Formulation, in vitro release and transdermal diffusion of azelaic acid with topical niacinamide

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(B.Pharm.)

Dissertation approved in the partial fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

(PHARMACEUTICS)

in the

School of Pharmacy

at the

North-West University, Potchefstroom Campus

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POTCHEFSTROOM

2010

This dissertation is presented in the so-called article format, which includes an introductory chapter with sub-chapters, a full length article for publication in a pharmaceutical journal and appendix containing relevant experimental data. The article contained in this dissertation is to be published in the International Journal of Pharmaceutics of which the complete guide for authors is included in the appendix.

ABSTRACT

Acne is a common skin disease that affects the follicular unit of the skin. Inflammatory- and non-inflammatory forms of acne exist. The most affected areas on the body include the face, upper part of the chest and the back. These are the areas with the most sebaceous follicles. Acne occurs when hyperkeratinisation causes the cells of the hair follicle to shed too fast. These cells then block the follicle opening. Thus, sebum cannot pass through the hair follicle onto the skin.

The human skin is composed of three layers, namely the epidermis, which acts as a waterproof layer and a barrier to infections; the dermis, which contains the skin appendages; and the subcutaneous fat layer. Skin acts as a protective layer against pathogens and damage to the body. It also provides a semi-impermeable barrier to prevent water loss.

Azelaic acid and niacinamide are both currently used in the treatment of acne. Azelaic acid is a saturated dicarboxylic acid which is used to treat mild to moderate acne. It has antibacterial, keratolytic and comedolytic properties. Niacinamide, on the other hand, is the amide of nicotinic acid and is beneficial in the treatment of both papular and pustular acne. It has a demonstrated anti-inflammatory action and causes dose-dependent inhibition of sebocyte secretions.

The Pheroid™ delivery system is a colloidal system that consists of even lipid-based submicron- and micron-sized structures that are very unique in nature. This technology is able to improve the absorption and/or efficacy of various active ingredients, as well as other compounds.

In this study, a cream, Pheroid™ cream, a gel and a Pheroid™ gel were formulated, containing both azelaic acid and niacinamide. Stability tests were conducted on these formulations for six months, and it was established that none of the formulations were stable under the different storage conditions. Tests that were conducted during stability testing, as determined by the Medicines Control Council, included: assay, mass variation, appearance, viscosity, pH determination and confocal laser scanning microscopy (CLSM).

Diffusion studies (12 hours long in total) with vertical Franz cells were conducted with Caucasian female skin obtained after abdominoplastic surgery. Tape-stripping followed in order to establish the epidermis and dermis concentrations of azelaic acid and niacinamide. Significant concentrations of both active ingredients were found in the epidermis and the dermis after 12 hours.

Keywords: Azelaic acid, Niacinamide, Transdermal diffusion, Stability testing, Pheroid™.

OPSOMMING

Aknee is 'n algemene veltoestand wat die follikulêre eenheid van die vel affekteer. Daar bestaan inflammatoriese sowel as nie-inflammatoriese vorme van aknee. Die areas op die liggaam wat die meeste geaffekteer word sluit die gesig, boonste gedeelte van die borskas en die rug in. Hierdie areas bevat die meeste sebum-produserende follikels. Aknee kom voor wanneer hiperkeratinisering veroorsaak dat die selle van die haarfollikels te vinnig afskilfer, wat dus die follikel-opening blokkeer. As gevolg hiervan kan sebum nie deur die haarfollikel tot op die vel beweeg nie.

Die menslike vel bestaan uit drie lae, naamlik die epidermis, wat as water-bestande laag asook 'n beskermende laag teen infeksie dien; die dermis, wat die vel aanhangsels bevat; en laastens die subkutaneuse vetlaag. Die vel dien as 'n beskermende laag teen patogene en skade aan die liggaam. Dit verskaf ook 'n gedeeltelik-ondeurlaatbare beskerming om waterverlies te beperk.

Aselaïese suur en niasienamied word tans beide gebruik in die behandeling van aknee. Aselaïese suur is 'n versadigde dikarboksielsuur wat gebruik word om minder ernstige tot matige aknee te behandel. Dit besit antibakteriële, keratolitiese en komedolitiese eienskappe. Niasienamied, daarteenoor, is die amied van nikotiensuur en is voordelig in die behandeling van beide papulêre en postulêre aknee. Dit beskik ook oor anti-inflammatoriese werking en veroorsaak dosis-afhanklike inhibering van sebum sekresies.

Die Pheroid™ afleweringsisteem is 'n kolloïdale sisteem wat lipied-gebaseerde sub-mikron en mikron-grootte strukture bevat. Pheroid™ tegnologie het die vermoë om die absorpsie en/of die effektiwiteit van verskeie aktiewe bestanddele sowel as ander bestanddele te verbeter.

Gedurende hierdie studie is 'n room, 'n Pheroid™ room, 'n jel en 'n Pheroid™ jel, wat beide aselaïese suur en niasienamied bevat, geformuleer. Stabiliteitstoetse is gedoen op bogenoemde formulerings oor 'n tydperk van ses maande. Dit is bevind dat geen van die formulerings stabiel was onder die verskeie bergingstoestande nie. Die toetse wat uitgevoer is tydens die stabiliteitstoetsing, soos vasgestel deur die Medisynebeheerraad, sluit in: geneesmiddelkonsentrasiebepaling, massavariasie, voorkoms, viskositeit, pH-bepaling en konfokaallaseraftastingsmikroskopie.

Diffusie studies (12 uur lank in totaal) is uitgevoer met behulp van vertikale Franz selle op blanke vroulike vel wat verkry is na abdominoplastiese chirurgie. Die kleefband-afstropingstegniek is daarna gebruik om die konsentrasies van aselaïese suur en niasienamied, wat vasgevang is in die epidermis en dermis, vas te stel. Betekenisvolle konsentrasies van beide aktiewe bestanddele was teenwoordig na 12 uur in die epidermis en die dermis.

Sleutelwoorde: Aselaïese suur, Niasienamied, Transdermale diffusie, Stabiliteitstoetsing, Pheroid™.

ACKNOWLEDGEMENTS

First, all of my gratitude goes to the Lord my God, for the opportunity, the privilege and the ability to complete this dissertation.

Thank you to my parents, sisters and grandparents for their love and support throughout my studies, I love you very much. I dedicate this dissertation to you.

To all of my friends, you mean a lot to me, thank you very much for all your help, motivation and support. You were always there for me through the laughter and the tears. Thank you.

To Prof. Jan du Preez, my supervisor, thank you for all your help and support. It was a privilege working with you.

Prof. Jeanetta du Plessis, thank you for your help and support. Working with you was also a great privilege.

Dr. Joe Viljoen, thank you very much for your motivation, help and support, it is highly appreciated. You really helped me through difficult times, thank you.

Dr. Minja Gerber, you helped me a lot, thank you very much for your contribution to this dissertation.

Prof. Jan du Plessis, thank you for your help with the statistical analysis and processing of the data.

To the National Research Foundation and the North West University, thank you for financing this project.

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CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

Acne is a well-known skin disease that is commonly found in young and old. People suffering from acne usually experience a sense of despair about their appearance. Although acne does not kill, some people can suffer from psychological disability and it can cause significant discomfort and disfigurement (Frank, 1971:vii).

Varieties of acne can be identified and it is rarely found in a pure form. These varieties are identified by the predominant lesion which may include comedo-, papular-, pustular-, cystic-miliary-, indurata-, tropical acne and many more (Frank, 1971:12-27).

In earlier days, acne was seen as a problem that could be ignored. The viewpoint was that it could be outgrown or washed away. However, later on it was discovered that acne must be treated (Frank, 1971:173). Before treating a patient for acne, it is necessary to explain the reasons for their condition. The factors that must be taken into account when deciding on the type of therapy include the type of acne as well as the severity thereof. Both topical and oral treatments are available. Topical treatment is sufficient for mild acne, but more severe acne requires oral treatment (Cunliffe, 1989:252-253).

Azelaic acid proved to be very beneficial in the treatment of acne. It is an anti-inflammatory, antioxidant, anti-keratinising and bacteriostatic agent, thus it is a very good option for the treatment of acne (Draelos & Kayne, 2008:AB40).

Niacinamide is used in the treatment of different skin problems, including acne. It is beneficial in the treatment of both papular and pustular acne (Draelos, 2000:237).

For this reason, azelaic acid and niacinamide were combined into a single topical acne product during this study. No similar products are currently available. Both azelaic acid and niacinamide need to be present within the epidermis and the dermis of the skin and also in the pilosebaceous unit of the skin. These two main ingredients were formulated into a cream and a gel. Both of these formulations were also formulated with the Pheroid™, which is a carrier system and this system will be discussed in section 2.3.7.

The formulated products underwent numerous studies which included stability testing at specified conditions as well as *in vitro* diffusion studies with vertical Franz diffusion cells.

The aim of this study was to determine whether the Pheroid™ formulations were more efficient in delivering azelaic acid and niacinamide transdermally than the non-Pheroid™ formulations. It was also determined whether these formulations were stable under different storage conditions.

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CHAPTER 2: TOPICAL DELIVERY OF AZELAIC ACID IN COMBINATION WITH NIACINAMIDE IN THE TREATMENT OF ACNE

2.1 Acne

Acne vulgaris is a common skin disease, a chronic inflammatory condition (Bershad, 2001:279). It affects almost 80% of adolescents and young adults (Krautheim & Gollnick, 2004:398). According to Adebamowo *et al.* (2008:787), acne is more common in girls, in the age group 12 years or younger. However, in adolescents 15 years or older, acne presents more in boys.

As mentioned above, the frequency and severity of acne, as well as its tendency toward scarring, is greater in males than females within the adolescent group. However, the persistence of acne into adulthood is more common in females (Bershad, 2001:279). Acne usually diminishes over time and will most probably decrease or disappear in the early twenties of a person's life. It can cause significant embarrassment and anxiety in affected patients (Feldman *et al.*, 2004:2123). Teenage acne causes discomfort, disfigurement, emotional distress and sometimes permanent scarring. Besides diminishing the patient's social and psychological wellbeing, acne in the paediatric age group and in pregnant and lactating women, presents therapeutic challenges according to the few drug studies in such patients (Akhavan & Bershad, 2003:474). Smoking, the male sex, genetics and youth are some of the risk factors for the development of, or increased severity of acne (Krautheim & Gollnick, 2004:398). Other potential factors that may contribute to acne development and severity include stress and exposure to comedogenic substances including tars, polyvinyl chloride and certain medications, i.e., corticosteroids, androgens and halogens. Other medications that may induce or worsen acne are bromides, iodides, lithium and vitamin B_{12} (Olutunmbi *et al.*, 2008:172).

Previous studies conducted by Adebamowo *et al.* (2008:788) depicted a positive association with milk consumption and prevalence of acne among a prospective cohort of girls in the USA aged 9 to 15. In another study by Adebamowo *et al.* (2008:789) to examine the association between dietary dairy intake and teenage acne among boys, they found that 79% of the boys reported that a few or more pimples occurred sometimes, whereas 44% reported that usually a few or more pimples occurred. It was concluded that the most consistent factors associated with acne were age, height and intake of skimmed milk. No association with total fat, dairy fat, total vitamin A and vitamin A from foods were found. Thus, Adebamowo *et al.* (2008:790) suggested that neither vitamin A nor the fat component of milk are important for comedogenicity. Milk intake may, however, influence comedogenesis due to the fact that it

contains androgens, 5α-reduced steroids and other nonsteroidal growth factors that affect the pilosebaceous unit (Adebamowo *et al.*, 2008:791).

Acne can be described as a disease that affects the pilosebaceous unit of the skin (Webster, 2002:475). Patients presenting with acne usually have a variety of lesions in various stages, along with post-inflammatory acne scars and hyperpigmentation (Olutunmbi *et al.*, 2008:172). The typical clinical picture of acne is an eruption located on the face, with the upper trunk often being affected as well (Bershad, 2001:279). According to Webster (2002:475), acne is limited to the more active pilosebaceous glands of the head and upper body, whereas Olutunmbi *et al.* (2008:172) stated that acne lesions are mostly present on the face, chest, upper arms and upper back. The following figure depicts the presentation of acne on the face and upper body.

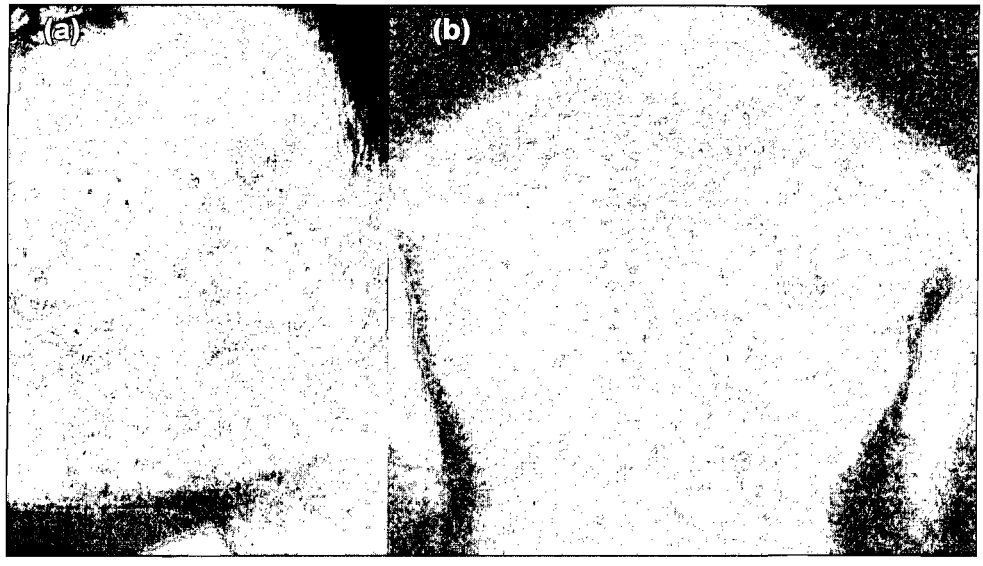

Figure 1: The presentation of acne on **(a)** the face and **(b)** the upper body.

Androgens, which appear at the beginning of adolescence, increase the production of sebum and also enlarge the sebaceous glands (Krautheim & Gollnick, 2004:398) by filling up pre-existing comedones with lipids (Webster, 2002:475). The pilosebaceous ducts are blocked, which leads to the development of microcomedo's as primary lesions (Krautheim & Gollnick, 2004:398). Thus, acne occurs when sebum is not able to pass through the hair follicle onto the skin. This happens when the cells of the hair follicle shed too fast, due to hyperkeratinisation which prevents normal shedding (Feldman *et al.*, 2004:2123) and blocks the opening of the follicle with the result that sebum cannot pass through. Lipids and cellular debris accumulate (Feldman *et al.*, 2004:2123) and the mixture of sebum and cells causes bacterial growth. The micro-environment enhances the colonisation of *Propionibacterium acnes*. *P. acnes* colonises when the shedded cells mix with the sebum and inflammatory reactions occur (Krautheim & Gollnick, 2004:398). This organism consumes glycerol fractions and discards the free fatty acids after metabolising sebaceous triglycerides (Webster, 2002:475).

Microcomedos, also known as "blackheads", are the impaction and distension of follicles with keratinocytes and sebum (Webster, 2002:475). The "blackhead" is an open comedo, whereas the "whitehead" is a closed comedo. Typical acne lesions are called comedones, inflammatory papules, pustules and nodules. When a closed comedo causes the follicular wall to rupture, an inflammatory reaction occurs. Due to this, papules, pustules, nodules and cysts form. A cyst is a pus-filled acne lesion greater than 5 mm in diameter, in which the wall is composed of inflammatory cells and scar tissue (Bershad, 2001:279).

Inflammation occurs when *P. acnes* is brought into contact with the immune system (Webster, 2002:475). Inflammation is enhanced by follicular rupture and leakage of lipids, fatty acids and bacteria onto the dermis (Feldman *et al.*, 2004:2123). Non-inflammatory forms of acne also exist (Webster, 2002:475).

The diagnosis of acne is generally straightforward. Differential diagnosis includes rosacea, which lacks comedones; perioral dermatitis; folliculitis and drug-induced acneform eruptions (Olutunmbi *et al.*, 2008:172).

Contrary to popular belief, hygiene plays, at most, a minor role in the etiology of acne and diet appears to have little or no influence. However, true acne can be exacerbated by external factors such as friction and pore-clogging cosmetics. The etiology of acne lies in a confluence of several factors which together produce clinical acne. Research suggests that genetic control, along with the stimulation of androgenic hormones, are responsible for abnormal sebum production (Bershad, 2001:280). According to Akhavan and Bershad (2003:474), only heredity and hormones are involved, and neither diet nor hygiene plays a meaningful role.

2.2 The current treatment of acne

According to Feldman *et al.* (2004:2129), the goals of acne therapy include controlling acne lesions, preventing scarring and minimising morbidity. They also state that the patient should be informed that the goal is to prevent new lesions and that current lesions must heal on their own.

When deciding on the regimen for treating acne, individual patient factors should be taken into account. These factors include disease state (predominant lesion type and severity), pre-existing medical conditions, and desired treatment mode (topical or systemic – thus oral) as well as endocrine history. Successful treatment is often achieved by targeting more than one of the known mechanisms involved in the pathogenesis of acne with combination therapy. Patients are typically evaluated on a quarterly basis and the regimen is adjusted based on the clinical response (Olutunmbi *et al.*, 2008:173).

In the treatment of acne, there are topical- and systemic agents. Acne may be treated through the use of topical agents, systemic agents or a combination of both.

2.2.1 Topical agents

Selection of topical therapy should be based on the severity and type of acne. Effective treatment for mild acne includes topical retinoids, benzoyl peroxide and azelaic acid. Topical antibiotics and medications with bacteriostatic- and anti-inflammatory properties on the other hand, are effective for treating mild to moderate inflammatory acne. Selected topical medications for the treatment of acne are as follows (Feldman *et al.*, 2004:2124):

Retinoids

- *Adapalene*

This is the most commonly used topical retinoid agent (Akhavan & Bershad, 2003:480). Adapalene is a topical synthetic retinoid analogue that normalises differentiation of follicular epithelial cells (Feldman *et al.*, 2004:2126). Thus it inhibits comedo formation and it demonstrates direct anti-inflammatory properties (Bershad, 2001:280). The anti-inflammatory effect is due to a more significant inhibition of lipoxygenase activity and subsequent eicosatetraenoic acid production by human leucocytes (Krautheim & Gollnick, 2003:1292). This is a reasonable choice as a first-line topical retinoid (Feldman *et al.*, 2004:2126).

- *Tazarotene*

Tazarotene is the newest topical agent in the retinoid class for acne. It is a synthetic acetylenic molecule that is rapidly converted to its active metabolite, tazarotenic acid, in keratinocytes (Akhavan & Bershad, 2003:481). It is usually considered a second-line retinoid option, due to its ability to cause an increase in skin irritation in patients who have not responded to topical tretinoin or adapalene therapy. It is not recommended for use during pregnancy as it is a category X agent (Feldman *et al.*, 2004:2126), which means foetal abnormalities may occur.

- *Tretinoin*

This is a naturally occurring form of vitamin A (Akhavan & Bershad, 2003:477). Tretinoin is a comedolytic agent that normalises desquamation of the epithelial lining, thereby preventing obstruction of the pilosebaceous outlet (Feldman *et al.*, 2004:2125). It has been a mainstay in the topical treatment of acne vulgaris for more than three decades (Krautheim & Gollnick, 2004:1290). Tretinoin appears to have direct anti-inflammatory effects (Feldman *et al.*, 2004:2125).

Antibiotics

- *Clindamycin*

Clindamycin is a lincosamide antimicrobial agent and is a semi-synthetic derivate of lincomycin. This antibacterial inhibits bacterial protein synthesis by attaching to the 50S subunit of the bacterial ribosome (Akhavan & Bershad, 2003:482). Therefore, it reduces the population of *P. acnes* on the skin surface and especially within the follicles (Krautheim & Gollnick, 2003:1293).

- *Erythromycin*

This antibiotic is a macrolide that attaches to the 50S subunit of bacterial ribosomes (Akhavan & Bershad, 2003:483). It is well established as an effective topical antibacterial in acne therapy (Krautheim & Gollnick, 2003:1294). Erythromycin prevents the effective progression of the translocation reaction necessary for bacterial protein synthesis (Akhavan & Bershad, 2003:483). It, just like clindamycin, reduces the population of *P. acnes* on the skin surface and particularly within the follicles (Krautheim & Gollnick, 2003:1293).

