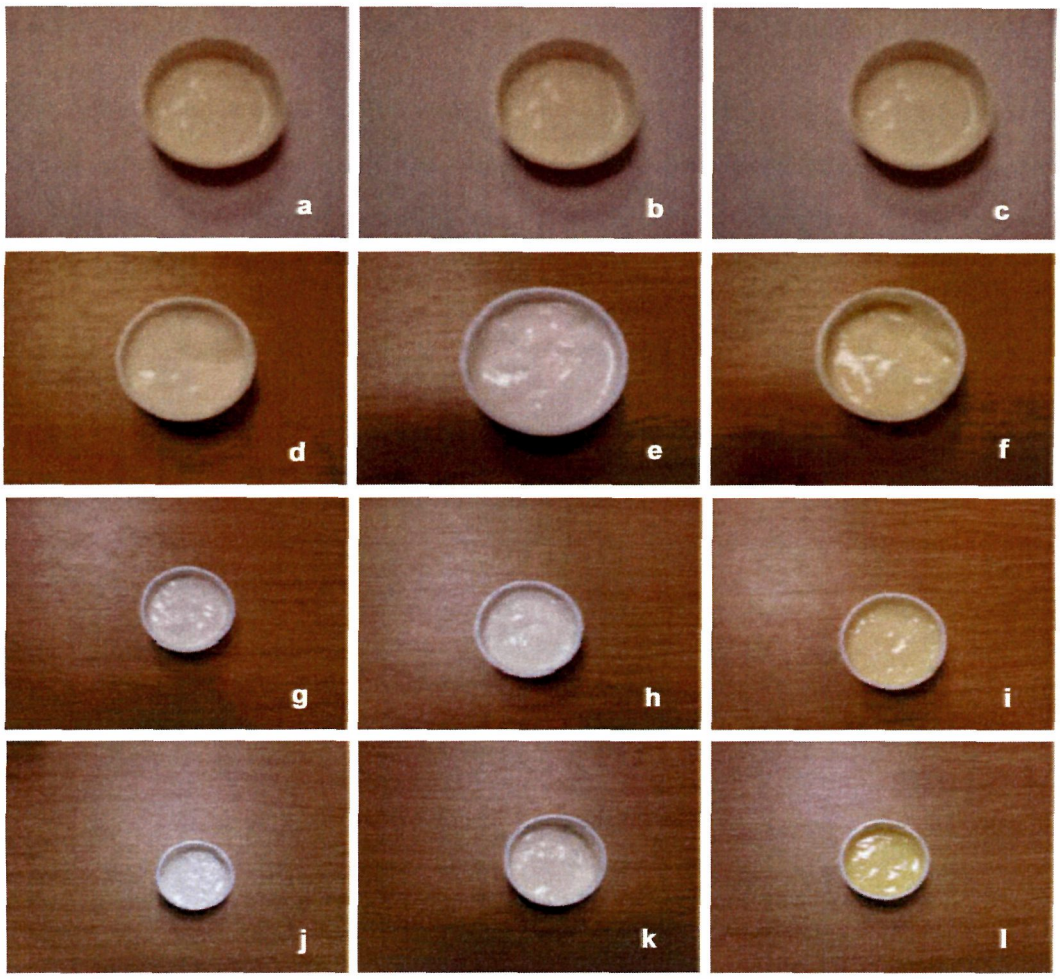

Figure C.9 Physical appearance of gel at **(a)** Month 0 (25°C/60% RH), **(b)** Month 0 (30°C/60% RH), **(c)** Month 0 (40°C/75% RH), **(d)** Month 1 (25°C/60% RH), **(e)** Month 1 (30°C/60% RH), **(f)** Month 1 (40°C/75% RH), **(g)** Month 2 (25°C/60% RH), **(h)** Month 2 (30°C/60% RH), **(i)** Month 2 (40°C/75% RH), **(j)** Month 3 (25°C/60% RH), **(k)** Month 3 (30°C/60% RH) and **(l)** Month 3 (40°C/75% RH).

C.2.6 Confocal laser scanning microscopy (CLSM)

The purpose of confocal laser scanning microscopy is to determine the size distribution of the Pheroid™ sponges. The phospholipid components of the Pheroid™ were stained with phenoxazine dye nile red (Haugland, 2005). The sample was then placed on a glass slide and covered with a glass cover-slip and sealed with an adhesive to prevent the Pheroid™ from drying out. The CLSM used to capture the images was a Nikon PCM 2000 confocal laser scanning microscope, with a DXM 1200 digital camera for real-time imaging and ApoPlanar oil immersion objective with numerical aperture (NA) of 1.4. The CLSM is equipped with a He/Ne laser with an emission of 543 nm which detects at 605-675nm and Argon ion laser with an emission of 457 nm which detects at 515-530nm.

Discussion

Visual assessment of the Pheroid™ sponges on microscopic level was done with CLSM. No crystals or impurities were observed in any of the formulations at all three temperatures. No change in the microscopic consistency of the Pheroid™ cream or cream was observed over the three month period at all three temperatures. The size of oil droplets in Pheroid™ gel as well as gel seemed to increase after one month at 40°C/75% RH

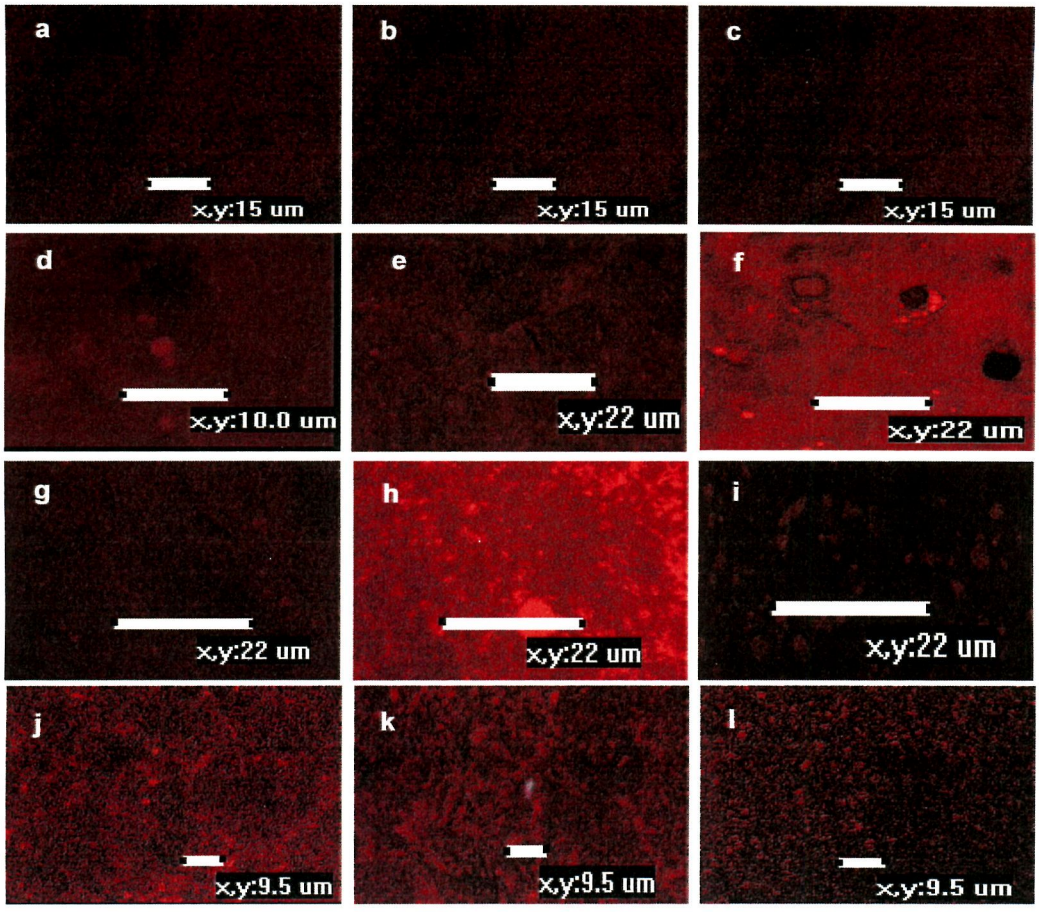

Figure C.10 CLSM results of Pheroid™ cream at **(a)** Month 0 (25°C/60% RH), **(b)** Month 0 (30°C/60% RH), **(c)** Month 0 (40°C/75% RH), **(d)** Month 1 (25°C/60% RH), **(e)** Month 1 (30°C/60% RH), **(f)** Month 1 (40°C/75% RH), **(g)** Month 2 (25°C/60% RH), **(h)** Month 2 (30°C/60% RH), **(i)** Month 2 (40°C/75% RH), **(j)** Month 3 (25°C/60% RH), **(k)** Month 3 (30°C/60% RH) and **(l)** Month 3 (40°C/75% RH).