Other

- *Benzoyl peroxide*

Benzoyl peroxide is an agent with both antibacterial and comedolytic action. It has a potent bactericidal effect against *P. acnes*. The mechanism of action is thought to be degradation of bacterial proteins via release of free-radical oxygen (Akhavan & Bershad, 2003:482). There is evidence that it reduces comedones, in addition to improving inflammatory acne (Bershad, 2001:282). Combinations of topical antibiotics and benzoyl peroxide increase efficacy and reduce antibiotic resistance in patients with *P. acnes* colonisation (Feldman *et al.*, 2004:2126). Benzoyl peroxide has no effect on sebum production (Krautheim & Gollnick, 2003:1295).

- *Salicylic acid*

This is a topical keratolytic agent that dissolves the intercellular cement which holds epithelial cells together (Akhavan & Bershad, 2003:485-486). It increases penetration of other substances, has a slight anti-inflammatory effect, and is bacteriostatic and fungistatic in low concentrations as a result of competitive inhibition of pantothenic acid (Krautheim & Gollnick, 2003:1296). It is a component of a variety of over-the-counter acne remedies. Salicylic acid causes severe stomach irritation and is therefore not prescribed for oral use (Akhavan & Bershad, 2003:485-486).

- *Sodium sulfacetamide*

Sodium sulfacetamide is a bacteriostatic antibacterial in the sulphonamide group. It displays activity against several gram-negative and gram-positive organisms. Sulphonamides act through competitive antagonism of para-aminobenzoic acid (PABA), halting bacterial DNA synthesis (Akhavan & Bershad, 2003:486). These products are generally not considered first-line therapies, however, they may be used in patients who cannot tolerate other topical agents (Feldman *et al.*, 2004:2127).

- *Sulphur*

The chemical element sulphur is considered a mild keratolytic and bacteriostatic agent. In keratinocytes, sulphur is reduced to form hydrogen sulphide by an unknown mechanism. The formed hydrogen sulphide is thought to break down keratin and it is also believed that sulphur has activity against *P. acnes* (Akhavan & Bershad, 2003:485).

- *Azelaic acid*

Azelaic acid, also known as 1,7-heptanedicarboxylic acid, lepargylic acid or anchoic acid, is a naturally occurring straight-chained, 9 carbon atom dicarboxylic acid (Thiboutot *et al.*, 2003:837).

Azelaic acid is currently being used in the treatment of acne. It shows anti-inflammatory, antioxidant, anti-keratinizing and bacteriostatic properties which makes it a very good option for acne (Draelos & Kayne, 2008:AB40). The effect against *P. acnes* is initiated by the inhibition of protein synthesis (Shemer *et al.*, 2002:178). In higher concentrations, azelaic acid is also effective against *Staphylococcus epidermis* (Manosroi *et al.*, 2005:236). Bacterial resistance to azelaic acid have not been reported (Webster, 2000:S49).

- *Niacinamide*

Niacinamide is the physiologically active amide of Vitamin B3 (Namazi, 2007:1229). It is a combination of niacin, also known as nicotinic acid, and its amide. Therefore niacinamide is also called nicotinamide. Niacinamide is a hydrophilic compound (Barai, 2001:10).

Niacinamide is being used in different skin problems, including atopic dermatitis, rosacea, hyperpigmentation, skin aging and acne. Application of 4% topical niacinamide has led to a global reduction in acne (Namazi, 2007:1230). It is beneficial in the treatment of both papular and pustular acne (Draelos, 2000:237). According to *in vitro* studies that were conducted, niacinamide causes dose-dependent inhibition of sebocyte secretions (Namazi, 2007:1230). Draelos, Matsubara and Smiles (2006:99-100) stated that niacinamide causes less sebum

production and reduction of facial shine and oiliness. Niacinamide has an anti-inflammatory effect and can therefore be used to reduce inflammatory papules (Gehring, 2004:92). As mentioned, niacinamide can also be used in the reduction of hyperpigmentation and improvement of the barrier function (Bisset *et al.*, 2005:P32). According to Barai (2001:18), niacinamide changes and maintains skin texture and properties.

Azelaic acid and niacinamide have not yet been combined into a topical product for the treatment of acne. Both of these active ingredients are currently used in the treatment of acne. Therefore, the purpose of this study was to combine these two active ingredients in a cream or gel that can be used for the treatment of mild to moderate acne.

2.2.2 Systemic agents

Acne that is resistant to topical treatment or that manifests as scarring or nodular lesions typically requires oral antibiotics (Webster, 2002:476). According to Katsambas and Papakonstantinou (2004:412) systemic treatment is necessary to prevent significant psychological and social impairment in acne patients. The choices of systemic agents include oral antibiotics, isotretinoin and hormonal treatment (Katsambas & Papakonstantinou, 2004:412).

Oral antibiotics

Oral antibiotics in acne are intended for long-term use (Katsambas & Papakonstantinou, 2004:413). It is indicated for the management of moderate and severe acne, acne that covers large parts of the body surface and acne that is resistant to topical treatment. Oral antibiotics suppress the growth of *P. acnes* as well as the inflammatory mediators synthesised and released by this pathogen (Katsambas & Papakonstantinou, 2004:412).

The following table lists examples of oral antibiotics, including their name, dosage, duration of treatment and drawbacks.

Table 1: Oral antibiotics for systemic treatment of acne (Katsambas & Papakonstantinou, 2004:413).

Antibiotic	Name	Dose	Duration	Drawbacks
Tetracyclines	Tetracycline Oxytetracycline	250-500 mg twice daily	4-6 months	• Gastrointestinal upset • Vaginal candidiasis • Need to take on empty stomach, can decrease compliance
	Doxycycline	50-100 mg twice daily	4-6 months	• Gastrointestinal upset • Photosensitivity
	Minocycline	• 50-100 mg twice daily • 100 mg once daily (slow-release)	4-6 months	• Vertigo • Hyperpigmentation of skin and oral mucosa • Expensive • Uncommonly significant systemic adverse effects
Macrolides	Erythromycin	500 mg twice daily	4-6 months	• Gastrointestinal upset • Vaginal candidiasis • Emergence of resistance of *P. acnes* is common
New antibiotics				
Tetracyclines	Lymecycline	150-300 mg daily	4-6 months	
Macrolides	Azithromycin	250 mg three times a week	4-6 months	• Gastrointestinal upset

Isotretinoin

Isotretinoin is a vitamin A derivative that is being used for severe, often nodulistic and inflammatory acne (Feldman *et al.*, 2004:2128). This systemic agent is indicated as a first-line agent for patients with severe nodulistic acne and can also be beneficial to patients with moderate or even mild acne who are resistant to long-term oral or topical treatment. It is also the first line treatment for acne associated with severe scarring or significant psychological complications, and extensive acne involving the face and trunk (Katsambas & Papakonstantinou, 2004:414). Isotretinoin acts against the four pathogenic factors that contribute to acne (Feldman *et al.*, 2004:2128-2129). It causes de-differentiation of the sebaceous gland, suppressing sebum production to pre-adolescent levels, thus the colonisation of *P. acnes* subsides. It also promotes shedding of keratinocytes (Bershad, 2001:283) and is the only medication with the potential to suppress acne in the long term (Feldman *et al.*, 2004:2128-2129). Patients using isotretinoin should be monitored routinely due to possible side-effects (Webster, 2002:478).

Hormonal

Hormonal treatment is a useful alternative to isotretinoin for all types of acne in adult and adolescent females. Its effectiveness is based on decreasing androgen-induced sebum production. The most common choice for hormonal treatment is oral contraceptives. Oral contraceptives reduce the availability of biologically active free testosterone by increasing hepatic synthesis of sex hormone-binding globulin. In addition, they inhibit the ovarian production of androgens by suppressing ovulation. These effects result in decreased sebum production. All oral contraceptives are effective in treating hormonal-related acne, although those containing progestins with no inherent androgenic activity, are generally preferred (Katsambas & Papakonstantinou, 2004:415). An alternative drug for treating hormonal acne in women is spironolactone, which can be combined with oral contraceptive therapy (Bershad, 2001:283). Spironolactone is an androgen receptor blocker, effective in treating inflammatory acne (Katsambas & Papakonstantinou, 2004:416).

2.3 Transdermal Drug Delivery

2.3.1 Structure and barrier functions of the skin

The skin is the largest organ in the system that protects the body from damage, called the integumentary system. It forms the body's defensive outer layer (Flynn, 2002:187) and therefore protects the body against harmful external factors. Through interfacing with the environment the skin plays a significant role in protecting the body against pathogens. Human skin regulates heat and water loss from the body. It can be categorised into four main layers,

namely: the subcutaneous tissue, dermis, viable epidermis and the stratum corneum (Williams, 2003:1-2).

The subcutaneous tissue is a fat layer between the overlying dermis and the underlying body constituents. In most areas of the body this layer is relatively thick (millimetres), however, there are some areas where it is not present, i.e., the eyelids. The primary purpose of this fatty layer is to insulate the body and also to give mechanical protection against physical shock. This layer carries the main blood vessels and nerves that innervates the skin (Williams, 2003:2).

The dermis is the major component of human skin (Williams, 2003:2). This layer exists between the viable epidermis and the subcutaneous fatty layer. The dermis is a complex structure held together by structural fibres, collagen, reticulum and elastin (Flynn, 2002:192). Oil- and sweat glands, nerve endings and blood vessels are present in the dermis. The subcutaneous fat layer underneath the dermis helps the body to stay warm and to absorb shocks. Furthermore it helps to "hold" the skin to the tissues. The dermis ranges from approximately 1–5 mm in thickness. The upper one fifth of this layer is named the papillary layer. This layer is the support for the subtle capillary plexus which nurtures the epidermis. It merges into the reticular dermis, which is a far coarser matrix. This is the deepest layer of the true skin and thus the main structural element of the skin (Flynn, 2002:192).

According to Williams (2002:2), in transdermal drug delivery the dermis is often viewed as essentially gelled water. Therefore it provides a minimal barrier to the delivery of most polar drugs. However, when delivering highly lipophilic molecules, the dermal barrier may be significant. Within this layer, numerous structures are embedded, such as blood and lymphatic vessels, nerve endings, pilosebaceous units and sweat glands.

The vasculature of the skin is essential for regulation of body temperature. Furthermore, it delivers oxygen and nutrients to the body tissue while removing toxins and waste products. The vasculature is also important in wound repair. Blood flow is approximately 0.05 ml/min per mg of skin. The rich blood flow is very efficient in the removal of molecules that have traversed the outer skin layers. Capillaries reach within 0.2 mm of the skin surface. Thus, molecules are removed (*in vivo*) from near the dermo-epidermal layer. This ensures that dermal concentrations of most permeants are very low. For the transdermal delivery of most drugs, the blood supply maintains a concentration gradient between the applied formulation on the skin surface and the vasculature across the skin membrane. It is this concentration gradient that is the driving force behind drug permeation (Williams, 2003:4).

The viable epidermis is a multilayered mass. It varies in thickness from 0.06 mm on the eyelids to approximately 0.8 mm on the palms and the soles of the feet. No blood vessels are present in this layer and thus, nutrients and waste products must diffuse across the dermo-epidermal

layer in order to maintain tissue integrity. Molecules permeating across the epidermis must cross the dermo-epidermal layer in order to be cleared into the systemic circulation (Williams, 2003:5). The interface between the stratum corneum and the viable epidermis is flat, whereas the interface of the epidermal mass with the dermis is mounded (Flynn, 2002:191). Almost 95% of the epidermal cells generate new skin cells, whereas the other 5% produces melanin, which provides colour to the skin.

Four histologicallly distinct layers may be identified in the epidermis, i.e., the stratum germinativum on the inside, the stratum spinosum, the stratum granulosum and on the outside the stratum corneum. This last layer mostly consists of dead cells. It provides the main barrier to transdermal delivery of drugs, and is therefore often treated as a separate membrane (Williams, 2003:5).

- Stratum germinativum

This is also known as the stratum basale. The cells of this layer are similar to those of other tissues within the body. These cells contain typical organelles, for example mitochondria and ribosomes; and are thus metabolically active. The stratum germinativum is the only layer in the epidermis that contains cells which undergo cell division via mitosis (Williams, 2003:7).

- Stratum spinosum

The stratum spinosum is also known as the spinous layer or the prickle cell layer. It is found on top of the stratum germinativum. These two layers together are called the Malpighian layer. The stratum spinosum consists of 2-6 rows of keratinocytes. Keratinocytes change morphology from columnar to polygonal cells. They begin to differentiate and synthesise keratins that combine to form tonofilaments. The tonofilaments condensate to form desmosomes. These desmosomes connect the cell membranes of adjoining keratinocytes, and maintain a distance of about 20 nm between the cells (Williams, 2003:8)

- Stratum granulosum

From the stratum spinosum through to the stratum granulosum, the keratinocytes continue to differentiate, synthesise keratin and start to flatten. The stratum granulosum is only 1 - 3 cell layers thick. It contains enzymes that begin the degradation of the viable cell components like the nuclei and organelles (Williams, 2003:8).

- Stratum corneum

The stratum corneum is the outermost layer of the skin. This horny layer is continuously under formation (Flynn, 2002:189). Although it is an epidermal layer, it is often, in topical and transdermal drug delivery, viewed as a separate membrane. It consists of approximately 10 - 15 cell layers and is approximately 10 µm thick when dry. When wet, the stratum corneum can swell several times in thickness. It is thickest on the palms and the soles of the feet, and thinnest on the lips. The stratum corneum regulates water loss from the body and also prevents the entry of harmful materials, including micro-organisms. The barrier nature of the stratum corneum depends on its constituents. It consists of 75 - 80% protein, located within the keratinocytes; 5 - 15% lipid and 5 - 10% of this layer is unidentified (Williams, 2003:9). Water plays a key role in maintaining the stratum corneum barrier integrity. It may mediate the activity of some hydrolytic enzymes within the stratum corneum since environmental humidity affects the activities of enzymes involved in the desquamation process. Water is also a plasticizer and thus, prevents the stratum corneum from cracking due to mechanical assault (Williams, 2003:13). The stratum corneum is in contact with the viable epidermis and is externally exposed to the environment (Flynn, 2002:189).

Skin appendages include hair follicles with their associated sebaceous glands, apocrine glands, eccrine glands, as well as finger- and toenails. As mentioned, each hair follicle contains one or more sebaceous glands. The hair follicles with its sebaceous glands are called a pilosebaceous unit. The pilosebaceous duct leads from the sebaceous gland to the open space around a hair shaft (Flynn, 2002:193). Hair follicles are found over the entire surface of the skin, except for the soles of the feet, the palms of the hands and the lips (Williams, 2003:4). Characteristics of the skin differ on different areas of the body, i.e. the head contains the most hair follicles on the body where, as mentioned, the soles of the feet contain none. Sebaceous glands produce sebum, which is composed of free fatty acids, waxes and triglycerides (Williams, 2003:4). It rises to the skin's surface to lubricate it and furthermore it makes the skin waterproof.

See figure 2 for the different layers of the skin.

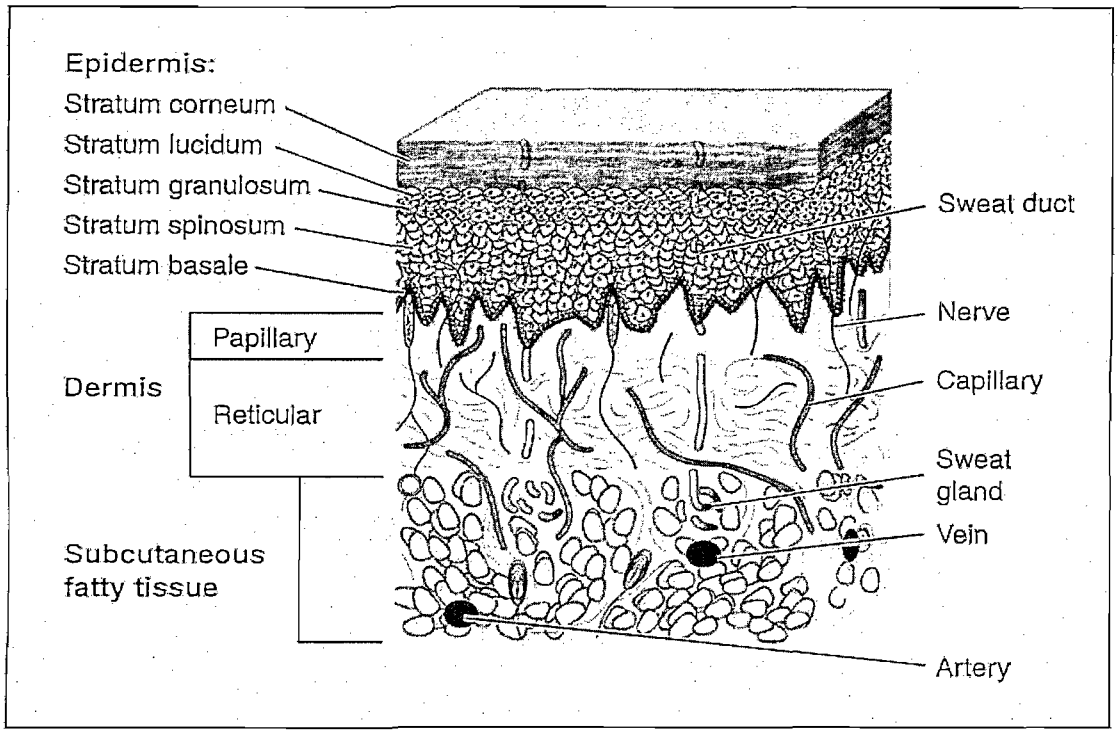

Figure 2: The layers of the skin (Anon. 2006).

The pH of the skin is approximately 4.2 - 5.6 (Yu & Van Scott, 2004:78). Sebum contributes in maintaining the surface pH at approximately 5. Sweat glands (eccrine glands) and apocrine glands are present in the skin. They originate in the dermal tissue. Eccrine glands are found over most of the body surface. They secrete sweat which is a diluted salt solution with a pH of approximately 5. The glands are stimulated in response to heat and emotional stress (Williams, 2003:4).

In transdermal drug delivery, skin appendages may offer a potential route by which molecules could enter the lower layers of the skin without having to cross the stratum corneum, which as mentioned previously, provides a barrier.

According to Flynn (2002:194), normal stratum corneum is a dense molecular continuum penetrable only by molecular diffusion. It is virtually an absolute barrier to microbes, preventing them from reaching the viable tissues and an environment suitable for their growth.

Other than physical barrier protection, several natural processes lead to skin surface conditions that are unfavourable to microbial growth. As mentioned previously, both sebaceous and eccrine secretions are acidic, lowering the surface pH of the skin below that welcomed by most pathogens. This acid layer, with a pH of approximately 5, is somewhat bacteriostatic. Also mentioned before, the sebum contains a number of short-chain fungistatic and bacteriostatic fatty acids. The dry surface of the skin also provides a level of protection. In general, infections

of the skin are more common in skin folds during warm weather. Intensified sweating leaves the skin continually moist in these regions (Flynn, 2002:194-195).

Pilosebaceous glands seem more vulnerable to infection, especially those on the forehead, face and upper back. Glands in these areas are especially prone to occlusion followed by infection, for example acne form pimples and blackheads. These infections are usually limited to a small area. However, if the infected gland ruptures and spews its contents internally, deep infection is possible. The body defends against this by walling off the lesion, forming a sac or cyst, and then destroying or eliminating the infected tissue. The destruction caused by cystic acne is deep, so much so that facial scarring is associated with it. In hair follicles containing prominent hairs, the growing hair shaft acts as a sebum conveyor, which unblocks the orifice. Strictly speaking, it may be simultaneous, but such follicles seem less prone to clogging and infection (Flynn, 2002:195).

According to Flynn (2002:195), the intact stratum corneum also acts as a barrier to chemicals brought into contact with it. Its diffusional resistance is significantly greater than that found in other barrier membranes of the body. Externally contacted chemicals can, in principle, bypass the stratum corneum by diffusing through the ducts of the appendages.

Azelaic acid regularises the excess shedding of skin cells in order to prevent blockage of the gland opening (Mohatta, 2007:1), whereas niacinamide causes less sebum production (Draelos *et al*, 2006:99-100). Thus, both azelaic acid and niacinamide need to be present within the epidermis and the dermis of the skin and also in the pilosebaceous unit.

2.3.2 Routes of drug permeation across the skin

Drug delivery through the skin is a concept that is a practical reality. As mentioned before, the stratum corneum has a natural barrier function. This limits the passive transdermal delivery of many drugs (Essa *et al.*, 2002:1481).