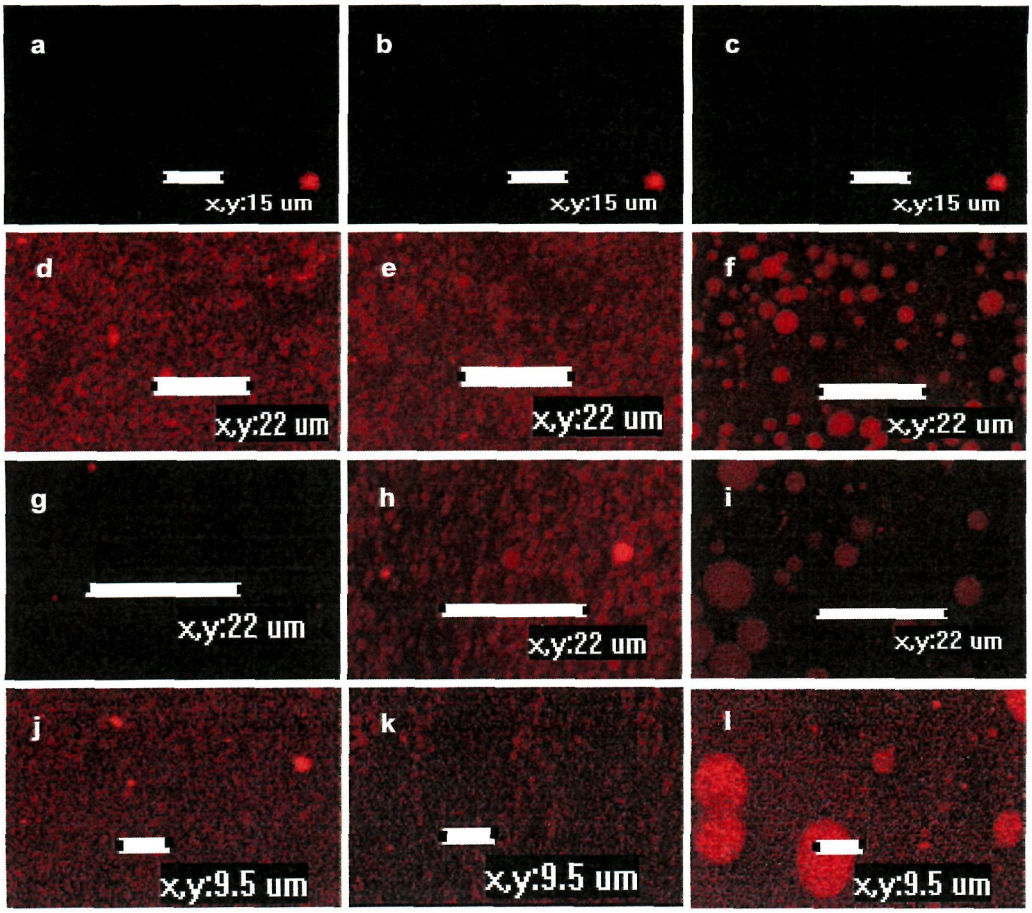

Figure C.11 CLSM of Pheroid™ gel at **(a)** Month 0 (25°C/60% RH), **(b)** Month 0 (30°C/60% RH), **(c)** Month 0 (40°C/75% RH), **(d)** Month 1 (25°C/60% RH), **(e)** Month 1 (30°C/60% RH), **(f)** Month 1 (40°C/75% RH), **(g)** Month 2 (25°C/60% RH), **(h)** Month 2 (30°C/60% RH), **(i)** Month 2 (40°C/75% RH), **(j)** Month 3 (25°C/60% RH), **(k)** Month 3 (30°C/60% RH) and **(l)** Month 3 (40°C/75% RH).

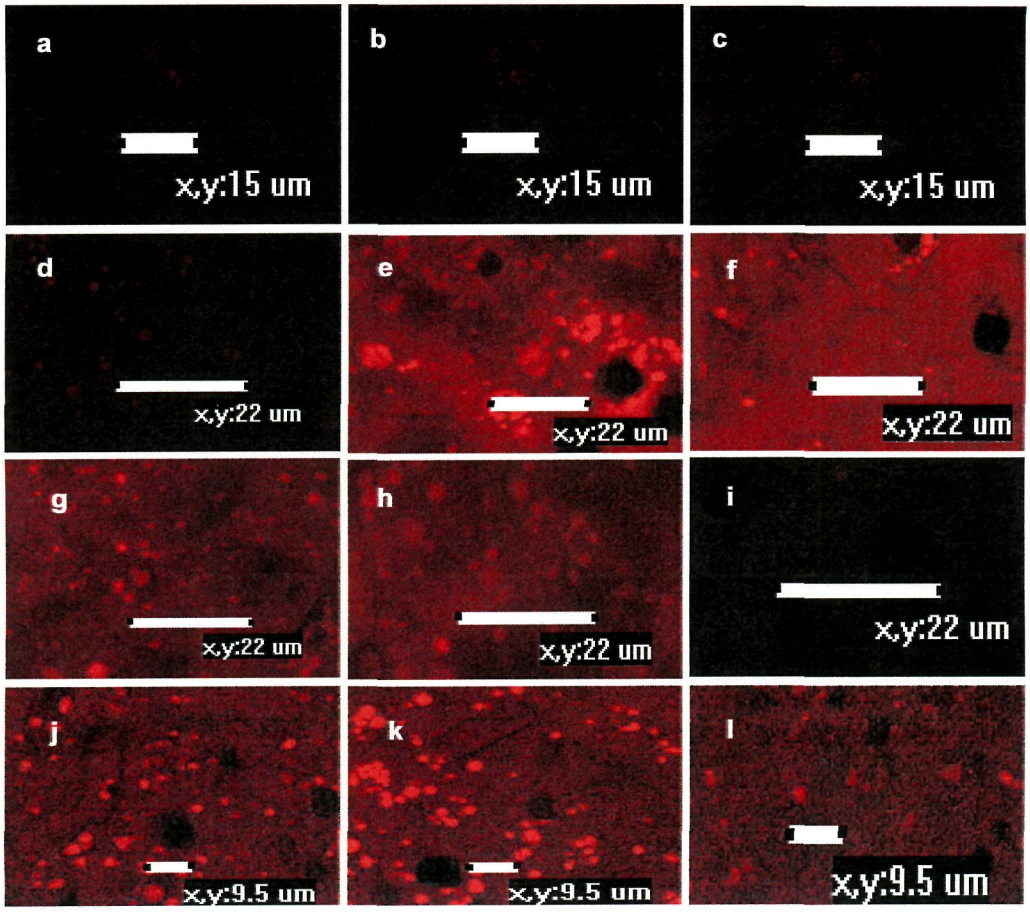

Figure C.12 CLSM results of cream at **(a)** Month 0 (25°C/60% RH), **(b)** Month 0 (30°C/60% RH), **(c)** Month 0 (40°C/75% RH), **(d)** Month 1 (25°C/60% RH), **(e)** Month 1 (30°C/60% RH), **(f)** Month 1 (40°C/75% RH), **(g)** Month 2 (25°C/60% RH), **(h)** Month 2 (30°C/60% RH), **(i)** Month 2 (40°C/75% RH), **(j)** Month 3 (25°C/60% RH), **(k)** Month 3 (30°C/60% RH) and **(l)** Month 3 (40°C/75% RH).

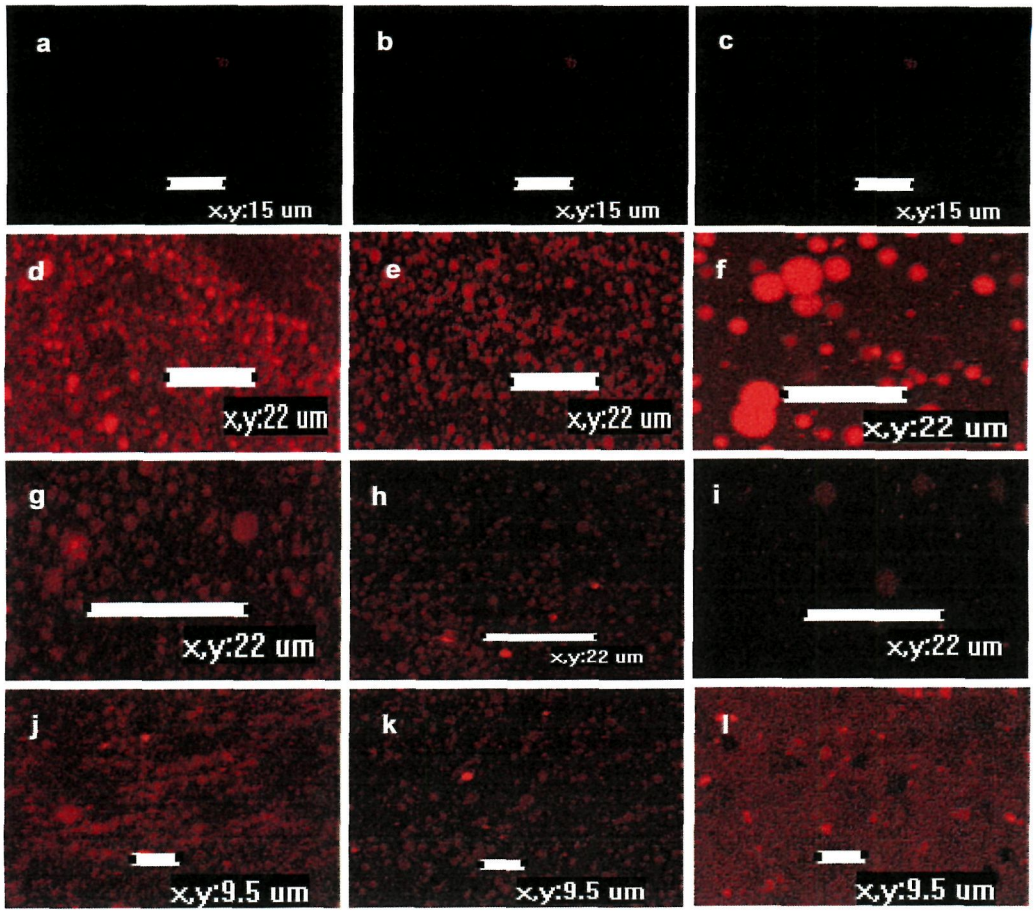

Figure C.13 CLSM results of gel at **(a)** Month 0 (25°C/60% RH), **(b)** Month 0 (30°C/60% RH), **(c)** Month 0 (40°C/75% RH), **(d)** Month 1 (25°C/60% RH), **(e)** Month 1 (30°C/60% RH), **(f)** Month 1 (40°C/75% RH), **(g)** Month 2 (25°C/60% RH), **(h)** Month 2 (30°C/60% RH), **(i)** Month 2 (40°C/75% RH), **(j)** Month 3 (25°C/60% RH), **(k)** Month 3 (30°C/60% RH) and **(l)** Month 3 (40°C/75% RH).

C.3 CONCLUSION

Assay results revealed that methylparaben showed significant change over the three-month period. This indicates that it is insufficient to use methylparaben as the only preservative. Preservation may be improved by combining methylparaben with another preservative. Further investigation into the preservation of the formulations is necessary.

HPLC analysis showed that BHT only exhibited sufficient anti-oxidant properties period at 30 °C/60 % RH in the gel formulation after 3 months. In all the other formulations it exhibited insufficient anti-oxidant properties. BHA and *d-l*-alpha tocopherol failed to comply with the stability test standards and are thus not usable for anti-oxidant purposes. Further investigation into the improvement of the anti-oxidants is necessary.

Alpha-lipoic acid was found to be unstable in al four of the formulations over the three-month period. It is thus necessary to improve the stability of alpha-lipoic acid in future.

According to Naik *et al* (200:319), none of the pH values of the formulations were ideal for transdermal delivery (pH 5-9). The pH values were however ideal for the transdermal permeation of Alpha-lipoic acid (pKa 5.4) as it is predominantly unionised at the pH values obtained.

The small changes in the viscosity over the three-month period in formulations without Pheroid™ when compared to the large differences in viscosity in formulations containing Pheroid™ revealed that the formulations without Pheroid™ were more stable. This was observed when the % RSD values were compared to each other.

Colour changes were clear in the Pheroid™ cream, Pheroid™ gel and gel formulations over the 3 month period, especially at 40°C/75% RH. No apparent change in colour was observed in the cream formulaton. The changes in colour may be due to oxidation of certain components. Further investigation is necessary to find the cause of the discolouration and a method to prevent it.

Confocal laser scanning microscopy revealed that the formulations were homogenous. At 40 °C/75 % RH there was a clear increase in the oil droplet size of the Pheroid™ gel and gel formulations. The Pheroid™ cream and cream formulations remained the same throughout the three-month period.

ANNEXURE D: TRANSDERMAL DIFFUSION OF ALPHA-LIPOIC ACID

D.1 INTRODUCTION

Permeation across human skin can occur via several pathways, none of which is mutually exclusive, and all of which probably operate for most molecules traversing the skin (Williams, 2003:38). The penetrant has three potential pathways to the viable tissue – through hair follicles with associated sebaceous glands, via sweat ducts, or across continuous stratum corneum between these appendages (Barry, 2001:101).

Skin penetration studies play an essential role in the optimisation of drug and formulation design in dermal and transdermal delivery. Therefore, the experimental use of in vitro permeation techniques such as Franz type diffusion cells (FC) is very important. The FC system is widely used because of its low cost. It is less time consuming and it is reproducible (Leveque et al., 2004:323).

The Franz cell is composed of two compartments: donor and receptor (El-Kattan et al., 2000:426). The receptor compartment has a volume of approximately 2 ml and an effective surface area of approximately 1.0 cm^2. The diffusion buffer is continuously stirred at 750 rpm by a magnetic bar. The temperature in the bulk of the solution is maintained by circulating thermostated water through a water jacket that surrounds the receptor compartment (El-Kattan et al.m 2000:428). The temperature of the water was maintained at 37 °C.

A tape stripping technique was used to investigate the amount of alpha-lipoic acid that was present in the dermis and epidermis of excised human skin after the application of the Pheroid™ cream, Pheroid™ gel, cream and gel formulations.

D.2 SKIN PREPARATION FOR DIFFUSION STUDIES

Excised human skin from female patients, who had undergone abdominal plastic surgery, was used (Leveque et al., 2004). The skin was frozen at -20 °C within 24 hours after removal. At this temperature the skin is stable with regards to the penetration of drugs, as well as the thickness of the stratum corneum, over a time period of 3 and 6 months respectively (Leveque et al., 2004). Full thickness skin (containing the overlying dermis, the viable epidermis and the outermost stratum corneum) (Williams, 2003) was used during this study. The innermost subcutaneous fat layer (hypodermis) (Williams, 2003) was carefully separated from the skin with a scalpel to avoid damage to the skin. Damaged or ruptured skin may lead to incorrect results.