There are multiple potential steps between a molecule's first application to the skin surface and until it appears in the systemic circulation. The permeation process is very complex (Williams, 2003:28).

Drugs are applied to the skin in a vehicle. It may be a simple vehicle, for example an aqueous solution, or it may be more complex, i.e. an emulsion. The molecules closest to the stratum corneum surface will partition into the membrane dependent upon their physicochemical properties (Williams, 2003:28).

The first step in transdermal delivery is partitioning of the therapeutic agent into the outermost layer of the stratum corneum. Only molecules closest to the skin can partition from the vehicle

into the tissue. Further drug delivery depends on molecules inside the vehicle redistributing at random to become the molecules closest to the stratum corneum surface (Williams, 2003:28-30).

After the permeant has partitioned into the outer layer of the stratum corneum, the drug diffuses through this layer to the stratum corneum/viable epidermis junction, where partitioning into the viable tissue occurs. Diffusion through the membrane to the epidermis/dermis junction follows, thereafter partitioning by diffusion through the dermal tissue to the capillaries occurs. Another partitioning occurs when molecules enter the blood vessels before removal by the systemic circulation.

In addition to these numerous partitioning and diffusion processes for transdermal drug delivery, there are other possible fates for molecules entering human skin. Permeants may bind with various elements of the skin (Williams, 2003:30).

The skin is metabolically active. Thus, the potential exists for drugs to be degraded at metabolic sites and they may also bind to receptors within the skin. Depending on the character of the drug, the permeant may not enter the systemic circulation, but may partition into the subcutaneous fatty layer (Williams, 2003:30).

There are essentially three pathways by which a molecule can cross the intact stratum corneum. These pathways are: (a) the passage through the skin appendages (shunt routes); (b) the intercellular route along the lipid domains; or (c) the transcellular route (Schnetz & Fartasch, 2001:166).

Transappendageal route (shunt route)

The appendages offer pores that bypass the barrier of the stratum corneum. This is a limited route for the uptake of substances as the surface area of the skin appendages is less than 1% of the skin surface (Schnetz & Fartasch, 2001:166). Eccrine glands, more commonly known as sweat glands, may be numerous in several areas of the body. Their openings, however, onto the skin surface, are still very small. Beyond the small surface area, these ducts are either evacuated or are actively secreting sweat. One would expect that to reduce the inward diffusion of topically applied agents. The opening of the follicular pore to the skin surface is considerably larger than that of the eccrine glands, though they are less abundant. The duct of the sebaceous gland again is filled, but rather than with aqueous sweat, the sebum in these follicular glands are lipoidal (Williams, 2003:31).

Intercellular route

This route was seen to be the most important pathway (Schnetz & Fartasch, 2001:166). The lipid bilayers cover approximately 1% of the stratum corneum diffusional area. It provides the only constant phase within the membrane. It has long been established that regulating the loss of water from the body and controlling the penetration of materials into the skin by the stratum corneum lipids, are important. Except for some specific cases it is now generally accepted that the intercellular lipid route provides the main pathway by which most small, uncharged molecules cross the stratum corneum (Williams, 2003:34).

According to Williams (2003:34) the exact nature of the intercellular pathway is still open to discussion. What is known is that the lipid bilayers offer the most important 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 stratum corneum, and therefore the path length of permeation is usually regarded as the thickness of the stratum corneum. In contrast, the intercellular route is highly complex, with permeants moving through the continuous lipid domains between the keratinocytes. In this case the path length taken by the molecule is significantly longer than the thickness of the stratum corneum (Williams, 2003:34-35). It is generally considered that lipophilic substances penetrate through this pathway (Schnetz & Fartasch, 2001:166).

Transcellular route

This pathway is often regarded as providing a polar route through the membrane. Hydrophilic molecules prefer to penetrate via this route through the protein-enriched corneocytes (Schnetz & Fartasch, 2001:166).

A molecule that crosses the intact stratum corneum via the transcellular route faces numerous repeating hurdles. First of all, there is the partitioning of the molecule into the keratinocyte, followed by diffusion through the hydrated keratin. 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, one after another, partition into and diffuse across the hydrophobic chains and the hydrophilic head groups of the lipids. Apparently, the process of multiple partitioning and diffusion steps between hydrophilic and hydrophobic domains is generally unfavourable to most drugs (Williams, 2003:33).

The character of the permeant will influence the relative importance of the transcellular route to the observed flux. For highly hydrophilic molecules the transcellular route may be more important at pseudo-steady state. However, the rate-limiting barrier for permeation via this route remains the multiple bilayered lipids that the molecule must cross between the

keratinocytes. The use of solvents to remove lipids from the stratum corneum consistently increases drug flux for even highly hydrophilic molecules (Williams, 2003:33).

2.3.3 Physiological factors affecting transdermal drug delivery

It goes without saying that skin disorders will affect the nature of the skin barrier and will thus influence topical and transdermal drug delivery (Williams, 2003:14). In the following section, the physiological factors that can influence the rate of drug delivery to and through healthy skin will be described.

Skin age

The most widely investigated physiological factor affecting transdermal drug delivery is skin ageing (Williams, 2003:14). In the dermis of the skin, age-related changes occur (McLauglin & Holick, 1985:1536). Williams (2003:14) stated that the moisture content of the skin decreases with age. Transdermal drug delivery is affected by tissue hydration; therefore drug permeation could be changed. According to Tanner and Marks (2008:251), percutaneous penetration does not change much with age, thus age does not itself influence penetration.

Temperature

According to Wiechers (1989:194), alterations in the permeability coefficient, in *in vitro* experiments, appear to be small at temperatures up to 70 °C. At higher temperatures, permanent denaturation occurs. Significant increases in the permeability coefficient can then be observed. Tanner and Marks (2008:251) stated that heat increases skin permeation by numerous mechanisms.

Body site

The skin of the palms, face and genetalia are areas more easily penetrated due to thinner skin (Tanner & Marks, 2008:251). The trunk of the body is intermediately permeable, whereas the arms and legs are the least permeable. Absorption of sites with similar stratum corneum thickness can differ, whereas similar levels of absorption occur in sites with different stratum corneum thickness (Williams, 2003:16).

Sex

Williams (2003:17) states that keratinocytes in females tend to be slightly larger than in males. In females it ranges from 37 - 46 µm, whereas in males it varies between 34 - 44 µm. There are, however, no reports of considerable differences in drug delivery between corresponding sites in the two sexes.

Race

Williams (2003:17) mentioned that previous studies depicted no differences in transepidermal water loss between African, Asian and European skin. However, there are considerable differences in the stratum corneum water content between races. It is predictable that this would be evident through differences in drug absorption.

2.3.4 Influence of permeant physico-chemical properties on absorption

The virtual contribution of the three pathways by which a molecule can cross the intact stratum corneum will differ depending on the character of the permeant. It is expected that all permeants make use of all three of the pathways to some extent (Williams, 2003:35). Some properties will be discussed in this section.

Partition coefficient

Partitioning into the membrane by the permeant must take place before it can cross the stratum corneum. This partitioning into the skin can be the rate-limiting step in the permeation process (Williams, 2003:35).

The pathway that a permeant will follow through the skin is usually determined by the partition coefficient of the permeant. It can be expected that a hydrophilic molecule will preferably partition into the hydrated keratin-filled keratinocytes rather than into the lipid bilayers, whereas lipophilic permeants will preferably partition into the lipoidal domains. As a result, it is expected that hydrophilic molecules mainly permeate via the intracellular pathway whereas lipophilic molecules will permeate mainly via the intercellular pathway (Williams, 2003:35).

The intercellular pathway is also the main pathway with which molecules with intermediate partition coefficients, molecules that show some solubility in both water and oil phases, will permeate the skin. This includes most molecules with a log $P_{(octanol/water)}$ of 1 to 3. For molecules with a log $P_{(octanol/water)}$ greater than 3 (more highly lipophilic), the intercellular pathway will be almost the only route used to cross the stratum corneum (Williams, 2003:36).

For molecules with a log $P_{(octanol/water)}$ less than 1 (more hydrophilic), the transcellular route is more important, however, there are still lipid bilayers to cross between the keratinocytes. (Williams, 2003:36).

Molecular size

The size and shape of a molecule are also key factors in determining the flux of a substance through the skin. When the influence of molecular size on permeation is considered, molecular volume is the most appropriate measure of permeant bulk (Williams, 2003:36).

According to Williams (2003:36) there is an inverse relationship between molecular weight and transdermal flux of the permeant. It is also said that small molecules cross human skin faster than larger molecules.

Solubility/melting point

At normal temperatures and pressures, which are the typical conditions in transdermal drug delivery, the majority of organic substances with high melting points and high enthalpies of melting have fairly low aqueous solubility. Consequently, there is an apparent relationship between melting point and solubility (Williams, 2003:37).

Williams (2003:37) states that lipophilic molecules most likely permeate through the skin faster than more hydrophilic molecules. Therefore, the partition coefficient (solubility within the intercellular lipids) can be associated with the permeability coefficient for a homologous sequence of compounds. Yet, at the same time as lipophilicity is usually a preferred characteristic of transdermal candidates, the molecule needs to display some aqueous solubility seeing as topical medications are normally applied in an aqueous formulation. The lipophilicity of lipophilic permeants that may provide a fairly high permeability coefficient will normally dictate that the aqueous solubility will be rather low, with a resulting impact upon drug flux through the tissue (Williams, 2003:37).

A drug with poor water solubility that is delivered from a saturated or sub-saturated aqueous formulation will cause a small quantity of drug to be present in the formulation. Due to poor water solubility of the drug, its molecules will cross into the stratum corneum rather rapidly, therefore, possibly resulting in a fast reduction of drug within the formulation. As the concentration of the drug reduces, the thermodynamic activity of the drug in the formulation will also decrease (Williams, 2003:37-38).

Ionisation

Ionisable drugs are seen as poor transdermal permeants. Undeniably, many of the opinions against using weak acids and weak bases that will dissociate to unreliable degrees depending on the pH of the formulation and on the pH of the stratum corneum, are found in the pH-partition hypothesis. According to this hypothesis, only a drug in its unionised form can permeate

through the lipid barrier in considerable amounts. This model cannot be rigidly applied to human skin due to its complex structure (Williams, 2003:38).

It is possible that ionised drugs can cross the membrane via the shunt route, but the amounts of these permeants may be less than if they were unionised and were to pass by the lipoidal intercellular route (Williams, 2003:38).

Lag time

An important result of binding between the drug applied and skin components, is the effect on lag time. A delay exists between applying a drug to the outer surface of the tissue and its appearance in a receptor solution. This delay, consequential from the time taken for the molecules to cross the stratum corneum, or skin membrane, is related to the lag time. It is noticeable that if the drug binds on its permeation through the tissue, the lag time will be extended (Williams, 2003:39).

2.3.5 Mathematics of skin permeation

Fairly simple mathematical actions can be applied to data gained from experiments with human skin. Fick's second law of diffusion is one of them. Drug absorption across human skin is passive and can thus be illustrated in physical terms (Williams, 2003:41).

Fick's laws are generally viewed as the mathematical description of the diffusion process through membranes. According to Schaeffer and Redelmeier (1996:178), Fick's first and second laws describe diffusion of uncharged compounds across a membrane or any homogenous barrier. Fick's first law is used in steady state diffusion, when the concentration within the diffusion volume does not change with respect to time.

It furthermore explains the diffusive flux to the concentration field. The flux proceeds from a region of high concentration to regions of low concentration, with a magnitude proportional to the concentration gradient.

$$J = -D\frac{dC}{dx}$$

Where

- J is the diffusion flux
- D is the diffusion coefficient

- c is the concentration
- x is the position (Williams, 2003:41)

Fick's Second Law is used in non-steady state diffusion when the concentration within the diffusion volume changes with respect to time. It predicts how diffusion causes the concentration field to change with time.

$$\frac{\partial C}{\partial x} = D\frac{\partial^2 C}{\partial x^2}$$

Where

- c is the concentration
- D is the diffusion coefficient
- x is the position

The most important factors that determine the flux of a compound between two points in an isotropic medium include the concentration gradient, the path length, and the diffusion coefficient (Schaeffer & Redelmeier, 1996:178).

2.3.6 Penetration enhancers

According to Benson (2005:25), skin permeation enhancement or optimisation techniques can be conducted in two different ways. Firstly, there is the drug/vehicle based option, which includes:

- drug selection,
- prodrugs & ion-pairs,
- drug – vehicle interactions,
- chemical potential of the drug,
- eutectic systems,
- complexes,
- liposomes, and
- vesicles and particles.

The second option is stratum corneum modification, which includes:

- hydration,
- lipid fluidisation,
- bypass/removal, and
- electrical methods.

Chan (2005:18) described percutaneous penetration enhancement technology as the breaking down into three essential alternates. These are physical-, chemical- and combinations of physical and chemical enhancement. Physical enhancement includes iontophoresis, electroporation, sonoporation, thermal poration and microneedles. Chemical enhancers include organic solvents, fatty acids and alcohols, detergents and surfactants, and proprietary chemical enhancers.

2.3.5.1 Physical enhancement

Physical enhancement includes:

- Iontophoresis,
- Electroporation,
- Sonophoresis, and
- Microneedles.

Iontophoresis

This method uses an electric field to move charged and uncharged species across the skin (Prausnitz *et al.*, 2004:118). The current at which molecules are driven into the skin is approximately 0.5 mA/cm² (Benson, 2005:31). This current is passing through an electrode which is in contact with a drug and the skin. A grounding electrode then completes the circuit (Barry, 2002:S36).

In iontophoresis it is expected that most ions would follow the path of least resistance. It is also expected that they then diffuse through the damaged areas of the skin and down the shunts of hair follicles and sweat glands (Barry, 2002:S36).

In the long term, iontophoresis promises to deliver hydrophilic drugs and even macromolecules across the skin (Prausnitz *et al.*, 2004:118).

According to Prausnitz *et al.* (2004:118), transdermal transport rate can be increased enormously, relative to passive diffusion-based methods. It can be altered by adjusting electrical parameters.

Electroporation

Electroporation is the application of short micro- to milli-second electrical pulses of approximately 100 - 1000 V/cm in order to create transient aqueous pores in lipid bilayers (Benson, 2005:31). During these pulses, molecules progress mainly because of iontophoresis and/or electro-osmosis (Barry, 2002:S37).

A significant increase in transdermal transport was shown with the use of electroporation. Partial reversibility occurred within seconds, and full reversibility within minutes to hours (Prausnitz *et al.*, 2004:119).

The pulses of electroporation are expected to be safe. These pulses can be administered painlessly using closely spaced electrodes to limit the electric field within the nerve-free stratum corneum (Prausnitz *et al.*, 2004:119).

Sonophoresis

This method uses ultrasound at various frequencies in the range of 20 kHz - 16 MHz (Prausnitz *et al.*, 2004:119). These frequencies and intensities of as much as 3 W cm^{-2} have been used in an effort to increase transdermal drug delivery (Naik *et al.*, 2000:324). Several studies have shown the ability of low-frequency ultrasound to deliver macromolecules across the skin (Ogura *et al.*, 2008:1219). Especially at low frequencies (20-100 kHz) it has been revealed that ultrasound significantly enhances skin permeability (Paliwal *et al.*, 2006:1095).

According to Prausnitz *et al.* (2004:120) several mechanisms have been investigated for sonophoresis. Firstly, there are thermal effects due to absorption of ultrasound by the skin. Secondly, acoustic streaming caused by development of time-independent fluid velocities in the skin due to ultrasound was investigated. Then there are also cavitational effects. It is accepted that cavitation, which is the violent growth and collapse of oscillating bubbles, is the principle behind low-frequency sonophoresis (Paliwal *et al.*, 2006:1095).

Microneedles

This method to deliver drugs into the skin includes a minimum invasive approach (Gill & Prausnitz, 2008:1537). Microneedles are pierced into the skin surface to create large enough holes for molecules to enter, however, these are small enough not to cause any pain or damage. *In vitro* studies have shown that the use of microneedles increases skin permeability enormously (Prausnitz *et al.*, 2004:120).

When it comes to using microneedles to enhance skin penetration, there are different ways of doing so. The first is to pretreat the skin with microneedles, and then a transdermal patch is applied on the particular skin area. Another method is to coat or encapsulate a drug onto or within the microneedles (Lee *et al.*, 2008:2113).

2.3.5.2 Chemical enhancement

Some chemical penetration enhancers include:

- Sulfoxides,
- Fatty Acids,
- Fatty Alcohols,

- Pyrrolidones, and
- Terpenes.

Sulfoxides

One of the most widely studied penetration enhancers is Dimethylsulfoxide (DMSO). It is an aprotic solvent that would rather form hydrogen bonds with itself than with water. DMSO as a penetration enhancer is useful for both hydrophilic and lipophilic permeants (Williams, 2003:87).

The effects of DMSO depend upon its concentration. For best possible enhancement, co-solvents that contain more than 60% DMSO are needed. The problem with this is that DMSO is an irritant at such high concentrations. It can cause erythema and wheals. Delamination of the stratum corneum and denaturation of proteins are other side effects that may occur (Williams, 2003:87-88).

Fatty Acids

Both saturated and unsaturated fatty acids are being used as effective skin penetration enhancers (Kanikkannan *et al.*, 1999:597). Fatty acids can undoubtedly be used to enhance the penetration of hydrophilic and lipophilic permeants, however, the flux of hydrophilic drugs appear to be higher than that of lipophilic drugs (Williams, 2003:92).

Oleic acid ($CH_3(CH_2)_7CH=CH(CH_2)_7COOH$)) is usually one of the main enhancers selected for investigations. This acid's mechanism of action is typically that of a long-chain fatty acid. From thermal analysis it can be seen that this acid interrelates with the lipid domains inside the stratum corneum (Williams, 2003:93).

Fatty Alcohols

The most commonly used alcohol for penetration enhancement is ethyl alcohol (Kanikkannan *et al.*, 1999:603). Also known as ethanol, this enhancer permeates rapidly through human skin with a steady-state flux of approximately 1 $mg/cm^2/h$ (Williams, 2003:94).

Fatty alcohols are usually applied in a co-solvent at 1 - 10%. This co-solvent normally is propylene glycol (Williams, 2003:95).

Pyrrolidones

Pyrrolidones together with their derivates are known to be potential penetration enhancers. They affect hydrophilic permeants to a larger extent than lipophilic permeants (Williams, 2003:91).

The pyrrolidones partition well into the stratum corneum where they adjust the solvent character of the tissue and then generate a permeant reservoir within the tissue. This can potentially lead to sustained release (Williams, 2003:92).

Terpenes

Terpenes are a more recent addition to the penetration enhancers. Monoterpenes are likely to be more active penetration enhancers than sesquiterpenes. Hydrocarbon terpenes, or non-polar group-containing terpenes, are better enhancers for lipophilic permeants than the polar terpenes. Just like the polar terpenes are better enhancers for hydrophilic permeants (Williams, 2003:98,101).

Terpenes disturb the lipid structure of the stratum corneum. Thus, they increase the diffusion coefficient of a polar drug in the membrane (Kanikkannan *et al.*, 1999:605)

2.3.6.3 Water

According to Williams (2003:84), increasing the water content of tissue is the safest and most generally used method for increasing skin penetration of drugs. In general, increasing stratum corneum hydration tends to increase transdermal delivery of both hydrophilic and lipophilic permeants.

The exact mechanism of action is not known, however, it is accepted that free water in the tissue modifies the solubility of the permeant in the stratum corneum and thus, modifies the partitioning of the drug from its vehicle into the membrane (Williams, 2003:85).

Research on penetration enhancers was included in this study because of their potential to increase the penetration of the drugs if slow absorption occurred.

2.3.7 Pheroid™ as Drug Delivery Vehicles

The Pheroid™ technology is based on what was previously called the Emzaloid™ technology. This technology is able to improve the absorption and/or efficacy of various active ingredients as well as other compounds. Significant improvements in the management of size, charge and the hydrophilic-lipophilic characteristics have also been depicted, when compared to other systems (Grobler *et al.*, 2008:284).

- *Structural characteristics*

The Pheroid™ delivery system is a colloidal system that has even lipid-based submicron- and micron-sized structures that are very unique in nature. These are called Pheroid™. Pheroid™

is homogeneously dispersed in a dispersion medium. The particles can be manipulated in terms of size, structure, morphology and function (Grobler *et al.*, 2008:285).

Pheroid™ is normally formulated to have a diameter of between 200 nm and 2 µm. When deciding on the type and diameter of the Pheroid™ particles, different parameters such as the required capacity, rate of delivery and the administration route should be taken into account (Grobler *et al.*, 2008:285).