The skin was punched into circles with a diameter of approximately 15 mm and placed onto Whatman® filter paper with the stratum corneum side facing upwards and left to air dry. The skin circles on the filter paper were then covered with aluminium foil and kept frozen at -20 °C until used. The frozen skin samples were thawed at room temperature prior to the diffusion study and thoroughly examined for any defects before being placed on the Franz cells.

Ethical approval for the procurement and exploitation of the skin was provided by the Research Ethics Committee of the North-West University under reference number 04D08. The identities of the skin donors were confidential and informed consent was obtained beforehand.

D.3 FRANZ CELL DIFFUSION METHOD

Vertical Franz cells with 2 ml receptor compartments and 11.7 cm^2 effective diffusion areas were used for the permeation studies. These Franz cells consist of a donor (top) and a receptor (bottom) compartment where the drug solution and the receptor fluid (PBS, pH 7.4) were placed, respectively. A small magnetic stirring bar was placed in each receptor compartment in order to maintain stirring throughout the experiment.

The full-thickness skin was thawed and mounted between the receptor and donor compartments with the stratum corneum (SC) facing upwards. Dow Corning high vacuum grease was applied to each Franz cell in order to prevent any leakage. The receptor compartments were filled with PBS (pH 7.4). Special care was taken to ensure that no air bubbles were trapped beneath the skin. The donor compartments were filled with 1 ml of the alpha-lipoic acid formulation under investigation and subsequently covered with Parafilm® to prevent evaporation for the duration of the experiment. A horseshoe clamp was used to secure the donor and receptor compartments.

In order to control the temperature, the receptor compartments were placed directly in a 37 °C water bath in order to attain a skin temperature of 32 °C (Cleary, 1993). Only the receptor compartments (equipped with stirring magnets) were submerged in the water. The cells were stirred by using a magnetic stirrer plate at a speed of 750 rpm. Ten Franz cells were used for each experiment.

The entire receptor compartment was emptied and replaced with fresh 37 °C PBS (pH 7.4) after 0.30, 0.60, 1.00, 1.30, 1,60, 2.00, 4.00, 6.00, 8.00, 10.00 and 12.00 hours. The entire receptor volumes were withdrawn to mimic sink conditions as they occur in the human body. The withdrawn

samples were directly assayed by HPLC to determine the drug concentration which had permeated through the stratum corneum.

D.4 TAPE STRIPPING METHOD

At 12 hours, after the removal of the donor and receptor phases, the diffusion cells were carefully dismantled, and the skin was pinned to a piece of Parafilm™ which was stapled to a solid surface. The skin was dabbed dry with tissue paper, and pieces of tape were cut to a length that covered the diffusional area but did not overlap the areas outside of the diffusion cell imprints. The exposed diffusional area (≈11.7 cm^2) was visibly marked by the groove from the diffusion cells (≈5 mm diameter). The first tape strip was discarded and the following 15 tape strips were placed in a vial containing 5 ml PBS. Complete removal of the stratum corneum was indicated by the glistening of the viable epidermal layer after 15 strips (Pellet, 1997). The excess skin was trimmed away from the flange imprints of the diffusion cells, and the remaining skin (dermis) was cut into pieces to enlarge the surface area. It was then placed in 2 ml of PBS (pH 7.4). Samples were filtered, prepared where necessary and assayed.

D.5 CHROMATOGRAPHIC CONDITIONS

Analytical instrument: The HPLC analysis of Alpha-lipoic acid was performed by using an Agilent® (Agilent Technologies, Palo Alto, (A)) 1100 Series HPLC (High Performance Liquid Chromatograph). The instrument consisted of a G1311A quaternary pump, G1315A diode array detector, G1313A autosampler injection mechanism, G1322A vacuum degasser, solvent module and HP Chemstation Software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25°C.

Column: A high performance silica based, reversed phase Phenomenex® Luna C18 (2) column, (150 x 4.6 mm) with a 5 µm particle size was used (Phenomenex, Torrance, CA).To maintain the condition of the column, it was rinsed each time before storage with HPLC grade water at a flow rate of 1.0 ml/min for 20 minutes, then with 70 % CH_3CN for a

	further 20 minutes and finally with $(CH_3)_2CHOH$ for the last 20 minutes.
Mobile phase:	The mobile phase consisted of 50 volumes of acetonitrile and 50 volumes of distilled water containing 0.1 % ortho-phosphoric acid.
Flow rate:	1 ml/min
Injection volume:	50 µl
Retention time:	The analyte elutes at approximately 5.5 minutes.
Detection:	Diode array detector at 210 nm

D.6 RESULTS

D.6.1 PERMEATION PROFILES

Formulations containing Pheroid™ were compared to those without Pheroid™ to determine whether Pheroid™ enhanced the flux of alpha-lipoic acid. The following figures illustrate the cumulative amount per area plotted against time for every individual Franz cell used for Pheroid™ formulations during the diffusion studies. No flux could be calculated for the formulations without Pheroid™ as cream remained in the epidermal layer and the gel diffused mostly into the dermis.

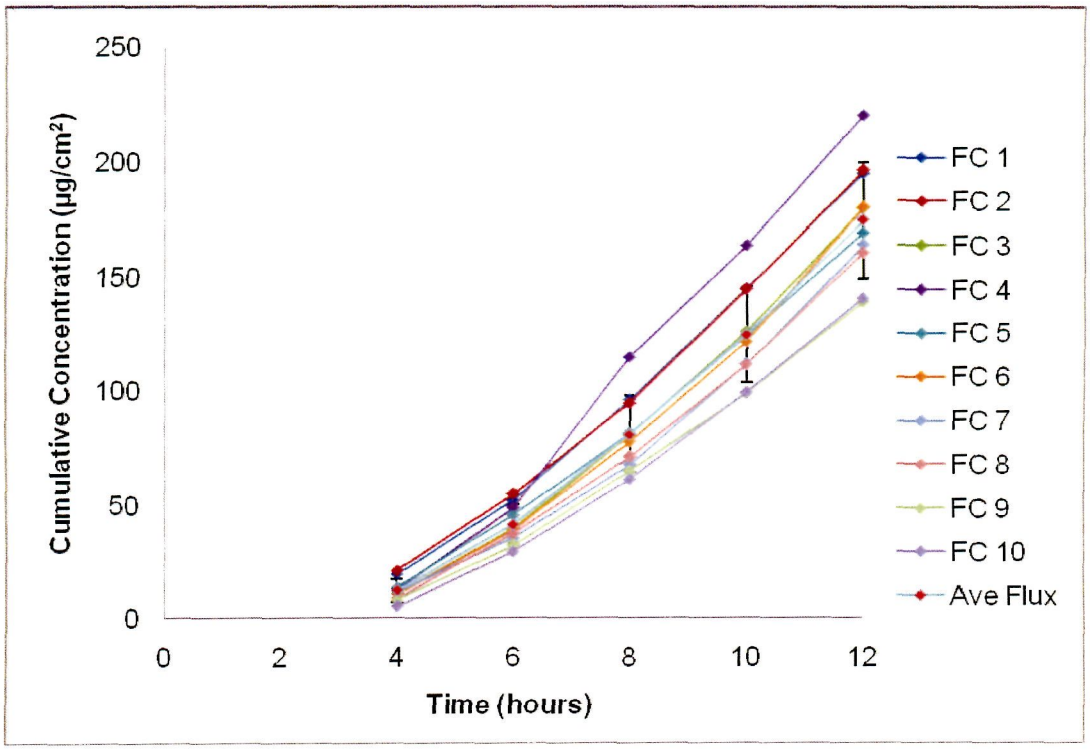

Figure D.3 Cumulative amount Pheroid™ gel per area applied to the skin plotted against time for each individual Franz cell, illustrating the average flux.

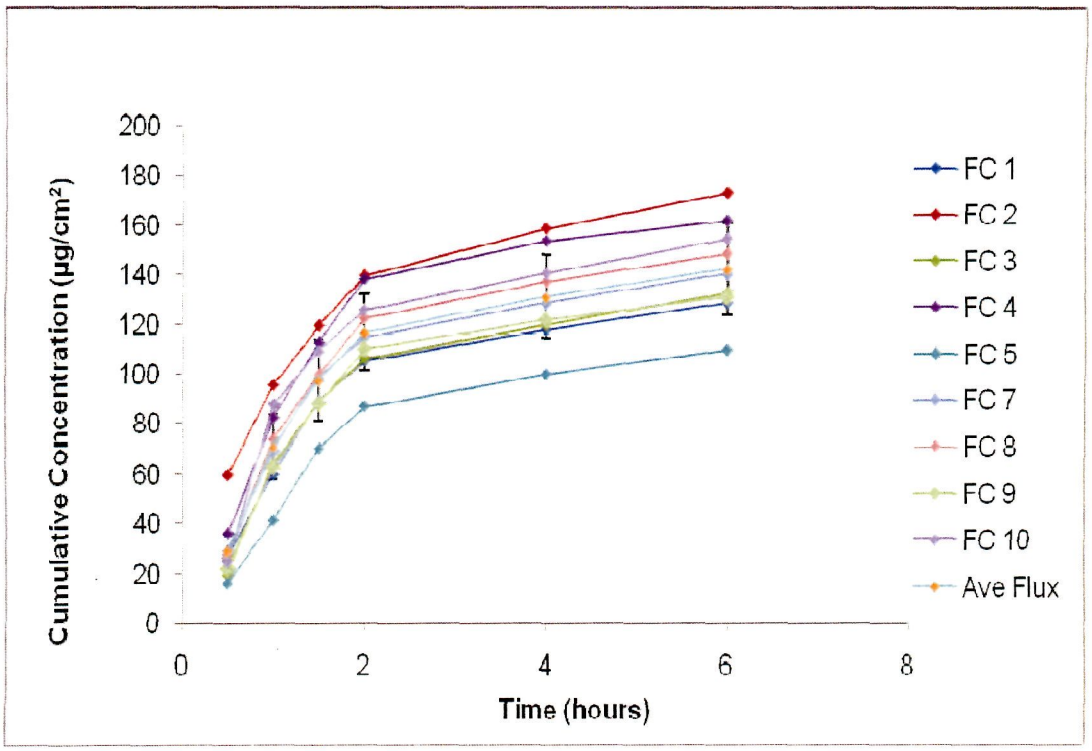

Figure D4 Cumulative amount Pheroid™ cream per area applied to the skin plotted against time for each individual Franz cell, illustrating average flux.

Figure D.5 illustrates the average cumulative concentration of alpha-lipoic acid in Pheroid™ cream that permeated through the epidermis was plotted against time. The average flux was as being the slope of the straight line. The profile of the Pheroid™ cream chart exhibited a biphasic flux pattern. A clear steady state flux from 0 to 2 hours was observed, after which the cumulative concentration of alpha-lipoic acid remained the same from 3 to 6 hours indicating that the entire amount of alpha-lipoic acid in Pheroid™ lotion had diffused through the skin. The average flux from 0 to 2 hours is 58.01 ± 6.63 µg/cm^2.h.

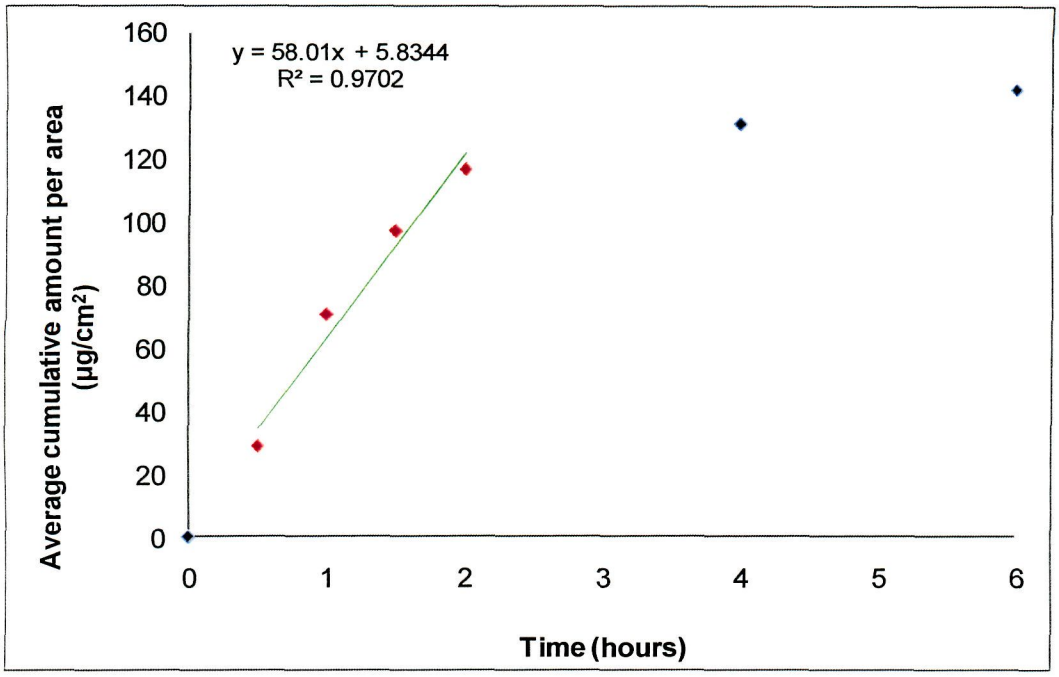

Figure D.5 The average cumulative amount of alpha-lipoic acid that penetrated the skin after application of the Pheroid™ cream as a function of time. The slope of the graph can be used in order to determine the average flux.

Figure D.6 illustrates the average cumulative concentration of alpha-lipoic acid that permeated through the epidermis was plotted against time. Average flux was obtained by the slope of the straight line. Pheroid™ gel exhibited a linear flux pattern from 4 to 12 hours. The average flux of Pheroid™ gel from 4 to 12 hours is 22.18 ± 3.33 µg/cm^2.h.

Table D.1 Average and median flux values of the formulations that permeated through the skin.

Formulation	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)
Pheroid™ cream	58.01 ± 6.63	57.8314
Pheroid™ gel	22.18 ± 3.33	20.299

The average flux for alpha-lipoic acid in neither the cream nor the gel could not be calculated, as none of the active diffused into the receptor phase of the Franz cells. The alpha-lipoic acid in cream was detected in the epidermal layer and the gel in the dermis during tape stripping.

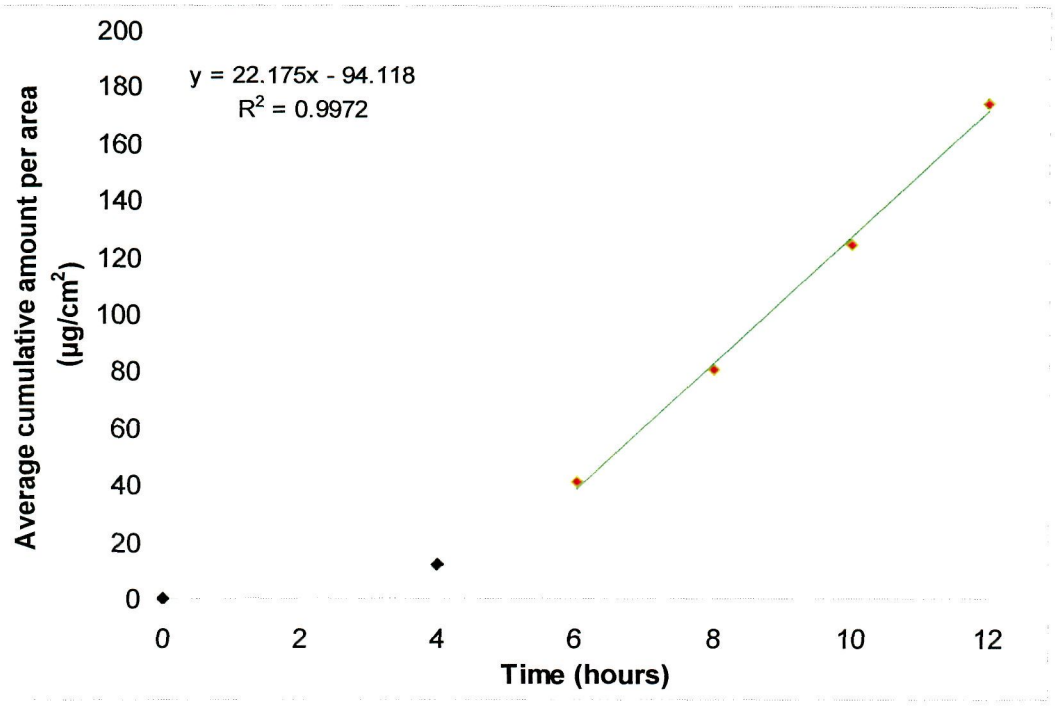

Figure D.6 The average cumulative amount of alpha-lipoic acid that penetrated the skin after application of the Pheroid™ gel as a function of time. The slope of the graph can be used in order to determine the average flux.