Colloidal systems are used as carriers of drugs or cosmetic compounds to enhance the efficacy of the administered compounds while reducing unwanted side effects (Grobler *et al.*, 2008:285).

- *Ingredients of Pheroid™*

Pheroid™ mainly consists of ethylated and pegylated polyunsaturated fatty acids, including omega-3 and omega-6 fatty acids, but not arachidonic acid (Grobler *et al.*, 2008:286).

Colloidal dosage forms frequently used include liposomes, emulsions and micro-emulsions, polyeric microspheres and macromolecular microspheres. One or more of these features may be integrated in the design of a formulation consisting of Pheroid™ (Grobler *et al.*, 2008:287).

Pheroid™ usually has a lipid bilayer with no phospholipids or cholesterol. It is created by a self-assembly process similar to that of low-energy emulsions and micro-emulsions. No lyophilisation or hydration of the lipid components is required. Similar to emulsions, Pheroid™ is dispersed in a dispersion medium, but differs in the fact that it also contains a dispersed gas phase which is associated with the fatty acid dispersed phase (Grobler *et al.*, 2008:288).

Pheroid™ contains one exclusive component, namely nitrous oxide (N_20), which is found distributed, with the dispersed phase, throughout the continuous phase. Adding the dispersed gas phase to the respective oil and water phases, thus adds another dimension to the basic Pheroid™ (Grobler *et al.*, 2008:289). The association of N_20 with the dispersed phase has been shown to have at least three functions:

- It contributes to the miscibility of the fatty acids in the dispersal medium.
- It contributes to the self-assembly process of Pheroid™, as determined by the zeta-potential and particle size analysis.
- It contributes to the stability of the formed Pheroid™, as shown by accelerated and formal stability studies, as well as to its stability at a high and low pH (Grobler *et al.*, 2008:289).

N_2O is a volatile anaesthetic compound that is both water- and fat-soluble. This characteristic enables the gas to move freely through the epidermal and dermal layers of the skin (Grobler *et al.*, 2008:289).

Manipulation of both the structural and functional features of Pheroid™ is allowed for by its design. The surface charge of the Pheroid™ particles can be modified by the degree of hydrogenation of the fatty acids. Manipulation of the mean particle size can be conducted by changing the composition and ratio of the fatty acids (Grobler *et al.*, 2008:292). Various investigations into different Pheroid™ formulations have shown that the structural and functional characteristics of the Pheroid™ particles may be manipulated by:

- changing the fatty acid composition or concentrations,
- adding non-fatty acids or phospholipids such as cholesterol,
- adding cryo-protectants,
- adding 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, and
- adding sunscreen formulations (Grobler *et al.*, 2008:292).

Unlike most lipid-based delivery systems which are sterically stabilised by cholesterol, Pheroid™ is sterically stabilised by electro-chemical interaction. Pheroid™ has been shown to cross capillary walls. The dense stratum corneum offers a considerable challenge and it is thought that the flexibility of the Pheroid™ membrane contributes to efficient dermal and transdermal delivery. Uptake of Pheroid™ by cells may be influenced by the Pheroid™ formulation and by the mechanism of uptake by the cells (Grobler *et al.*, 2008:299). The permeation of the Pheroid™ formulation is determined by one or more factors, which include:

- the size of the Pheroid™ particles,
- morphology of the Pheroid™ particles,
- molecular geometry of the fatty acids themselves,
- concentrations and ratios of the various fatty acids,

- the hydration medium (ionic strength, etc.),
- pH of the preparation,
- the presence of charge-changing molecules,
- the presence of molecules that influence the electronic milieu,
- character and concentration of the active/drug, and
- the state of the Pheroid™ formulation (i.e., either gel state, fluid state or in between) (Grobler *et al.*, 2008:299)

2.4 Topical delivery of azelaic acid and niacinamide

2.4.1 Treatment of acne with azelaic acid and niacinamide

2.4.1.1 Azelaic acid

As mentioned in section 2.2.1, azelaic acid is an anti-inflammatory, antioxidant, anti-keratinising and bacteriostatic agent that is currently being used in the treatment of acne. Due to these properties, especially its antibacterial effect against *P. acnes,* azelaic acid is considered a good option for treating acne (Draelos & Kayne, 2008:AB40). Its antibacterial effect is initiated through inhibition of protein synthesis (Shemer *et al.*, 2002:178). Futhermore, in higher concentrations azelaic acid is effective against *Staphylococcus epidermis* (Manosroi *et al.*, 2005:236). Bacterial resistance to azelaic acid has not yet been reported (Webster, 2000:S49).

Figure 3 shows the structure of azelaic acid and Table 2 indicates the physio-chemical properties of azelaic acid.

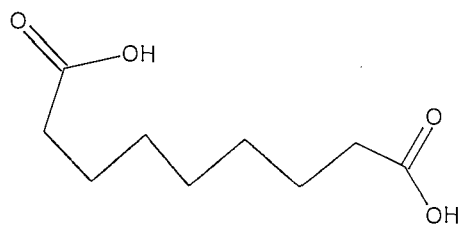

Figure 3: Structure of azelaic acid.

Table 2: Properties of azelaic acid.

Molecular formula (Anon., 1998a:1)	$C_9H_{16}O_4$
Molecular weight (Anon., 1998a:1)	188.22
Melting point (Anon., 1998a:1)	106.5 °C
Boiling point (Anon., 1998a:1)	286 °C
pKa (Anon., 1998a:1)	4.53 at 25 °C 5.33
Solubility (Anon., 1998a:1)	Freely soluble in boiling water and alcohol
Density (Anon., 2003a:1)	1.225 $g.cm^{-3}$
Stability (Anon., 1998a:1)	Stable
Incompatibilities (Anon., 2003a:1)	Bases & strong oxidizing agents
Elimination half-life (Akhavan & Bershad, 2003:488)	12 hours after topical application
Appearance (Anon., 2003a:1)	White to cram solid

Acne is caused through blockage of the gland opening by skin cells that shed too fast, and thus keeps the sebum from passing through. Azelaic acid regularises this excess shedding of skin cells to prevent blockage of the gland opening. Therefore this drug is used to treat infected acne, as well as preventing further formation of acne due to its ability to keep the pores open (Mohatta, 2007:1). According to Webster (2000:S47), azelaic acid is effective in reducing the number of inflammatory- as well as non-inflammatory lesions. In a study conducted by Bjerke *et al.* (1999:457), azelaic acid depicted significant reductions in total inflammatory lesion count as well as a mean overall decrease in papules. A reduction in erythema was also visible.

2.4.1.2 Niacinamide

Niacinamide is used in treating different skin problems, including atopic dermatitis, rosacea, hyperpigmentation, skin aging and also acne (section 2.2.1.). The application of 4% topical niacinamide caused a global reduction in acne (Namazi, 2007:1230). It is beneficial in the treatment of both papular and pustular acne (Draelos, 2000:237). According to *in vitro* studies that were conducted, niacinamide caused dose-dependent inhibition of sebocyte secretions (Namazi, 2007:1230). Draelos *et al.* (2006:99-100) stated that niacinamide caused less sebum production and reduction of facial shine and oiliness. It can be used to reduce inflammatory papules due to its anti-inflammatory action (Gehring, 2004:92). As mentioned, niacinamide can be used for the reduction of hyperpigmentation and improvement of the barrier function (Bisset *et al.*, 2005:P32). According to Barai (2001:18), niacinamide changed and maintained skin texture and properties.

Figure 4 shows the structure of niacinamide and Table 3 indicates the physio-chemical properties of niacinamide.

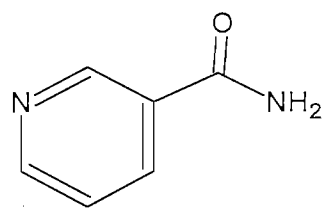

Figure 4: Structure of Niacinamide.

Table 3: Properties of Niacinamide.

Molecular formula (Moety, Tariq, & Al-Badr, 1991:479), (Anon., 1998b:1)	$C_6H_6N_2O$
Molecular weight (Anon., 2003b:1)	122.13
Melting point (Anon., 2003b:1)	130 °C
pK (Moety, Tariq, & Al-Badr, 1991:481)	3.3 (20 °C)
pKa (Anon., 2003b:1)	0.5 and 3.35
pH (Moety, Tariq, & Al-Badr, 1991:481)	1.3 % solution – 3-3.5 5.0 % solution – 6-7.5 10.0 % solution – 7 Saturated – 2.7
Solubility (Moety, Tariq, & Al-Badr, 1991:480) (Anon., 2003b:1)	Freely soluble in water and alcohol Soluble in glycerine
Density (Anon., 2003b:1)	1.44 $g.cm^{-3}$
Stability (Draelos, 2000:237)	Extraordinarily stable to heat, light and oxygen
Incompatibilities (Anon., 2003b:1)	Strong oxidizing agents
Appearance (Moety, Tariq, & Al-Badr, 1991:480)	Colourless crystals/white crystalline powder, salty and bitter taste

2.4.2. Problems associated with the absorption of Azelaic acid and Niacinamide

2.4.2.1 Azelaic acid

Azelaic acid is not chemically related to any other acne treatment and the mechanism of therapeutic effect is still unknown. What is known is that azelaic acid does not decrease the size of the sebaceous gland or the production of sebum. It also does not affect testosterone levels (Mackrides & Shaughnessy, 1996:2457).

Azelaic acid has an acceptable safety profile (Webster, 2000:S48), although a few side-effects have been reported. These side-effects include skin- and eye irritation (Anon, 1998a:1), skin dryness, scaling, pruritis, erythema, burning, and rarely, hypo-pigmentation (Feldman *et al.*, 2004:2125). According to Mackrides and Shaughnessy (1996:2458) the side-effects that occurred most, were mild transient erythema and cutaneous irritation. These side-effects are characterised by scaling, pruritis and a mild burning sensation.

Azelaic acid is classified as category B during pregnancy (Mackrides & Shaughnessy, 1996:2458). Therefore, it may be used during pregnancy if needed (Thiboutot *et al.*, 2003:845). It is not expected that the use of azelaic acid will affect the infant of a nursing mother. The effect, however, on children under the age of 12 years has not yet been studied (Mackrides & Shaughnessy, 1996:2458).

Akhavan and Bershad (2003:488) stated that 3 - 5% of a single dose azelaic acid applied topically was retained in the stratum corneum and approximately 4% was absorbed systemically. With the use of gel formulations, this percentage increased to 8%.

The pH of the skin is approximately 4.2-5.6 (Yu & Van Scott, 2004:78). Azelaic acid is active at a low pH. Therefore, an increase in skin pH would reduce the antibacterial effect of Azelaic acid (Charnock *et al.*, 2004:594-595).

2.4.2.2 Niacinamide

Niacinamide is converted to nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These are coenzymes of dehydrogenases. They are involved in electron transfer reactions in the respiratory chain. NADP and NADPH inhibit glycation (Namazi, 2007:1230). This is an oxidative cross-linking reaction that occurs between protein and endogenous sugar during the skin aging process (Bisset *et al.*, 2005:P32). Thus, niacinamide is effective against aging of the skin. It furthermore increases the production of keratin, filaggrin and involucrin (Namazi, 2007:1230).

Soma *et al.* (2005:197-198) stated that niacinamide decreased transepidermal water loss (TEWL) and increased hydration of the stratum corneum. Although it has been safely used topically, side-effects that may occur with the use of niacinamide include local irritation and itching (Soma *et al.*, 2005:201). Niacinamide may cause dryness of the skin, and irregularly also erythema and a burning sensation (Anon, 2006:1373).

2.5. Conclusion

Acne vulgaris is a common skin disease, a chronic inflammatory condition (Bershad, 2001:279). It affects almost 80% of adolescents and young adults (Krautheim & Gollnick, 2004:398). Acne can be described as a disease that affects the pilosebaceous unit of the skin (Webster, 2002:475).

Azelaic acid is currently being used in the treatment of acne. It shows anti-inflammatory, antioxidant, anti-keratinizing and bacteriostatic properties which make it a very good option for the treatment of acne (Draelos & Kayne, 2008:AB40). Niacinamide is being used for different skin problems, including atopic dermatitis, rosacea, hyperpigmentation, skin aging and acne (Namazi, 2007:1230). It is beneficial in the treatment of both papular and pustular acne (Draelos, 2000:237).

The goal of this study was to combine azelaic acid and niacinamide into a product that can be applied topically for the treatment or prevention of acne. Both of these active ingredients are currently used in the treatment of acne. A cream and gel were formulated, as well as a Pheroid™ cream and a Pheroid™ gel. In the following chapter the experimental methods, results and discussion thereof will be provided, as well as in Appendix B, D, and E.

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

FORMULATION AND TRANSDERMAL DIFFUSION OF NIACINAMIDE IN COMBINATION WITH AZELAIC ACID

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Abstract

The aim of this study was to combine azelaic acid and niacinamide into a single product and to determine whether these two active ingredients can be delivered transdermally. It was also determined whether the Pheroid™ formulations delivered azelaic acid and niacinamide more efficiently transdermally than the non-Pheroid™ formulations and whether these formulations were stable under different storage conditions.

Acne vulgaris is a common skin disease, a chronic inflammatory condition that is very common amongst adolescents. It can be described as a disease that affects the pilosebaceous unit of the skin. Azelaic acid regulates the excess shedding of skin cells in order that blockage of the gland opening does not occur, whereas niacinamide causes less sebum production. Thus both azelaic acid and niacinamide need to be present within the pilosebaceous unit of the skin in order to control or prevent acne. Formulations containing azelaic acid and niacinamide, with and without the Pheroid™ delivery system, were prepared. These formulations were used to conduct diffusion studies in Caucasian female skin. Accelerated stability testing of these formulations at different storage conditions was also performed.

The Pheroid™ formulations did not prove to be more advantageous overall for transdermal diffusion of azelaic acid and niacinamide and these formulations were not more stable than non-Pheroid™ formulations.

Keywords: Azelaic acid, Niacinamide, Transdermal diffusion, Stability testing, Pheroid™

1. Introduction

Acne vulgaris is a common skin disease; a chronic inflammatory condition (Bershad, 2001) that affects approximately 80% of adolescents and young adults (Krautheim and Gollnick, 2003). According to Adebamowo et al. (2008), acne is more common in girls, especially in the age group 12 years or younger. However, in adolescents 15 years or older, acne presents more regularly in boys.

Acne usually diminishes over time and will most probably decrease or disappear in the early twenties of a person's life. It can, however, cause significant embarrassment and anxiety in affected patients (Feldman et al., 2004). Teenage acne causes discomfort, disfigurement, emotional distress and sometimes, permanent scarring (Akhavan and Bershad, 2003). Smoking, the male sex, genetics and youth are some of the risk factors for the development, or increased severity of acne (Krautheim and Gollnick, 2004).

Acne can be described as a disease that affects the pilosebaceous unit of the skin (Webster, 2002). Patients presenting with acne usually have a variety of lesions in various stages, along with post-inflammatory acne scars and hyper-pigmentation (Olutunmbi et al., 2008). Androgens, which appear at the beginning of adolescence, increase the production of sebum and also enlarge the sebaceous glands (Krautheim and Gollnick, 2004) by filling up pre-existing comedones with lipids (Webster, 2002). The pilosebaceous ducts are blocked, which leads to the development of microcomedos as primary lesions (Krautheim and Gollnick, 2004). Thus, acne occurs when sebum is not able to pass through the hair follicle onto the skin. This occurs when the cells of the hair follicle shed too fast due to hyperkeratinisation which prevents normal shedding (Feldman et al., 2004) and blocks the opening of the follicle with the result that sebum is not able to pass through. Lipids and cellular debris accumulate (Feldman et al., 2004) and the mixture of sebum with cells causes bacterial growth. The micro-environment enhances the colonisation of *Propionibacterium acnes (P. acnes)*. *P. acnes* colonises when the shedded cells mix with the sebum and inflammatory reactions occur (Krautheim and Gollnick, 2004). This

organism consumes glycerol fractions and discards the free fatty acids after metabolising sebaceous triglycerides (Webster, 2002).

According to Feldman et al. (2004), the goals of acne therapy include controlling acne lesions, preventing scarring and minimising morbidity. They also stated that the patient should be informed that the goal is to prevent new lesions from forming and that current lesions must heal on their own. Acne treatment include topical- and systemic agents. Therefore, acne may be treated through the use of topical agents, systemic agents or a combination of both.

Azelaic acid is currently used in the treatment of acne. It shows anti-inflammatory, antioxidant, anti-keratinising and bacteriostatic properties which makes it a preferred option for acne treatment (Draelos and Kayne, 2008). Niacinamide, on the other hand, is used in treating different skin problems; including atopic dermatitis, rosacea, hyper-pigmentation, skin aging and acne (Namazi, 2007). It is beneficial in the treatment of both papular and pustular acne (Draelos, 2000). According to *in vitro* studies that were conducted, niacinamide caused dose-dependent inhibition of sebocyte secretions (Namazi, 2007). Thus, both azelaic acid and niacinamide need to be present within the epidermis and the dermis of the skin as well as in the pilosebaceous unit, as these are the target areas.

According to Grobler et al. (2008), the Pheroid™ delivery system is a colloidal system that consists of even lipid-based submicron- and micron-sized structures. Grobler et al. (2008) also stated that the Pheroid™ technology is able to improve the absorption and/or efficacy of various active ingredients as well as other compounds.

The aim of this study was to combine azelaic acid and niacinamide in topical formulations and test the stability of these products as well as their transdermal diffusion and penetration into the skin.

2. Materials and methods

2.1. Materials

Azelaic acid and niacinamide were purchased from Sigma-Aldrich (Johannesburg, South Africa). Methanol (HPLC grade) was purchased from Merck chemicals (Wadeville, Gauteng, South Africa). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Formulation

In this study, a gel and a cream containing azelaic acid and niacinamide were formulated with and without Pheroid™.

A cream was formulated with the following ingredients: polyethylene glycol (PEG) 400 (20.0%), cetyl alcohol (15.0%), methyl paraben (0.1%), azelaic acid (20.0%), niacinamide (4.0%) and water (40.9%). All the ingredients were weighed. Azelaic acid and PEG 400 were heated to 80 °C and stirred until the azelaic acid dissolved. Cetyl alcohol and methyl paraben, as well as water and niacinamide were heated to 70 °C. PEG 400 and azelaic acid were added to the cetyl alcohol and methyl paraben. This mixture was added to the water and niacinamide while homogenising at 13 500 rpm until 38 °C was reached. Thereafter, homogenisation at 200 rpm was conducted until room temperature (25 ± 0.5 °C) was reached.

A gel was formulated with the following ingredients: PEG 400 (20.00%), span 60 (0.50%), tween 80 (4.50%), propyl paraben (0.08%), methyl paraben (0.40%), xanthan gum (1.50%), azelaic acid (15.00%), niacinamide (4.00%) and water (54.02%). All the ingredients were weighed. Azelaic acid and PEG 400 were heated to 80 °C and stirred until the azelaic acid dissolved. Span 60, tween 80, propyl paraben and methyl paraben were heated to 70 °C. PEG 400 and azelaic acid were then added to the aforementioned mixture. Approximately a quarter of the water and niacinamide were heated to 70 °C. The remaining water was heated to 80 °C after which xanthan gum was added while homogenising at 13 500 rpm. Dissolved niacinamide was

added to the homogenising mixture and the dissolved azelaic acid, span 60, tween 80, propyl paraben and methyl paraben mixture were added to xanthan gum in water mixture while still homogenising at 13 500 rpm until 38 °C. Subsequently, homogenisation at 200 rpm was then conducted until room temperature (25 ± 0.5 °C) was reached.

The formulas used for compounding the cream and gel were utilised in compounding a Pheroid™ cream and a Pheroid™ gel, however, butylhydroxytoluene (BHT), vitamin E and Pheroid™ were added to the formulations. The Pheroid™ were manufactured by the North-West University, Potchefstroom, South Africa.

2.2.2. Stability testing

Three Labcon humidity chambers (South Africa) with conditions: 25 °C/60% RH (relative humidity); 30 °C/60% RH and 40 °C/75% RH, were used to conduct stability testing over a six month period (Medicines Control Council, 2009).