D.6.2 TAPE STRIPPING

The concentration of Alpha-lipoic acid in Pheroid™ cream found in the epidermis was 569.10 µg/ml and 0 µg/ml in the dermis. The concentration of Alpha-lipoic acid in Pheroid™ gel found in the dermis was 23.62 µg/ml and 0 µg/ml in the epidermis. The amount of Alpha-lipoic acid after application of the cream found in the epidermis was 764.93 µg/ml and 0 µg/ml in the dermis. The amount of Alpha-lipoic acid in the dermis after application of the gel was 61.06 µg/ml and in the epidermis 0 µg/ml. Alpha-lipoic acid in both the cream formulations favoured the epidermis while in the gel formulations it diffused into the dermis.

D.7 CONCLUSION

For statistical analysis, both the mean (synonym for average used to distinguish between the transdermal and statistical analysis) and the median (statistically calculated 50 percentile or centre

of a given set of data) of the flux values were examined. A Wilcoxon two-sample test (Steyn et.al., 1998) was performed to evaluate the differences in median value for two groups, Pheroid™ gel and gel after 12 hours, using the NPAR1WAY procedure of SAS (SAS Institute, Inc., 2005). A p-value less than 0.05 would indicate a statistical significance between the medians of the two groups. A p-value of 0.0002 was obtained, indicating that the data is highly statistically significant.

A Wilcoxon two-sample test was also performed to evaluate the differences in median value between the formulations that diffused into the dermis. A p-value of < 0.0001 was obtained. The same test was performed on the formulations that remained in the epidermis after diffusion. A p-value of < 0.0001 was obtained, rendering the data statistically meaningfully different.
A correlation value of -0.857 between flux and the concentration of Alpha-lipoic acid in the dermis was obtained after a CORR procedure of SAS. This means that the concentration of Alpha-lipoic acid in the dermis increases as the flux decreases. A p-value of < 0.0001 was obtained which indicates that the correlation is statistically significant. A correlation value of -0.824 between the concentration of Alpha-lipoic acid in the epidermis and Alpha-lipoic acid in the dermis was further obtained. This means that the concentration of Alpha-lipoic acid in the epidermis decreases as the concentration of Alpha-lipoic acid in the dermis increases. A p-value of < 0.0001 was obtained rendering the data statistically significant.

Alpha-lipoic acid was detected in the receptor compartments of the formulations containing Pheroid™ and was absent in the receptor compartments of the formulations not containing Pheroid™. This proves that the Pheroid™ was favourable for penetration of Alpha-lipoic acid through full-thickness skin. Tape stripping showed significant differences between the epidermis and dermis. The amount of Alpha-lipoic acid in the epidermis was higher in the Pheroid™ cream and the cream formulations, indicating that the creams formulations were more effective in delivering Alpha-lipoic acid to the target delivery site. When comparing the average (mean) and median flux values of Pheroid™ cream and Pheroid™ gel , the Pheroid™ cream proved to be have the better flux value.

ANNEXURE E: ANALYSIS OF FORMULATION COMPONENTS FOR ASSAY PURPOSES

E.1 THE HPLC SYSTEM

Analytical instrument: The HPLC analysis of Alpha-lipoic acid was performed by using an Agilent® 1100 Series HPLC (High Performance Liquid Chromatograph). The machine is designed with a G1311A quaternary pump, G1315A diode array detector, G1313A autosampler injection mechanism, G1322A vacuum degasser, solvent module and HP Chemstation Software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25°C.

Column: A high performance silica based, reversed phase Phenomenex® Luna C18 (2) column, (150 x 4.6 mm) with a 5 µm particle size was used. To maintain the condition of the column, it was rinsed each time before storage with HPLC grade water at a flow rate of 1.0 ml/min for 20 minutes, then with 70 % CH_3CN for a further 20 minutes and finally with $(CH_3)_2CHOH$ for the last 20 minutes.

Mobile phase: The mobile phase consisted of 75 volumes of methanol and 25 volumes of distilled water containing 10 % orhto-phosphoric acid.

Flow rate: 1 ml/min

Injection volume: 20 µl

Retention time: Approximately 30 minutes.

Detection: Diode array detector at 220 nm

E.2 PREPARATION OF THE STANDARD SOLUTION

Table E.1 illustrates the amounts of methylparaben, BHA, BHT, alpha-lipoic acid and *d-l*-alpha tocopherol that were accurately weighed in 50 ml volumetric flasks and filled with methanol. The amount of each of the five components in the standard solution resembles the amount which is found in 2 g of each formulation. These amounts represent a 100 % assay solution.

Table E.1 Amount of each component of the standard solution.

Component	Amount (mg)
Methylparaben	10.0
BHA	0.4
BHT	4.0
Alpha-lipoic acid	20.0
d-l-alpha tocopherol	4.0

E.3 VALIDATION OF THE HPLC METHOD

E.3.1 SPECIFICITY

The purpose of specificity is to provide an exact result which allows an accurate statement on the content or potency of the analyte in the sample (ICH Q2 (R1) 2005:4). 1 ml of the 100% standard solution was transferred into four vials. 1 ml of distilled water, H_2O_2, HCl and NaOH was added to each vial respectively. These vials were placed in an oven at 40 °C and left to stand overnight after which they were analysed in duplicate. HPLC analysis proved that the distilled water, H_2O_2, HCl and NaOH did not influence linearity of the analytical method.

E.3.2 LINEARITY

The linearity of an analytical method is its capacity (within a given range) to elicit test results which are directly proportional to the amount (concentration) of analyte in the sample. For the establishment of linearity, a minimum of 5 concentrations is recommended (ICH Q2 (R1) 2005:4). Five concentrations (70 %, 80 %, 100 %, 120 % and 130 %) of the standard solution were prepared for analysis. The linearity Methylparaben, BHA, BHT, of alpha-lipoic acid and *d-l*-alpha tocopherol was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml) of the standards

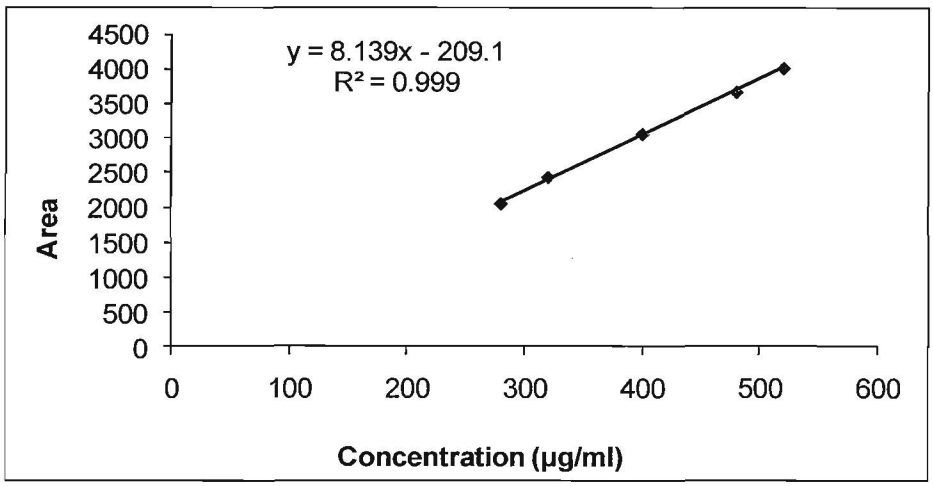

Figure E.1 Linear regression curve of alpha-lipoic acid.

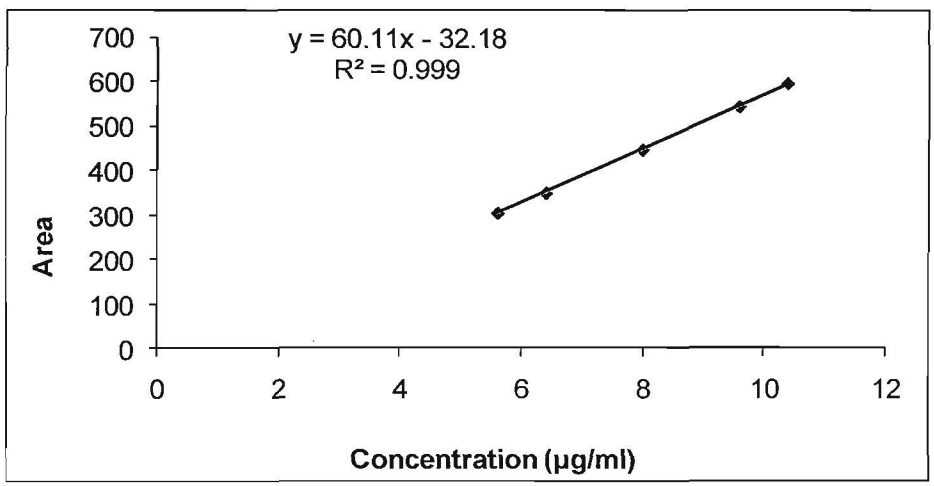

Figure E.2 Linear regression curve of BHA.

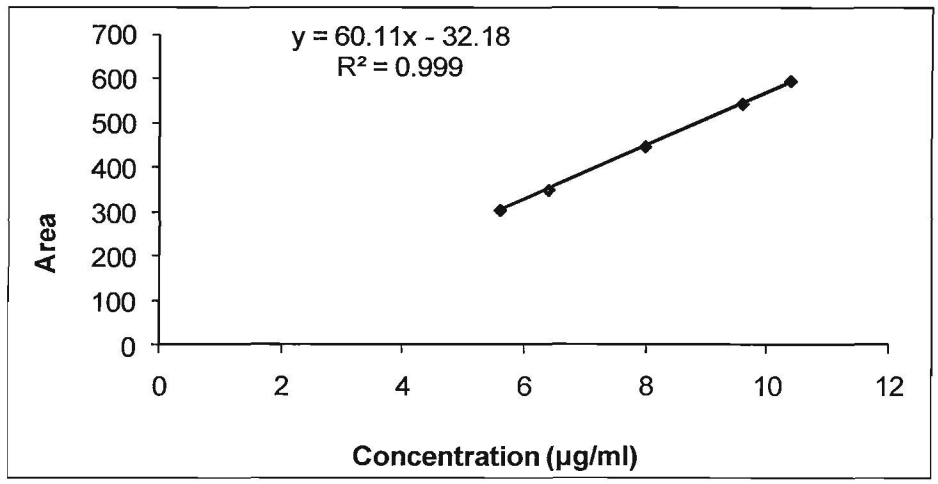

Figure E.3 Linear regression curve of BHT.

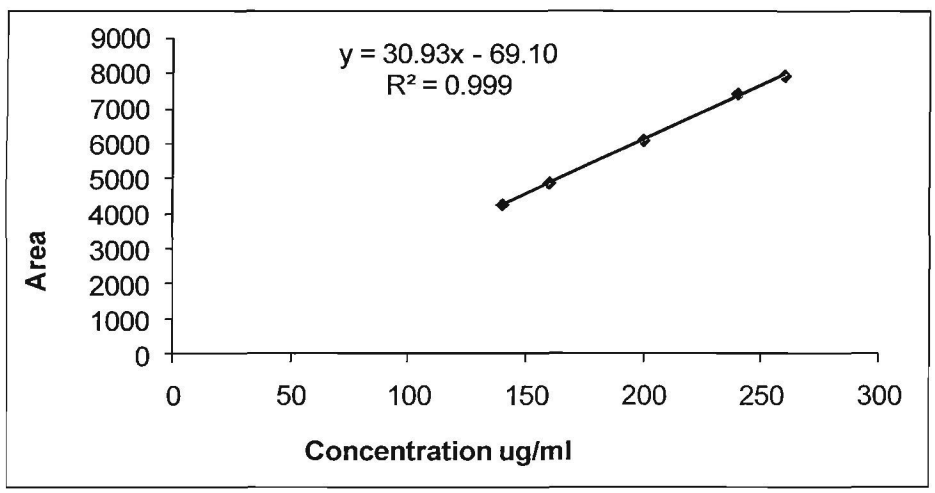

Figure E.4 Linear regression curve of methylparaben.

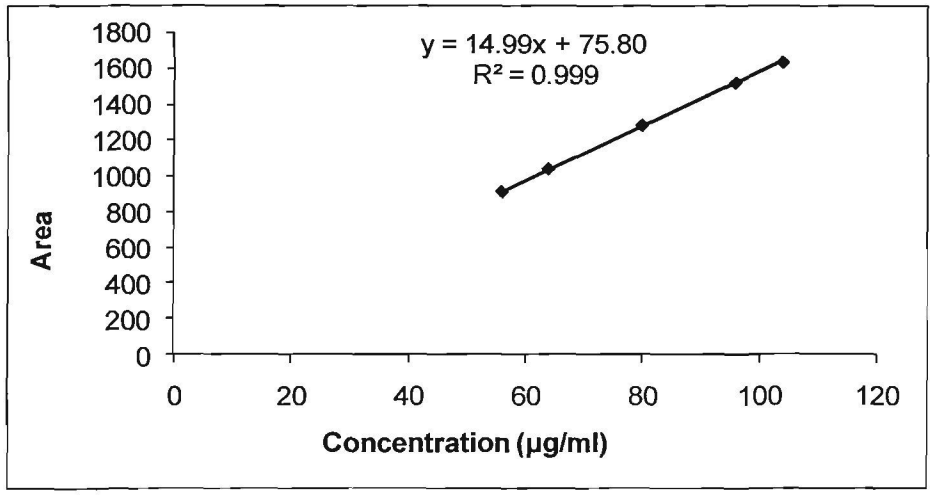

Figure E.5 Linear regression curve of of *d-l*-alpha tocopherol.

E.3.3 ACCURACY AND INTRA-DAY PRECISION

The accuracy of an analytical procedure represents the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

HPLC analyses was performed in triplicate on three different samples of known standards (80%, 100% and 120%) of the 5 formulation components during the same day (n = 3). Three concentrations of the standard solution were prepared for analysis. These concentrations were added to 1.6 g, 2.0 g and 2.4 g of a placebo cream (a cream containing none of the 5 components of the standard solution) respectively. The mixtures were transferred to 50 ml volumetric flasks, sonicated for 10 minutes, filtered and placed in vials for HPLC analysis. Each of the different concentrations were analysed in triplicate.

Recovery proved to be between 93.1 % and 103.34 % for methylparaben, between 97.83 % and 102.44 % for BHA, between 97.54 % and 102.79 % for BHT, between 99.03 % and 102.4 % for alpha-lipoic acid and between 97.90 % and 101.30 % for *d-l*-alpha tocopherol. According to the USP standards, accuracy of an analytical procedure should be established across its range.

Table E.2 Accuracy parameters for methylparaben.