2.2.2.1. Assay

A standard solution was prepared by weighing azelaic acid (160 mg) and niacinamide (32 mg) in a 20 ml volumetric flask (flask 1). Methyl paraben (4 mg), BHT (8 mg) and vitamin E (8 mg) were placed in another 20 ml volumetric flask (flask 2). Flask 2 was made up to volume with methanol (100%). 4 ml of flask 2 was withdrawn and added to flask 1. Flask 1 was then made up to volume with methanol (100%). The solution was transferred into vials for analysis with HPLC (high performance liquid chromatography).

To prepare samples, 2 g of each formulation, at each temperature, were weighed in duplicate in 50 ml volumetric flasks. No specific sampling technique was followed. Formulations were randomly sampled from containers. Both the cream and the Pheroid™ cream were made up to volume with water, whereas the gel and Pheroid™ gel were first dissolved in approximately 20 ml of methanol and then made up to volume with water. An Elma Transonic TS540 sonic bath (Germany) was used to enhance the dissolving process. Each solution was analysed in duplicate with HPLC. An Agilent 1100 series HPLC auto sampler, HP Agilent 1100 series variable detector and an HP Agilent 1100 series isocratic pump with a Phenomenex, Luna 5µ

C18, 250x4.60 mm column, 0.005 M okt.SO_3-Na in water, pH 3.5 (A) as mobile phase and methanol (B) as solvent were used at a flow rate of 1 ml/min. A linear gradient elution was employed starting at 25% B to 100 % B after 8 minutes.

2.2.2.2. Confocal laser scanning microscopy

This part of stability testing was conducted with the Nikon Digital Eclipse C1 si confocal laser scanning microscope (CLSM) (Japan), with violet diode laser 400-405 nm, a 543 nm He/Ne laser and an Argon ion laser 457-514 nm. It facilitates the capture of a three dimensional image. The samples were prepared by weighing 0.1 g of each formula, at each temperature, in an Eppendorf vial. As with the assay, no specific sampling technique was followed. Distilled water (1000 µl) was added and dissolved thoroughly with a Stuart Scientific Autovortex SA 6 mixer (UK) and Nile Red (2 µl) was added. Samples with Nile Red were left in a dark area for approximately 15 minutes. Nile Red is a phenoxazine dye which highlights lipids. Each sample (20 µl) was put on separate 76 x 26 x 1 mm microscope slides and each slide was covered with a 24 x 60 mm microscope cover glass. A drop of A nd = 1.515 (23 °C) immersion oil (Japan) was placed on each microscope slide so that it could be viewed with the CLSM. The homogeneity of each sample was determined by its micrograph.

2.2.2.3. Viscosity

To determine viscosity changes, a 125 ml jar of each formulation was kept at 25 ± 0.5 °C. Each month the viscosity was determined with a Brookfield DV-II+ Programmable Viscometer (USA). Temperature controller (water bath) was set at 25 ± 0.5 °C. The viscosity of the cream and Pheroid™ cream was determined at a speed of 0.3 rpm, whereas the viscosity of the gel and Pheroid™ gel was determined at a speed of 0.6 rpm. The smallest spindle (S96) was used for all formulations. For each formulation, 32 data points were taken and 30 of the 32 were used to determine an average viscosity. The time interval between each reading was 10 seconds.

2.2.2.4. Mass variation

Mass variation was performed with a Shimadzu AUW120D (Japan) scale. One container of each of the formulations, stored at the different storage conditions, was weighed every month.

2.2.2.5. pH

The pH of each formulation at the specified stability conditions was determined, every month, with a Mettler Toledo pH meter (China). Due to the fact that a big batch was formulated, all the samples of each formula had the same pH. Thus, the pH of only one sample of each formulation was determined for month 0.

2.2.2.6. Appearance

Determining appearance was based on the colour changes of each formulation due to time, temperature and humidity. This was conducted by taking a picture every month of every formulation at the different storage conditions with a Samsung S1060 camera (USA). Colour charts were used in order to establish whether there were any changes in colour.

2.2.3. Diffusion studies

2.2.3.1. Skin preparation

In order to conduct diffusion studies, human skin from Caucasian female patients who underwent cosmetic abdominoplastic surgery was obtained. The Research Ethics Committee approved the procurement and utilisation of skin (Reference number 04D08). Informed consent from the participating anonymous patients was obtained beforehand. The skin was frozen at -20 °C within 24 hours after removal (Henning et al., 2008). Excessive fat was removed with a scalpel to leave only the full thickness skin, containing the stratum corneum, epidermis and the dermis. Full thickness skin was then punched into circles with a diameter of approximately 15 mm.

2.2.3.2. Preparation of receptor solutions

The receptor solution, PBS (phosphate buffer solution), was prepared at a pH of 7.4 according to the British Pharmacopoeia (2007) and the receptor solution was kept at 32 ± 0.5 °C.

2.2.3.3. Franz cell diffusion method

For the *in vitro* diffusion studies, vertical Franz diffusion cells with a donor (top) and receptor (bottom) compartment were used. Franz cells have a receptor capacity of approximately 2 ml. PBS and a small magnetic stirring bar were placed in each Franz cell. The orifice (diffusion area) of the donor compartment was 1.075 cm^2, where approximately 1 ml of formulation was placed. Full thickness skin circles were placed on circular Whatman® filter papers (with a diameter of 15 mm) between the donor- and receptor compartments with the stratum corneum facing upwards. Ten cells per formulation were prepared. Dow Corning high vacuum grease was applied to each cell to prevent leakage. A horseshoe clamp was used to clamp the donor- and receptor compartments together after which the formulation was placed in the donor compartment. The donor compartment was covered with Parafilm® and a cap to prevent evaporation. The PBS was placed in the receptor compartment. A Franz cell stand containing 10 prepared cells was placed in a Grant water bath (England) at 37 °C with a Variomag magnetic stirring plate (Germany) stirring at 750 rpm. This was the starting time of the study. For the first two hours the withdrawal and replacement of the receptor solution was conducted every 20 minutes. Samples were then withdrawn every 2 hours up to 12 hours. Withdrawal and replacement of the receptor solution were done using a syringe with silicone tubing attached to the needle. After every withdrawal, the sample was placed in a vial to be analysed with LC/MS (liquid chromatography/mass spectrometry). An Agilent 1100 series HPLC with a binary gradient pump, auto sampler and vacuum degasser attached to an Applied Biosystems API 2000 triple quadruple mass spectrometer with a mobile phase of 0.1% formic acid (A), a Restek Ultra IBD 5 µm, 150 x 2.1 mm column and acetonitrile (B) as solvent, were used at a flow rate of 250 µl/min for analysis of samples. Linear gradient elution was employed starting at 10% B to 85% B after 2 minutes.

2.2.4. Tape stripping method

2.2.4.1. Epidermis

Skin circles were removed from the cells after the 12 hour Franz cell diffusion study and pinned on a piece of Parafilm® which was stapled to a solid surface (Pellet et al., 1997). Imprints on each of the skin circles due to the donor compartment, depicted the diffusion area. Tissue paper was used to slightly wipe the skin in order to remove excess formulation (Pellet et al., 1997). 16 strips of 3M Scotch tape per skin circle were cut to perform tape stripping. The first tape strip was disposed of, due to the fact that it was considered to be part of the cleaning procedure and might have been contaminated with drug from the formulation (Pellet et al., 1997). Therefore, 15 strips per skin circle were used and placed in separate polytops with 5 ml PBS at pH 7.4. These polytops were left overnight at 4 °C and then analysed with LC/MS.

2.2.4.2. Dermis preparation

Skin around the diffusion area were trimmed and the skin of the diffusion area were cut into smaller pieces and placed in separate polytops with 2 ml PBS at pH 7.4 (Pellet et al., 1997). These polytops were also left overnight at 4°C and subsequently it was analysed with LC/MS.

2.2.5. Aqueous solubility and log D value

The aqueous solubility and log D (octanol-buffer partition coefficient) values of azelaic acid and niacinamide were determined separately by preparing standard solutions in order to obtain a calibration curve. The standard solution for azelaic acid was prepared by weighing azelaic acid (10 mg) which was made up to 100 ml with PBS and diluted to concentrations of 1.00, 0.10, 0.04 and 0.02 µg/ml. A standard solution for niacinamide was prepared by weighing niacinamide (5 mg) which was made up to 50 ml and injected with different injection volumes in order to obtain a calibration curve.

A Labotec shaking water bath was preheated to 32 ± 0.5 °C. PBS (10 ml, pH 7.4) was placed in three test tubes with magnetic stirrers. An excess of the compound was added to each test tube in order to maintain saturation. These test tubes were stirred in the water bath for 24 hours and the contents were filtered and diluted before being inserted into vials for analysis with LC/MS.

The log D value was determined by adding 100 ml of *n*-octanol and 100ml PBS (pH 7.4) in a container whereafter it was stirred vigorously for 24 hours. The two phases were then separated. Niacinamide (50 mg) was placed in each of three test tubes and pre-saturated PBS (3 ml) was added which was then stirred in the water bath for 10 minutes at 32 ± 0.5 °C. *n*-Octanol (3 ml) was added to each test tube, and the tubes were stirred in the water bath for 45 minutes. An Eppendorf Centrifuge 5804R (South Africa) was used at 4000 rpm to centrifuge the tubes for 30 minutes. PBS was withdrawn and analysed with the HPLC (Leo et al., 1971).

2.2.6. Statistical analysis

Kruskal-Wallis ANOVA is the non-parametric equivalent of a one-way ANOVA (Steyn et al., 1994). A one-way ANOVA may yield inaccurate estimates of the p-value if the assumptions of normality and equal variances of the different groups are not met. The ANOVA is most commonly used when there is one nominal variable and one measurement variable, and the measurement variable does not meet the above mentioned assumptions of an ANOVA (McDonald, 2009). If data does not comply with the above mentioned assumptions, Kruskal-Wallis ANOVA tests must be used (McDonald, 2009). This test studies the nul hypothesis of (a) no difference between three or more group median (centre point of data) values against the alternative hypothesis that (b) a significant difference exists between the medians. A 5% level of significance was used to test the null hypothesis. A p-value less than 0.05 depicted that at least one of the groups' medians was significantly different (SAS Institute Inc., 2005).

3. Results and Discussion

3.1. Stability testing

3.1.1. Assay

Overall the recovery of the formulations depicted a decrease in all of the ingredients. The results obtained after a three month period are illustrated in figures 1 to 5. These figures show the percentage reductions in the ingredients in the different formulations at three temperatures.

The reductions of azelaic acid could be due to the fact that azelaic acid is unstable at higher temperatures/when excessively heated with excess heat (Material safety data sheet, 2005a). BHT reductions can be ascribed to the fact that BHT shows instability with excess heat (Material safety data sheet, 2005b). According to Weber et al. (1938), the destruction of vitamin E has been demonstrated before, which may explain the decrease thereof in the Pheroid™ formulations.

The Pheroid™ formulations depicted more significant decreases than the non-Pheroid™ formulations. According to the ICH Harmonised Tripartite Guideline (2003), a change of 5% in assay is seen as a significant change. Thus, all of the ingredients have shown significant change after a three month period.

3.1.2. Confocal laser scanning microscopy

Results obtained from CLSM depicted that waxes were present in the cream formulations. This could be due to the cetyl alcohol that was not spread homogeneously throughout the formulation. Little or no waxes were visible in the Pheroid™ cream , therefore it can be said that the Pheroid™ cream was more homogeneous than the cream. The gel appeared to be very homogeneous, no crystals could be seen. At month 3 the Pheroid™ gel formulation at 40 °C depicted large oil drops, this might be due to prolonged exposure to high temperatures.

3.1.3. Viscosity

Overall a reduction in viscosity could be established. In the Pheroid™ formulations, this reduction could be due to the decomposition of the Pheroid™ which caused oil to form on the surface of the formulations. Decreases ranged from 2-50%, which indicated that formulations were not stable due to the fact that all formulations were outside the experimental error of 20% (Ademola, 1997).

3.1.4. Mass variation

It could be established that both creams depicted a reduction in weight. The weight decreased as the temperature increased. Both the gels depicted a slight increase initially, however, after a period of approximately 2 months it almost stabilised or slightly reduced. This initial slight increase could be attributed to xanthan gum which was not fully hydrated during the formulation process, and only hydrated during storage.

3.1.5. pH

No specific pattern could be detected with change in the pH values, a reduction in pH only occurred in the Pheroid™ formulations, whilst the pH of the cream and gel increased. No significant pH changes occurred.

3.1.6. Appearance

No change in colour could be detected for the cream formulation throughout the testing period. However, the Pheroid™ cream underwent dramatic colour changes over time. The colour changed to darker shades of yellow with an increase in temperature. This could also be seen with the Pheroid™ gel. The gel formulation darkened slightly after a month and thereafter the colour stayed constant. The colour changes may be ascribed to the fact that incompatibilities occurred between the ingredients of the formulations and those of Pheroid™.

3.2. *Franz cell diffusion studies*

A linear regression line could not be observed in the average cumulative concentration curve of either azelaic acid or niacinamide. From the average cumulative concentration after 12 hours and the average percentage (%) diffused it was established that for niacinamide, the cream

formulations overall diffused more significantly than the gel formulations. This could be due to the fact that niacinamide is hydrophilic and its tendency to diffuse from a lipophilic medium (cream formulations) to the hydrophilic keratinocytes is higher than its diffusion from the more hydrophilic medium (gel formulations) to the keratinocytes. In the case of niacinamide, the cream formulation diffused more (65.312 µg/cm², 0.088% diffused) than the Pheroid™ cream (16.152 µg/cm², 0.022% diffused). The average concentration and percentage diffused of niacinamide in the gel formulation (7.704 µg/cm², 0.010% diffused) was double of that of the Pheroid™ gel (3.497 µg/cm², 0.005% diffused) formulation. In the case of the gel formulations, the Pheroid™ did not enhance the diffusion of niacinamide transdermally.

For the 12 hours cumulative concentrations of niacinamide, the median cumulative concentration values were established to be less than the average cumulative concentrations in all four of the formulations. In all the formulations, i.e. the cream (29.626 µg/cm²), Pheroid™ cream (7.567 µg/cm²), gel (3.123 µg/cm²) and the Pheroid™ gel (1.951 µg/cm²), the median cumulative concentration values were half of that of the average cumulative concentrations. The median values are expected to be a more accurate value, due to the fact that it is the centre of the data and are not influenced by outliers, as in the case of the average cumulative concentration (Gerber et al, 2008).

Figure 6: Box-plots indicating median cumulative concentration (µg/cm^2) of (a) niacinamide and (b) azelaic acid after 12 hours.

With azelaic acid, a specific pattern of diffusion was not observed. According to the 12 hours average cumulative concentrations, diffusion from of the Pheroid™ cream formulation (38.476 µg/cm², 0.010% diffused) was greater than the gel formulation (24.508 µg/cm², 0.009% diffused) which diffused better than from the Pheroid™ gel formulation (11.025 µg/cm², 0.004% diffused) as well as from the cream formulation (3.225 µg/cm², 0.001% diffused). The Pheroid™, in this case, were more efficient in the Pheroid™ cream than in the Pheroid™ gel, but also caused better diffusion of azelaic acid in the Pheroid™ cream formulation than the diffusion of azelaic acid in the non-Pheroid™ cream.

For the azelaic acid 12 hours concentrations, the median cumulative concentration values were less than the average cumulative concentrations in all four of the formulations. In all of them, i.e. the Pheroid™ cream (19.482 µg/cm²), gel (13.234 µg/cm²), Pheroid™ gel (5.603 µg/cm²) and the cream (1.611 µg/cm²), the median cumulative concentration values were half of that of the average cumulative concentrations. The median value is expected to be a more accurate value, due to the fact that outliers do not influence the data, as in the case of the average cumulative concentration (Gerber et al, 2008).

3.3. Tape stripping method

During tape stripping it was found that niacinamide had the highest average concentration in the cream formulation (11.482 µg/ml in the epidermis and 34.572 µg/ml in the dermis) and the Pheroid™ cream formulation (4.471 µg/ml in the epidermis and 15.742 µg/ml in the dermis) was second highest. However, the Pheroid™ gel formulation depicted a higher niacinamide concentration in the epidermis (2.903 µg/ml) and dermis (9.655 µg/ml) than the gel formulation (1.656 µg/ml in the epidermis and 7.078 µg/ml in the dermis). The ratio of the concentrations between the formulations is approximately similar to the 12 hours average cumulative concentrations. Pheroid™ proved to be more efficient in the Pheroid™ gel formulation, which made the diffusion of niacinamide out of this formulation bettwr than from the non-Pheroid™ gel. However, in the creams, it was depicted that the Pheroid™ were less efficient in transdermal diffusion of niacinamide.

Figure 7: The average concentration values (µg/ml) of niacinamide and azelaic acid in **(a)** the epidermis and **(b)** the dermis after tape stripping.

For azelaic acid, the Pheroid™ cream presented the highest average concentrations in the epidermis (6.906 µg/ml) and dermis (28.926 µg/ml) and the cream formulation (0.247 µg/ml in the epidermis and 1.844 µg/ml in the dermis) the second highest. The gel formulation had third highest concentrations in the epidermis (4.745 µg/ml) and dermis (23.083 µg/ml) and the Pheroid™ gel formulation (2.767 µg/ml in the epidermis and 13.113 µg/ml in the dermis)

showed the lowest concentrations. The ratio of the concentrations between the formulations is similar to the 12 hours average cumulative concentrations.

3.4. Aqueous solubility and log D

The aqueous solubility of azelaic acid was established as 2.837 mg/ml and the log D value was 0.105. For niacinamide, the aqueous solubility was 212.92 mg/ml and the log D value was -0.32. The aqueous solubility of both azelaic acid and niacinamide were more than 1 mg/ml, which indicated that both may be transdermally delivered through the skin (Naik et al., 2000). The log D values of azelaic acid and niacinamide fell outside the ideal range to be transdermally delivered, the log P (octanol-water partition coefficient) value needs to be between 1 and 2 (Sloan, 1989; Hadgraft, 1996; Roberts and Sloan, 2000).

3.5. Statistical analysis

Table 1: *Table indicating multiple p-value comparisons of niacinamide and azelaic acid.*

Statistical signifficant differences did not exist everywhere when the different formulations were compared. The p-values of the total cumulative amount of niacinamide diffused after 12 hours indicated that no statistical significant differences existed between the cream and Pheroid™ cream, the Pheroid™ cream and the gel, or the gel and Pheroid™ gel. The p-values of the total cumulative amount of niacinamide in the epidermis indicated no statistical significant differences between the cream and Pheroid™ cream, the Pheroid™ cream and Pheroid™ gel and the gel and Pheroid™ gel. The p-values of the total cumulative amount of niacinamide in the dermis indicated that no statistical significant differences existed between the cream and Pheroid™ cream, the Pheroid™ cream and gel, the Pheroid™ cream and Pheroid™ gel as well as the gel and Pheroid™ gel.

The p-values of the total cumulative amount of azelaic acid diffused after 12 hours indicated no statistical significant differences between the cream and Pheroid™ gel, the Pheroid™ cream and gel and the gel and Pheroid™ gel. In the case of the epidermis concentrations, no statistical significant differences existed between the Pheroid™ cream and gel, the Pheroid™

cream and Pheroid™ gel and the gel and Pheroid™ gel. The p-values of the total cumulative amount of azelaic acid in the dermis indicated no statistical significant differences between the cream and Pheroid™ gel, the Pheroid™ cream and gel and the gel and Pheroid™ gel.

4. Conclusion

According to the stability tests, the non-Pheroid™ formulations proved to be more stable under the different storage conditions. Assay results showed that the Pheroid™ formulations concentrations decreased more than the non-Pheroid™ formulations concentrations. Viscosity reduced overall over the time period tested, which in the Pheroid™ formulations, may be due to the decomposition of the Pheroid™ causing oil to form on the surface of the formulations. Drastic colour changes only occurred with the Pheroid™ formulations, which could be due to possible incompatibilities between the ingredients of the formulations and those of Pheroid™.

Both azelaic acid and niacinamide were found in the epidermis and the dermis. No flux values could be obtained; therefore, average total cumulative concentrations of the active ingredients after 12 hours were shown. All four formulations allowed the diffusion of azelaic acid and niacinamide through the skin. The Pheroid™ formulations did not prove to be more advantageous for transdermal diffusion or to be more stable than non-Pheroid™ formulations, except in the case of azelaic acid in the Pheroid™ cream.

Acknowledgements

Special thanks to the National Research Foundation and the North West University for funding this project. Also to Prof Jan du Plessis for your assistance with the statistical analysis. Thank you to Dr. A. Lamont (Carstenhoff clinic, Midrand, South Africa), Dr. D.A. Hoffman (Sunward Park Hospital, Boksburg, South Africa), Dr. G. Annandale (Brooklyn surgical centre, Pretoria, South Africa) and Dr. V. Jandera (Pretoria-East Hospital, Pretoria, South Africa) for donation of human skin samples obtained from abdominoplastic surgery.

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Figure legends:

Figure 1: Assay - Recovery percentage reductions of niacinamide

Figure 2: Assay - Recovery percentage reductions of azelaic acid

Figure 3: Assay - Recovery percentage reductions of methyl paraben

Figure 4: Assay - Recovery percentage reductions of BHT

Figure 5: Assay - Recovery percentage reductions of vitamin E

Figure 6: Box-plots indicating median cumulative concentration (μg/cm^2) of **(a)** niacinamide and **(b)** azelaic acid after 12 hours.

Figure 7: The average concentration values (μg/ml) of niacinamide and azelaic acid in **(a)** the epidermis and **(b)** the dermis after tape stripping.

Tables:

Table 1: Table indicating multiple p-value comparisons of niacinamide and azelaic acid.

		Niacinamide				Azelaic acid			
		Cream	Pheroid™ cream	Gel	Pheroid™ gel	Cream	Pheroid™ cream	Gel	Pheroid™ gel
12 h	Cream	-	*	°	°	-	°	°	*
	Pheroid™ cream	*	-	*	°	°	-	*	°
	Gel	°	*	-	*	°	*	-	*
	Pheroid™ gel	°	°	*	-	*	°	*	-
Epidermis	Cream	-	*	°	°	-	°	°	°
	Pheroid™ cream	*	-	*	*	°	-	*	*
	Gel	°	°	-	*	°	*	-	*
	Pheroid™ gel	°	*	*	-	°	*	*	-
Dermis	Cream	-	*	°	°	-	°	°	*
	Pheroid™ cream	*	-	*	*	°	-	*	°
	Gel	°	*	-	*	°	*	-	°
	Pheroid™ gel	°	*	*	-	*	°	*	-

° *indicates statistical signifficant difference* * *indicates no statistical significant difference*

Figures:

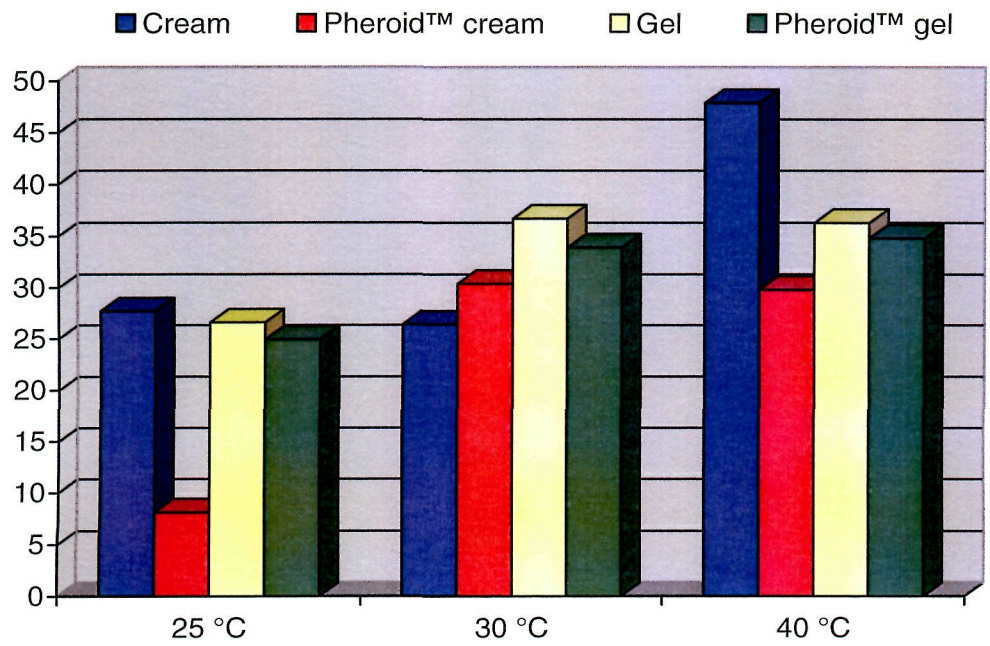

Figure 1: Assay - Recovery percentage reductions of niacinamide

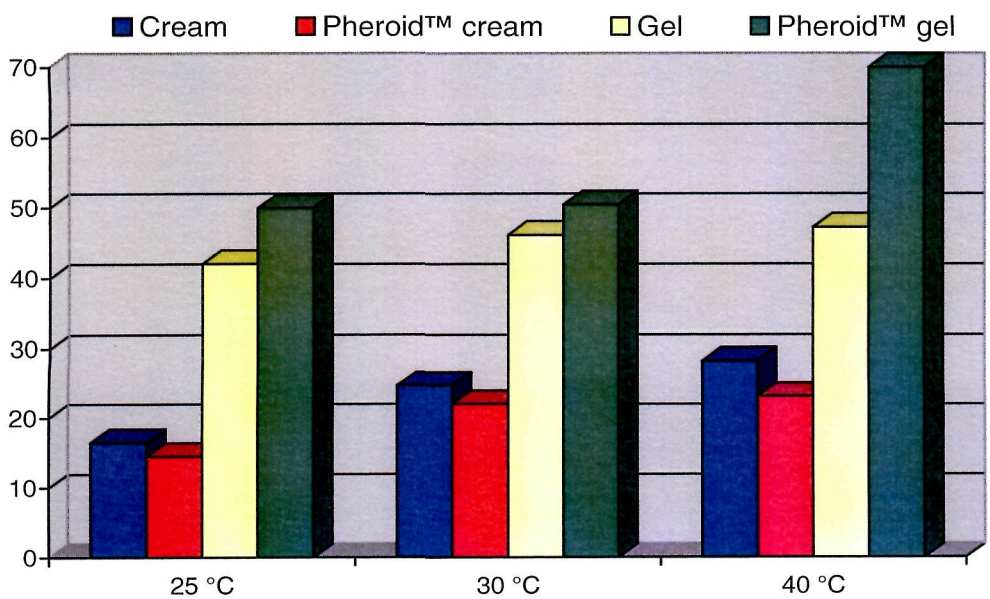

Figure 2: Assay - Recovery percentage reductions of azelaic acid

**

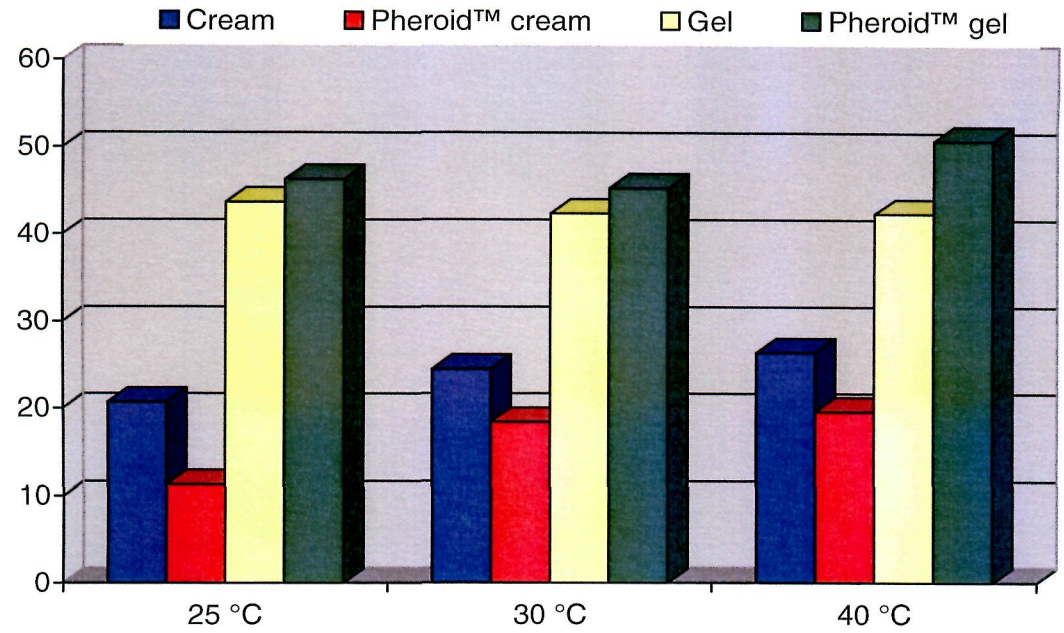

Figure 3: Assay - Recovery percentage reductions of methyl paraben

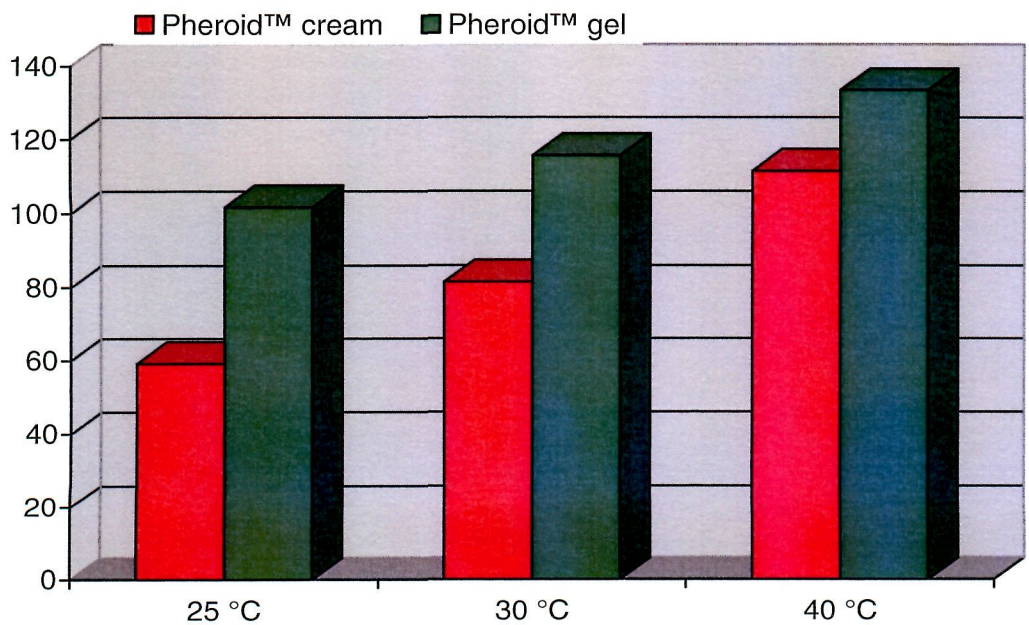

Figure 4: Assay - Recovery percentage reductions of BHT

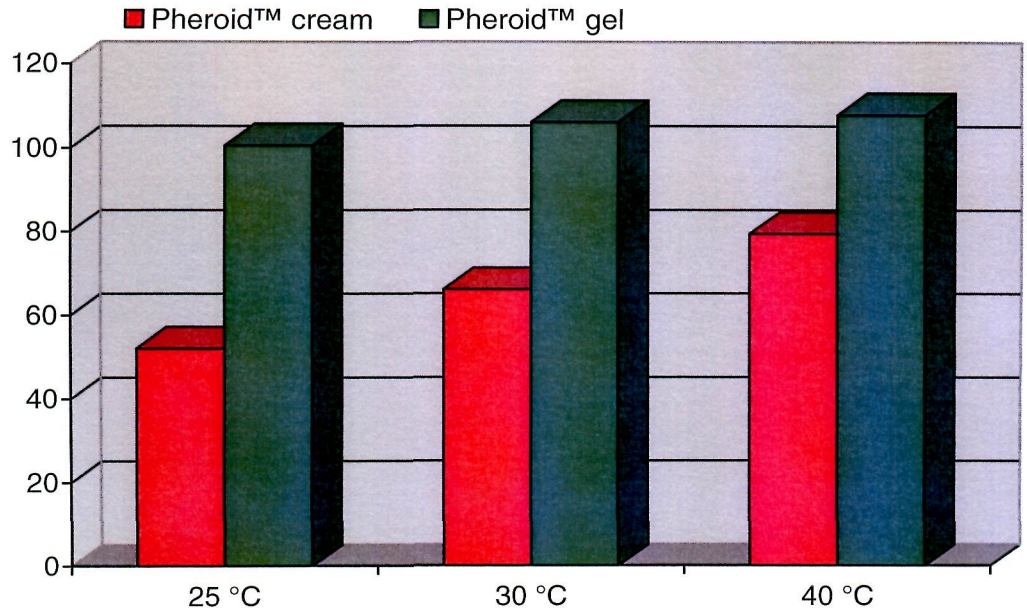

Figure 5: Assay - Recovery percentage reductions of vitamin E

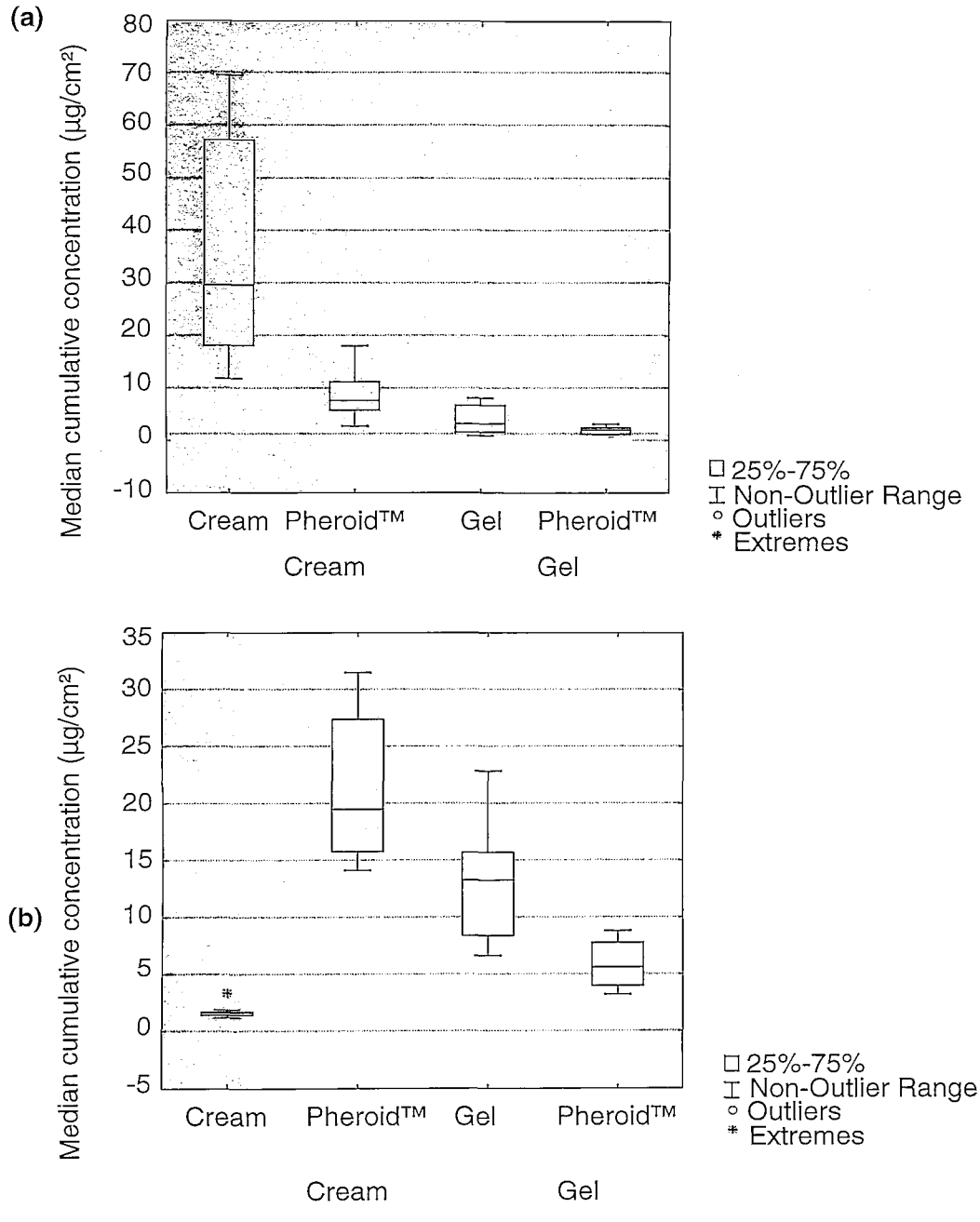

Figure 6: Box-plots indicating median cumulative concentration (µg/cm^2) of **(a)** niacinamide and **(b)** azelaic acid after 12 hours.

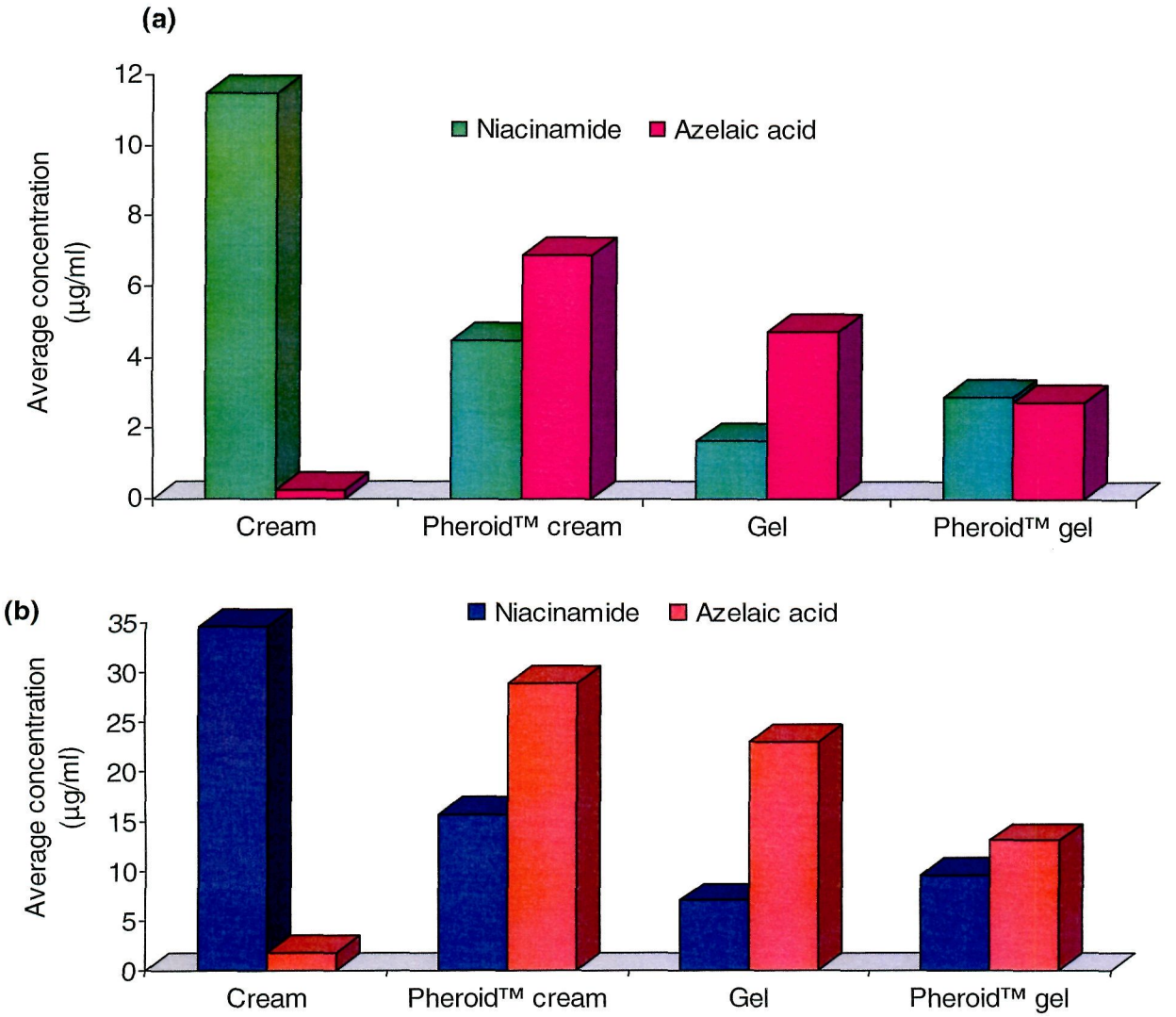

Figure 7: The average concentration values (µg/ml) of niacinamide and azelaic acid in **(a)** the epidermis and **(b)** the dermis after tape stripping.