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
161.40	4480.83	4461.81	4471.05	166.78	103.34
161.40	4454.56	4451.29	4452.93	166.11	102.91
161.40	4351.02	4428.00	4389.51	163.74	101.45
203.60	5432.96	5502.66	5467.81	203.97	100.18
203.60	5368.01	5422.12	5395.06	201.25	98.85
203.60	5389.41	5490.82	5440.12	202.93	99.67
241.10	6090.67	6128.35	6109.51	227.90	94.53
241.10	5989.26	6046.67	6017.97	224.49	93.11
241.10	6098.82	6049.43	6074.12	226.58	93.98

Mean	98.67
SD	3.67
%RSD	3.72

Table E.3 Accuracy parameters for BHA.

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
6.45	200.78	208.71	204.74	6.55	101.51
6.45	203.59	205.72	204.65	6.55	101.46
6.45	205.11	206.90	206.01	6.59	102.13
8.14	261.18	260.13	260.65	8.34	102.44
8.14	258.58	253.43	256.00	8.19	100.61
8.14	254.88	259.66	257.27	8.23	101.11
9.74	301.23	307.47	304.35	9.74	99.98
9.74	308.46	292.14	300.30	9.61	98.65
9.74	294.65	301.04	297.85	9.53	97.83

Mean	100.64
SD	1.47
%RSD	1.46

Table E.4 Accuracy parameters for BHT.

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
64.56	2404.73	2405.69	2405.21	65.92	102.10
64.56	2439.97	2403.28	2421.49	66.36	102.79
64.56	2434.19	2372.55	2403.37	65.87	102.02
81.44	2976.55	2975.27	2975.91	81.56	100.14
81.44	2947.74	2943.03	2945.38	80.72	99.12
81.44	2948.25	2974.62	2961.44	81.16	99.66
97.44	3498.30	3579.95	3539.13	96.99	99.54
97.44	3545.94	3507.41	3526.68	96.65	99.19
97.44	3455.54	3480.10	3467.82	95.04	97.54

Mean	100.23
SD	1.62
%RSD	1.61

Table E.5 Accuracy parameters for alpha-lipoic acid.

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
322.8	2305.05	2290.67	2297.86	330.55	102.40
322.8	2243.18	2255.45	2249.31	323.57	100.24
322.8	2253.59	2280.66	2267.13	326.13	101.03
407.2	2869.43	2869.01	2869.22	412.74	101.36
407.2	2885.67	2816.95	2851.31	410.17	100.73
407.2	2885.67	2865.23	2836.88	408.09	100.22
482.2	2808.53	3364.36	3390.41	487.72	101.14
482.2	3416.45	3310.66	3347.63	481.57	99.87
482.2	3332.57	3306.45	3319.51	477.52	99.03

Mean	100.67
SD	0.91
%RSD	0.91

Table E.6 Accuracy parameters for *d-l*-alpha tocopherol.

Conc. spiked µg/ml	Peak area 1	Peak area 2	Mean	Recovery µg/ml	%
64.56	1259.63	1254.36	1256.99	64.00	99.14
64.56	1243.12	1247.12	1245.12	63.40	98.20
64.56	1247.56	1235.05	1241.30	63.20	97.90
81.44	1622.98	1617.56	1620.27	82.50	101.30
81.44	1600.79	1591.05	1595.91	81.26	99.78
81.44	1592.89	1612.67	1595.91	81.26	99.78
97.44	1859.53	1859.02	1859.27	94.67	97.16
97.44	1898.61	1920.17	1909.39	97.13	99.77
97.44	1941.76	1925.49	1933.62	98.45	101.04

Mean	99.34
SD	1.31
%RSD	1.32

E.3.4 INTER-DAY PRECISION

HPLC analysis was performed in triplicate on 3 different samples of a known concentration of the 5 formulation components on three different days (n = 3). Table E.7 to E.11 reveal the results of the 5 different components.

Table E.7 Inter-day precision parameters for methylparaben.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	101.80	108.37	103.98	
	103.42	106.45	105.13	
	102.97	103.31	103.44	
	99.04	97.49	94.76	
	104.97	98.80	97.62	
	109.63	105.69	97.62	
	100.65	99.71	103.56	
	94.61	99.72	96.89	
	89.28	100.84	97.41	
Mean	100.71	102.26	100.05	101.00
SD	5.60	3.61	3.68	4.49
%RSD	5.56	3.53	3.68	4.45

Table E.8 Inter-day precision parameters of BHA.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	105.11	103.06	104.13	
	105.12	103.8	101.99	
	99.74	101.63	101.07	
	92.63	97.30	95.79	
	99.72	98.82	97.23	
	98.64	106.01	102.30	
	101.21	102.15	100.78	
	94.56	101.66	99.51	
	86.16	101.70	101.09	
Mean	98.10	102.12	100.43	100.27
SD	5.76	2.75	2.43	4.16
%RSD	5.78	2.70	2.42	4.15

Table E.9 Inter-day precision parameters of BHT.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	106.17	94.03	102.10	
	104.26	97.99	98.26	
	103.58	94.11	98.99	
	99.23	93.55	94.96	
	103.02	96.27	97.58	
	103.03	100.96	102.15	
	101.71	101.93	100.71	
	95.66	98.79	99.27	
	85.47	100.77	100.54	
Mean	100.24	97.60	99.40	99.08
SD	5.97	3.07	2.17	4.22
%RSD	5.95	3.15	2.18	4.26

E.4 CONCLUSION

The HPLC method developed has been found to be reliable and sensitive enough for the analysis of the formulations.

Table E.10 Inter-day precision parameters of alpha-lipoic acid.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	106.06	105.32	100.70	
	108.55	102.37	102.03	
	101.81	99.98	100.59	
	96.47	96.92	94.51	
	97.14	99.28	96.96	
	99.40	105.56	103.19	
	99.30	101.44	99.40	
	92.96	100.81	98.34	
	89.57	101.63	99.22	
Mean	99.03	101.48	99.44	99.98
SD	5.62	2.59	2.48	4.00
%RSD	5.68	2.56	2.49	4.00

Table E.11 Inter-day precision parameters of *d-l*-alpha tocopherol.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	103.67	105.15	103.53	
	97.43	104.22	103.07	
	104.59	101.02	100.16	
	99.44	97.62	95.23	
	102.25	99.64	98.41	
	103.46	106.84	104.06	
	101.41	103.49	100.79	
	94.20	102.83	99.81	
	91.27	103.95	100.57	
Mean	99.71	102.75	100.63	101.03
SD	4.31	2.70	2.61	3.55
%RSD	4.32	2.63	2.59	3.51

ANNEXURE F: INTERNATIONAL JOURNAL OF PHARMACEUTICS GUIDE FOR AUTHORS

Scope of the journal

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

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Note: In 2004, a new section was started on pharmaceutical nanotechnology. For more details, see Editorials in 279/1-2, 281/1, and 288/1.

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

Crowe, L.M., Crowe, J.H., Womersley, C., Reid, D., Appel, L., Rudolph, A., 1986. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. Biochim. Biophys. Acta, 861, 131-140.

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

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

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

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

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

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ANNEXURE G: PHOTOS OF APPARATUS USED DURING DIFFUSION STUDIES AND SAMPLE ANALYSIS AND STABILITY TESTING

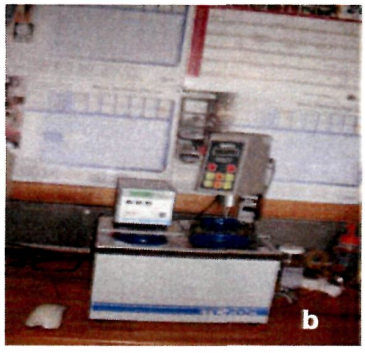

Figure G.1 The apparatus used during diffusion studies and stability testing were: **(a)** a climate chamber used to maintain the correct temperature and humidity for stability testing; **(b)** A Brookfield Model DV –II+ viscometer equipped with a water bath; **(c)** A Milli-Q water purifying system; **(d)** An Agilent® 1100 series HPLC; **(e)** A Mettler Toledo seveneasy pH meter; **(f)** Grant water bath; **(g)** A vertical Franz diffusion cell; **(h)** Variomag® magnetic stirrer plate; **(i)** Assembled Franz diffusion cells; **(j)** Vials used during HPLC analysis.