CHAPTER 4: FINAL CONCLUSION AND FUTURE PROSPECTS

Acne is a common disease that affects millions of people around the world. It presents as microcomedones which may enlarge to form open or closed comedones (Akhavan & Bershad, 2003:474). Topical therapy is the first line of treatment for patients presenting with non-inflammatory open or closed comedones. However, for more severe forms of acne, additional systemic treatment is necessary (Krautheim & Gollnick, 2004:398).

Azelaic acid and niacinamide were combined in this study due to their advantageous effects on acne. Azelaic acid is bacteriostatic against several microbes, including *P. acnes*. It decreases keratin production and can also provide an anti-inflammatory effect (Stein & Lebwohl, 2001:185). Niacinamide, on the other hand, has an anti-inflammatory effect and can therefore be used to reduce inflammatory papules (Gehring, 2004:92). It also causes dose-dependant inhibition of sebocyte secretions (Namazi, 2007:1230).

A cream, a Pheroid™ cream, a gel and a Pheroid™ gel were formulated in this study, each containing both azelaic acid and niacinamide. These formulations were exposed to stability testing in order to determine whether they were stable after a period of six months. Tests that were conducted during stability testing included assay, mass variation, viscosity, pH determination, appearance and confocal laser scanning microscopy. According to the Medicines Control Council requirements, none of these formulations were stable, as depicted by the results obtained. It was also established that Pheroid™ was not successful throughout in improving transdermal diffusion.

Franz cell diffusion studies were performed in order to establish the ability of azelaic acid and niacinamide to be released from the four formulations. The results of the average percentage diffused from the formulations showed that the release of niacinamide was more significant in case of the cream formulations overall, more so from the cream than the Pheroid™ cream. Azelaic acid, on the other hand, showed higher release concentrations from the Pheroid™cream and the gel. The concentrations measured after 12 hours showed that the niacinamide concentration was the highest in the cream and second highest in the Pheroid™ cream. Whereas the azelaic acid concentration was higher in the Pheroid™ cream and gel, which is apparently due to the average percentage diffused. Tape stripping was used to determine the concentrations of niacinamide and azelaic acid in the epidermis and dermis of the skin. The niacinamide concentration was higher in the cream and second highest in the Pheroid™ cream and the azelaic acid concentration was higher in the Pheroid™ cream and gel.

Aqueous solubility and log D values were determined. Naik *et al.* (2000:319) stated that the ideal solubility for a drug to permeate the skin is more than 1 mg/ml. It was established that the aqueous solubilities of azelaic acid (2.837 mg/ml) and niacinamide (212.92 mg/ml) were more than 1 mg/ml, which indicated that both may be transdermally delivered through the skin. The log P value of an active ingredient should be between 1 and 3 for it to be transdermally delivered (Hadgraft, 2004:292). From the results in the study, it was evident that both azelaic acid (0.105) and niacinamide (-0.32) are poorly delivered through the skin. This can be improved by using penetration enhancers or special delivery systems.

Future prospects include the following:

- Optimal formulations must be developed in order to maintain stability and acceptable shelf life.
- Different delivery systems should be attempted for more effective delivery of these cosmeceutical actives.
- Clinical testing must be performed in order to determine the possible beneficial and adverse reactions, which may be associated with the use of the products.

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APPENDIX A: INTERNATIONAL JOURNAL OF PHARMACEUTICS GUIDE FOR AUTHORS

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

Du Plessis, J., 1992. Topical liposomal delivery of biologically active peptides. Ph.D Thesis, Potchefstroom University for CHE, South Africa.

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APPENDIX B: FORMULATION OF COMPOUNDS CONTAINING AZELAIC ACID AND NIACINAMIDE

B.1. INTRODUCTION

Topical dosage forms have been used throughout history to deliver a drug topically to the skin to treat various disorders (Allen, 1998a:187). Topical dosage forms include ointments, creams, pastes, gels, etc. In this study, a gel and a cream containing azelaic acid and niacinamide were formulated with and without Pheroid™.

According to Allen (1998a:187), a cream is an opaque, soft solid or thick liquid intended for external application. In a cream, the active ingredient is being dissolved or suspended in a base to form either a water-in-oil (w/o) or an oil-in-water (o/w) emulsion.

Allen (1998b:201) also states that a gel is an excellent drug delivery system for various routes of administration. Gels are semisolid systems in which a liquid phase is trapped within an interlocking, three-dimensional polymeric matrix of a natural or synthetic gum (Flynn, 2002:216).

B.2. AIM

In this Appendix, the different formulas that were compounded will be given. In the formulation process, different formulas were compounded in order to formulate a cream and a gel containing azelaic acid and niacinamide, as well as Pheroid™ formulations for both. In each case, the formulation with the best qualities (eg appearance and viscosity) was chosen for the final formula of each compound. 100g of each formula was compounded. Both solids and liquids were weighed for perfection. The aim was to formulate a stable cream and gel dosage form, with and without Pheroid™.

B.3. MATERIALS AND METHODS

Equipment that were used in the formulation process include a Shimadzu AUW 120 scale (Japan), Heidolph DIAX 600 (Germany) and Wisestir Diahan Scientific cc (Korea) homogenisers, a hotplate and appropriate glassware. Protective clothing included sterilised coats, masks and gloves.

Azelaic acid and niacinamide was obtained from SIGMA-ALDRICH® (South Africa). All other ingredients were of analytical grade.

B.4. RESULTS

B.4.1. FORMULATION OF CREAM

B.4.1.1. Cream 1

1. Cetyl alcohol	15.0 g
2. Liquid paraffin	12.0 g
3. Cremophor EL	2.7 g
4. Methyl paraben	0.1 g
5. Water	38.2 g
6. Propylene glycol	8.0 g
7. Niacinamide	4.0 g
8. Azelaic acid	20.0 g
Total	**100 g**

Procedure

1. Dissolve the azelaic acid in the liquid paraffin. Heat to 80 °C if necessary.
2. Dissolve the niacinamide in the water.
3. Heat the cetyl alcohol, cremophor EL and methyl paraben to 80 °C.
4. Add propylene glycol to the above heated mixture.

5. Add all the ingredients together and homogenise at 13 500 rpm to room temperature (25 ± 0.5°C).

See section B.5. for discussion.

B.4.1.2. Cream 2

1. Polyethylene Glycol (PEG) 400		20.0 g
2. Cetyl alcohol		15.0 g
3. Methyl paraben		0.1 g
4. Water		36.4 g
5. Azelaic acid		20.0 g
6. Niacinamide		4.0 g
	Total	**100 g**

Procedure

1. Weigh and heat PEG 400 and azelaic acid to 80 °C. Stir until the azelaic acid is dissolved.
2. Weigh and heat cetyl alcohol and methyl paraben to 70 °C.
3. Weigh and heat water and niacinamide to 70 °C.
4. Add PEG 400 and azelaic acid to cetyl alcohol and methyl paraben.
5. Add the above mixture to the water and niacinamide while homogenising at 13 500 rpm until 38 °C is reached.
6. Homogenise at 200 rpm until room temperature (25 ± 0.5 °C).

See section B.5. for discussion.

B.4.1.3. Cream 3

1. Cetyl alcohol		17.0 g
2. Liquid paraffin		17.0 g
3. Span 60		0.5 g
4. Tween 80		4.5 g
5. Methyl paraben		0.1 g
6. Niacinamide		4.0 g
7. Azelaic acid		20.0 g
8. Water		36.9 g
	Total	**100 g**

Procedure

1. Dissolve the azelaic acid in the liquid paraffin. Heat to 80°C if necessary.
2. Dissolve the niacinamide in water.
3. Heat the cetyl alcohol, liquid paraffin and azelaic acid, span 60, tween 80 and methyl paraben to 80 °C.
4. Heat the water and niacinamide to 80 °C and add to the rest of the ingredients.
5. Homogenise at 13 500 rpm to room temperature (25 ± 0.5 °C).

See section B.5. for discussion.

B.4.2. FORMULATION OF GEL

B.4.2.1. Gel 1

1. Propan-2-ol	35 g
2. Propylene glycol	20 g
3. Azelaic acid	15 g
4. Niacinamide	4 g
5. Hydroxypropylmethylcellulose (HPMC)	2 g
6. Glycerin	10 g
7. Water	14 g
Total	**100 g**

Procedure

1. Mix propan-2-ol and the propylene glycol.
2. Dissolve the azelaic acid in the above mixture.
3. Dissolve the niacinamide in approximately a third of the water.
4. Wet the HPMC with the glycerin.
5. Mix all the ingredients and slowly add the remaining water.
6. Mix slowly at 200 rpm for 5 minutes to form a gel.

See section B.5. for discussion.

B.4.2.2. Gel 2

1. Propan-2-ol		50.71 g
2. Propylene glycol		15.87 g
3. Water		8.44 g
4. Isopropyl Myristate		4.21 g
5. Methyl paraben		0.08 g
6. Azelaic acid		15.00 g
7. Niacinamide		4.00 g
8. HPMC		1.69 g
	Total	**100 g**

Procedure

1. Mix together the propan-2-ol, propylene glycol and water to form a co-solvent system.
2. Dissolve azelaic acid, niacinamide, isopropyl myristate and methyl paraben into the co-solvent system.
3. Add HPMC to 50% solvent and homogenise at 13 500 rpm for more than 45 minutes to hydrate the gel.
4. Add the rest of the solvent to form the final product and homogenise at 200 rpm to room temperature (25 ± 0.5 °C).

See section B.5. for discussion.

B.4.2.3. Gel 3

1. Xantham gum		3.0 g
2. Glycerol		20.0 g
3. Azelaic acid		15.0 g
4. Niacinamide		4.0 g
5. Ethanol		44.8 g
6. Methyl paraben		0.2 g
7. Water		13.0 g
	Total	**100 g**

Procedure

1. Dissolve the azelaic acid in the ethanol.
2. Then dissolve the niacinamide in approximately a third of the water.
3. Dissolve methyl paraben in the glycerol.
4. Add the dissolved methyl paraben and niacinamide to azelaic acid in ethanol.
5. Add the remaining water.
6. Homogenise at 200 rpm to form a gel.

See section B.5. for discussion.

B.4.2.4. Gel 4

1. PEG 400		20.00 g
2. Span 60		0.50 g
3. Tween 80		4.50 g
4. Propyl paraben		0.08 g
5. Methyl paraben		0.40 g
6. Xantham gum		1.50 g
7. Azelaic acid		15.00 g
8. Niacinamide		4.00 g
9. Water		49.52 g
	Total	**100 g**

Procedure

1. Weigh and heat PEG 400 and azelaic acid to 80 °C. Stir until dissolved.
2. Weigh and heat span 60, tween 80, propyl paraben and methyl paraben to 70 °C.
3. Add PEG 400 and azelaic acid to span 60, tween 80, propyl paraben and methyl paraben mixture.
4. Weigh and heat approximately a quarter of water and niacinamide to 70 °C.
5. Heat the remaining water to 80°C and add xantham gum while homogenizing at 13 500 rpm.
6. Add dissolved niacinamide to above homogenising mixture.
7. Add dissolved azelaic acid, span 60, tween 80, propyl paraben and methyl paraben mixture to xantham gum in water mixture while still homogenizing at 13 500 rpm.
8. Homogenise at 13 500 rpm until 38 °C is reached.
9. Homogenise at 200 rpm until room temperature (25 ± 0.5 °C) is reached.

See section B.5. for discussion.

B.4.3. PHEROID™ CREAM

The formula used for compounding the cream (B.4.1.2. Cream 2) was utilised in compounding a Pheroid™ cream, except for the addition of butylhydroxytoluene (BHT), Vitamin E and Pheroids™ to the formulation. The Pheroids™ were manufactured by the North-West University.

B.4.4. PHEROID™ GEL

The formula used for compounding the gel (B.4.2.4. Gel 4) was utilised in compounding a Pheroid™ gel, except for the addition of butylhydroxytoluene (BHT), Vitamin E and Pheroids™ to the formulation. The Pheroids™ were manufactured by the North-West University.

B.5. DISCUSSION

Cream 2 was used as the final formula for a cream, due to the fact that Cream 1 appeared to be too thin and in contrast Cream 3 was too thick. This was judged by the ability of the cream to be applied on the skin.

Gel 4 was used as the final formula for a gel. Both Gels 1 and 2 appeared to be very thick and application to skin was difficult. Gel 3 was not homogeneous and was too watery.

As mentioned in B.4.3. and B.4.4., Cream 2 and Gel 4 were also used to formulate a Pheroid™ cream and a Pheroid™ gel, with the addition of the mentioned ingredients.

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APPENDIX C: VALIDATION OF THE HPLC EXPERIMENTAL METHOD FOR A PHEROID™ CREAM CONTAINING AZELAIC ACID AND NIACINAMIDE

C.1. PURPOSE OF THE VALIDATION

The objective of the validation of the HPLC experimental method was to ensure that the analytical method was sensitive and reliable for stability testing.

C.2. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: Hewlet Packard (HP) Agilent 1100 series HPLC, auto sampler, HP Agilent 1100 series variable detector and an HP Agilent 1100 series isocratic pump.

Column: Phenomenex, Luna 5µ C18, 250x4.60 mm.

Mobile phase: 0.005 M 1-Octanesulfonic acid/water, pH 3.5 balanced by adding Sodium Hydroxide (NaOH) and Phosphoric acid (H_3PO_4) dropwise.

Gradient:

Time (min)	A (%)	B (%)
1.00	75	25
8.00	0	100
25.00	0	100
25.10	75	25

Where: A = 0.005 M Octanesulfonic acid in water, pH 3.5

B = Methanol (100%)

Flow rate: 1 ml/min

Injection volume: 10 µl

Detection: UV detection at 220 nm

Retention time:	Niacinamide – 2.9 min
	Methyl Paraben – 7.2 min
	Azelaic acid – 7.5 min
	BHT – 12.3 min
	Vit E – 20.4 min
Runtime:	30 min
Solvent:	Methanol (100%), *gradient grade for HPLC.*

C.3. CALIBRATION CURVE

A calibration curve was obtained from injecting 2, 3, 5, 7, 10, 12 and 15 µl of the standard solution. The standard solution was prepared as follows:

1. In a 20 ml volumetric flask (flask 1), weigh 160 mg Azelaic acid and 32 mg Niacinamide
2. In another 20 ml volumetric flask (flask 2), weigh 4 mg Methyl paraben, 8 mg BHT and 8 mg Vitamin E
3. Make flask 2 up to volume with Methanol (100%), analytical grade
4. Withdraw 4 ml of flask 2 and add to flask 1
5. Make flask 1 up to volume with Methanol (100%), analytical grade
6. Transfer the standard into a vial for analysis.

C.4. VALIDATION PARAMETERS

C.4.1. LINEARITY

The linearity of an analytical method is the capability (within a particular range) to acquire test results that are directly proportional to the amount (concentration) of analyte present in the sample. Linear regression analysis should give a regression coefficient (R^2) of ≥ 0.99. The lowest and highest concentrations between which the response remains linear, and/or where acceptable precision is obtained, is determined as the range.

Preparation of standards were as follows:

1. Prepare standard solution as mentioned under Calibration curve.
2. Transfer the standard into a vial for analysis.
3. Inject 2, 3, 5, 7, 10, 12 and 15 µl of this standard solution in duplicate on HPLC.

C.4.1.1. RESULTS

C.4.1.1.1. Niacinamide

Table 1: Linearity of niacinamide.

Injection volume	µg/ml	Area			Mean
2 µl	329.00	12205.1	12204.6	12331.4	12247.0
3 µl	493.50	18082.4	18105.5	18056.0	18081.3
5 µl	822.50	28120.2	28317.7	28221.2	28219.7
7 µl	1151.50	36572.3	36661.1	36336.2	36523.2
10 µl	1645.00	47938.5	48184.6	47981.3	48034.8
12 µl	1974.00	56229.6	56061.3	55975.9	56088.9
15 µl	2467.50	68783.6	68161.3	68662.2	68535.7

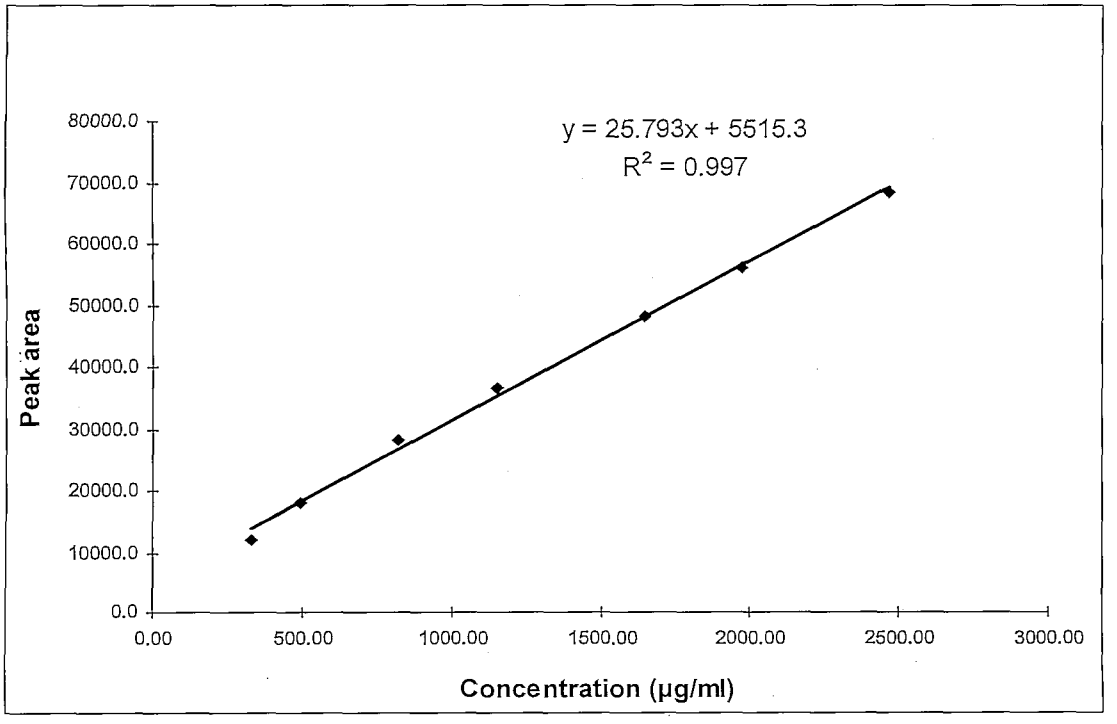

Figure 1: Linearity of niacinamide.

C.4.1.1.2. Azelaic acid

Table 2: Linearity of azelaic acid.

Injection volume	µg/ml	Area			Mean
2 µl	1626.90	234.1	250.4	243.1	242.5
3 µl	2440.35	355.3	360.0	364.0	359.7
5 µl	4067.25	578.9	577.6	576.3	577.6
7 µl	5694.15	773.1	781.2	783.7	779.3
10 µl	8134.50	1061.3	1078.7	1087.3	1075.8
12 µl	9761.40	1261.2	1260.0	1278.6	1266.6
15 µl	12201.75	1506.5	1548.3	1550.2	1535.0

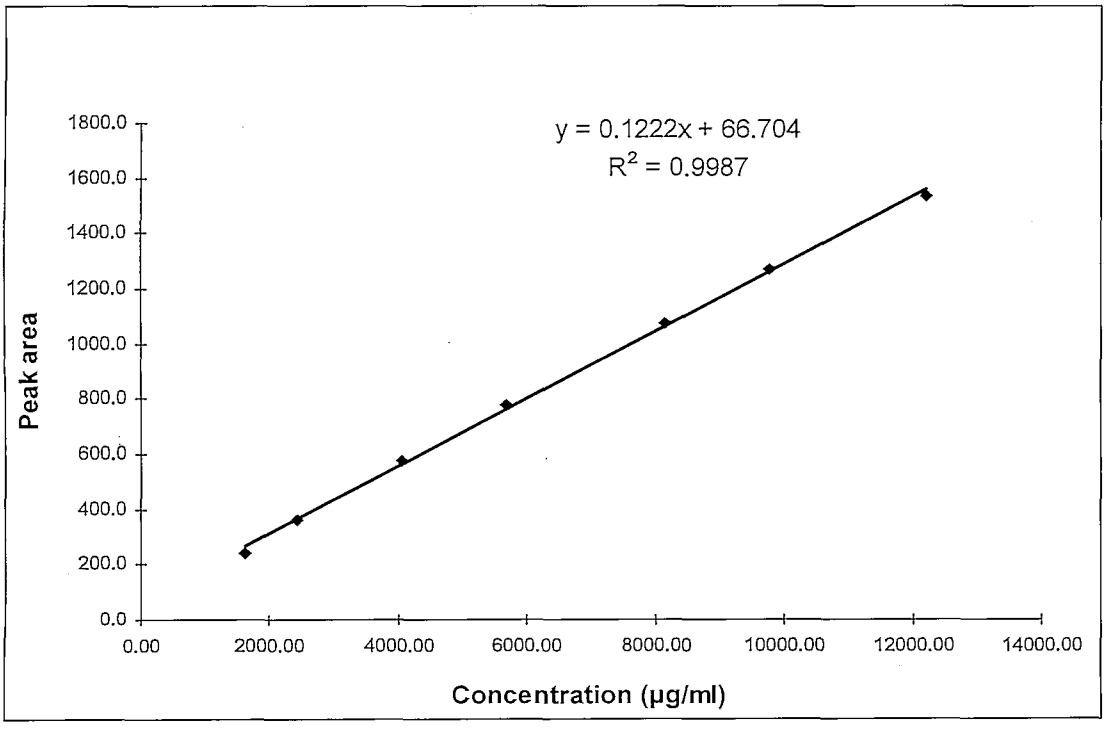

Figure 2: Linearity of azelaic acid.

C.4.1.1.3. Methyl paraben

Table 3: Linearity of methyl paraben.

Injection volume	µg/ml	Area			Mean
2 µl	9.42	109.3	89.7	143.5	114.2
3 µl	14.13	129.2	131.8	132.8	131.3
5 µl	23.55	192.5	186.2	191.8	190.2
7 µl	32.97	242.9	239.6	235.7	239.4
10 µl	47.10	284.6	275.2	300.2	286.7
12 µl	56.52	362.4	315.7	325.9	334.7
15 µl	70.65	375.2	382.8	381.2	379.7

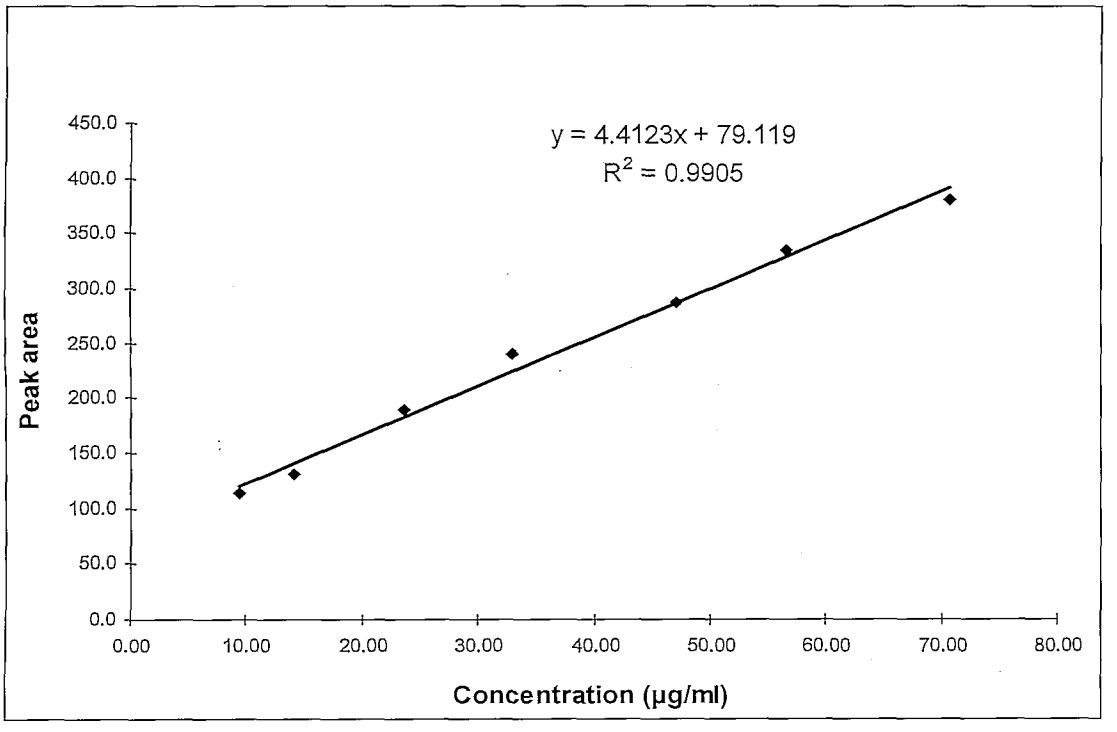

Figure 3: Linearity of methyl paraben.

C.4.1.1.4. BHT

Table 4: Linearity of BHT.

Injection volume	µg/ml		Area		Mean
2 µl	21.70	395.0	392.5	401.3	396.3
3 µl	32.55	600.6	595.1	595.6	597.1
5 µl	54.25	988.9	983.3	985.6	985.9
7 µl	75.95	1367.2	1377.7	1378.7	1374.5
10 µl	108.50	1914.9	1950.0	1945.7	1936.9
12 µl	130.20	2311.9	2333.4	2331.3	2325.5
15 µl	162.75	2906.1	2889.5	2912.7	2902.8

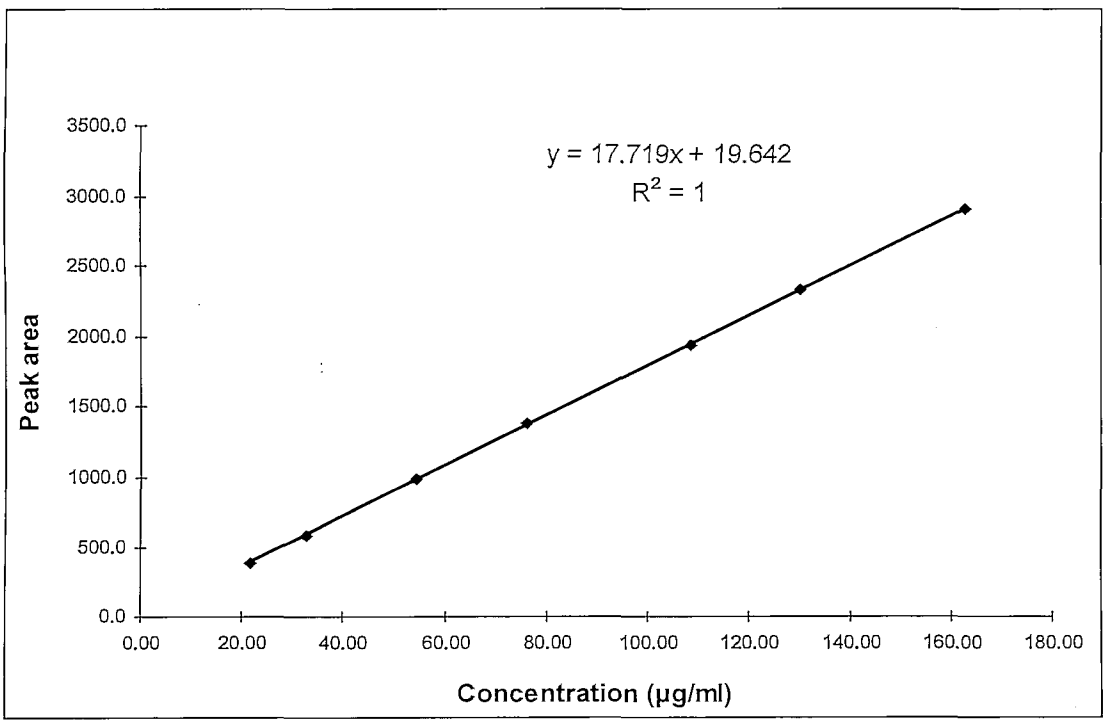

Figure 4: Linearity of BHT.

C.4.1.1.5. Vitamin E

Table 5: Linearity of vitamin E.

Injection volume	µg/ml	Area			Mean
2 µl	17.14	238.8	235.4	229.7	234.7
3 µl	25.71	372.3	369.0	370.4	370.6
5 µl	42.85	572.8	639.6	620.5	611.0
7 µl	59.99	832.1	862.8	857.5	850.8
10 µl	85.70	1192.5	1224.6	1228.0	1215.1
12 µl	102.84	1474.1	1474.5	1468.5	1472.4
15 µl	128.55	1832.1	1830.0	1838.6	1833.6

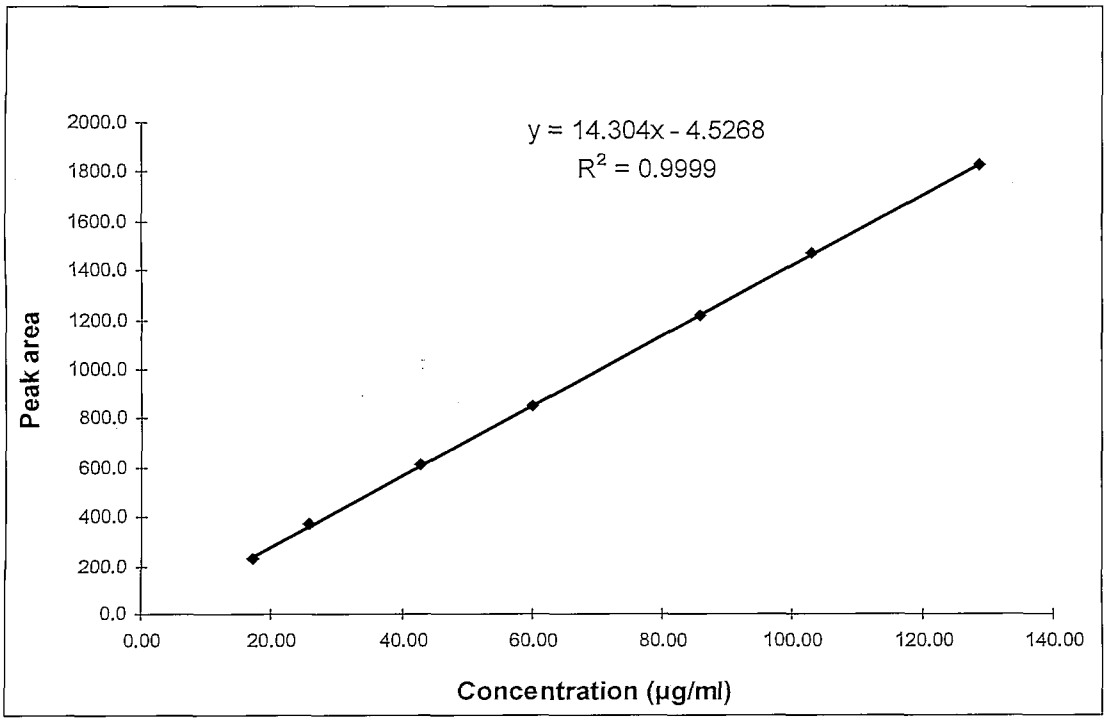

Figure 5: Linearity of vitamin E.

C.4.1.2. DISCUSSION

The regression coefficient (R^2) that were obtained for all the ingredients were ≥ 0.99, which indicates excellent linearity. The R^2 values were as follows:

	R^2
• Niacinamide	0.9970
• Azelaic acid	0.9987
• Methyl paraben	0.9905
• BHT	1
• Vitamin E	0.9999

C.4.2. ACCURACY AND PRECISION

The closeness of the value found to the true value is being expressed by the accuracy of an analytical method. It is assessed over a particular range, and 3 replicates at 3 concentrations must be analysed. The accuracy is expressed as the % recovery. Recovery should be between 98-102%.

Precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from numerous sampling of the same homogeneous sample under the prescribed conditions. Sub-divisions of Precision includes repeatability (Intraday)- and intermediate (Interday) precision. Repeatability must be better than 2%, (n=9) and Intermediate precision must be better than 5% (n=9).

C.4.2.1. Accuracy and Intraday precision

Accuracy and Intraday precision was determined as follows:

1. Prepare a placebo containing all matrix components except those to be tested.
2. Accurately weigh 3 times 80%, 100% and 120% of the sample amount of the placebo into 20 ml volumetric flasks.

3. Spike these with 80%, 100% and 120% of the analytical concentration of analyte.

4. Mix thoroughly and fill to volume with solvent.

5. Further dilute 10 ml of these solutions to 100 ml with solvent.

6. Transfer the samples into vials and analyse in duplicate.

C.4.2.1.1. RESULTS

C.4.2.1.1.1. Niacinamide

Table 6: *Accuracy and intraday precision of niacinamide.*

Conc.	Area			Mean	Recovery	
µg/ml					µg/ml	%
1350.0	42594.9	42406.1	43428.8	42810	1445.9	107.1
1655.0	48653.5	48844.6	48429.8	48643	1672.1	101.0
1935.0	53060.1	53992.9	53372.8	53475	1859.4	96.1
					Mean	101.4
					SD	4.5
					% RSD	4.4

C.4.2.1.1.2. Azelaic acid

Table 7: Accuracy and intraday precision of azelaic acid.

Conc.	Area			Mean	Recovery	
µg/ml					µg/ml	%
6465.0	543.4183	556.6	559.907	553	6576.6	101.7
8130.0	823.4728	836.8171	823.4157	828	8601.6	105.8
9640.0	948.2379	940.3477	934.3126	941	9435.4	97.9
					Mean	101.8
					SD	3.2
					% RSD	3.2

C.4.2.1.1.3. Methyl paraben

Table 8: Accuracy and intraday precision of methyl paraben.

Conc.	Area			Mean	Recovery	
µg/ml					µg/ml	%
34.0	225.6508	237.6044	238.3923	234	35.1	103.2
43.6	272.6021	269.0516	268.5989	270	43.3	99.3
51.0	305.1605	303.6783	290.2433	300	50.0	98.0
					Mean	100.2
					SD	2.2
					% RSD	2.2

C.4.2.1.1.4. BHT

Table 9: Accuracy and intraday precision of BHT.

Conc.	Area			Mean	Recovery	
µg/ml					µg/ml	%
65.8	1158.642	1152.3	1152.565	1154	64.0	97.3
84.0	1545.553	1534.451	1551.529	1544	86.0	102.4
98.8	1735.334	1738.175	1738.576	1737	96.9	98.2
					Mean	99.3
					SD	2.2
					% RSD	2.3

C.4.2.1.1.5. Vitamin E

Table 10: Accuracy and intraday precision of vitamin E.

Conc.	Area			Mean	Recovery	
µg/ml					µg/ml	%
68.6	979.8673	977.6	979.2878	979	68.8	100.3
86.2	1191.257	1193.829	1202.155	1196	83.9	97.3
102.8	1510.02	1512.673	1504.908	1509	105.8	102.9
					Mean	100.2
					SD	2.3
					% RSD	2.3

**

C.4.2.1.2. DISCUSSION

The recovery values for all five ingredients were within the limits of 98-102%.

% RSD values were all above 2%.

The values were as follows:

	Recovery	% RSD
• Niacinamide	101.4	4.4
• Azelaic acid	101.8	3.2
• Methyl paraben	100.2	2.2
• BHT	99.3	2.3
• Vitamin E	100.2	2.3

C.4.2.2. Interday precision

Analyse the same homogeneous sample in triplicate as described for intraday precision, at 100% of the sample concentration, on two more occasions, in duplicate, to determine the between-day variability of the method.

C.4.2.2.1. RESULTS

C.4.2.2.1.1. Niacinamide

Table 11: Interday precision of niacinamide.

	Conc. µg/ml	Area			Mean	Recovery µg/ml	Recovery %
Day 1	1645.00	46938.5	46184.6	47381.3	46834.8	1602.0	97.4
Day 2	1655.0	51653.5	52844.6	51429.8	51976	1801.3	108.8
Day 3	1645.0	47452.9	47091.1	46444.6	46996	1608.2	97.8
						Mean	101.3
						SD	5.3
						%RSD	5.2

C.4.2.2.1.2. Azelaic acid

Table 12: Interday precision of azelaic acid.

	Conc. µg/ml	Area			Mean	Recovery µg/ml	Recovery %
Day 1	8134.50	1061.3	1078.7	1087.3	1075.8	10429.5	128.2
Day 2	8130.0	773.5	776.8	773.4157	775	8208.2	101.0
Day 3	8134.5	757.6	762.2	766.6724	762	8116.7	99.8
						Mean	109.7
						SD	13.1
						%RSD	12.0

**

C.4.2.2.1.3. Methyl paraben

Table 13: Interday precision of methyl paraben.

	Conc.	Area			Mean	Recovery	
	µg/ml					µg/ml	%
Day 1	47.10	284.6	275.2	300.2	286.7	47.0	99.9
Day 2	43.6	305.7	317.6044	318.4	314	53.2	122.0
Day 3	47.1	320.7	320.662	319.6	320	54.7	116.1
						Mean	112.7
						SD	9.4
						%RSD	8.3

C.4.2.2.1.4. BHT

Table 14: Interday precision of BHT.

	Conc.	Area			Mean	Recovery	
	µg/ml					µg/ml	%
Day 1	108.50	1914.9	1950.0	1945.7	1936.9	108.2	99.7
Day 2	84.0	1545.6	1534.5	1551.5	1544	86.0	102.4
Day 3	108.5	1524.7	1517.4	1521.0	1521	84.7	78.1
						Mean	93.4
						SD	10.9
						%RSD	11.7

C.4.2.2.1.5. Vitamin E

Table 15: Interday precision of vitamin E.

	Conc. µg/ml	Area			Mean	Recovery µg/ml	%
Day 1	85.70	1172.5	1174.6	1178.0	1175.1	82.5	96.2
Day 2	86.2	1319.3	1321.8	1312.2	1318	92.4	107.2
Day 3	85.7	1162.4	1167.4	1167.2	1166	81.8	95.5
						Mean	99.6
						SD	5.4
						%RSD	5.4

C.4.2.2.2. DISCUSSION

All of the % RSD values were higher than 5%. The values were as follows:

	% RSD
• Niacinamide	5.2
• Azelaic acid	12.0
• Methyl paraben	8.3
• BHT	11.7
• Vitamin E	5.4

C.4.3. RUGGEDNESS

Ruggedness is sub-divided into Stability of sample solutions and System repeatability. For system repeatability, the peak area and retention times should have a RSD of 2% or less.

C.4.3.1. Stability of sample solutions

1. Weigh approximately 50 mg of analyte accurately and dissolve in 100 ml of the solvent.

2. Dilute 10 ml of this solution to 100 ml with solvent.

3. Transfer the standard into a vial for analysis.

4. Leave the sample in the tray and reanalyse at hourly intervals up to 12 hours to determine the stability of the sample.

5. Program the pump to reduce the flow rate to 0.1 ml/min after elution of the peak, and reset the flow rate to 1 ml/min 5 minutes before injecting the next sample.

C.4.3.1.1. RESULTS

C.4. 3.1.1.1. Niacinamide

Table 16: Ruggedness - Sample stability of niacinamide.

Time (hours)	Peak Area	%
0	45429.7	100.0
1	42429.6	93.4
2	44382.3	97.7
3	44675.2	98.3
4	45148.5	99.4
5	45801.2	100.8
6	45105.2	99.3
7	44866.7	98.8
8	44827.4	98.7
9	45456.8	100.1
10	45349.6	99.8
11	45307.5	99.7
12	45346	99.8
Mean	44932.7	98.9
SD	808.02	1.78
RSD %	1.80	1.80

C.4. 3.1.1.2. Azelaic acid

Table 17: Ruggedness - Sample stability of azelaic acid.

Time (hours)	Peak Area	%
0	940.2429	100.0
1	934.4282	99.4
2	935.6917	99.5
3	924.7912	98.4
4	912.6132	97.1
5	926.1444	98.5
6	904.9273	96.2
7	878.0573	93.4
8	898.346	95.5
9	848.1567	90.2
10	847.041	90.1
11	846.8435	90.1
12	837.4563	89.1
Mean	891.2	95.2
SD	36.20	3.95
RSD %	4.06	4.15

**

C.4. 3.1.1.3. Methyl paraben

Table 18: Ruggedness - Sample stability of methyl paraben.

Time (hours)	Peak Area	%
0	492.492	100.0
1	482.1924	97.9
2	481.7247	97.8
3	476.8572	96.8
4	469.2128	95.3
5	484.9273	98.5
6	491.8501	99.9
7	488.7216	99.2
8	488.1834	99.1
9	486.9298	98.9
10	485.7952	98.6
11	483.4554	98.2
12	489.2154	99.3
Mean	484.7	98.4
SD	6.11	1.24
RSD %	1.26	1.26

C.4. 3.1.1.4. BHT

Table 19: Ruggedness - Sample stability of BHT.

Time (hours)	Peak Area	%
0	1436.634	100.0
1	1429.766	99.5
2	1439.869	100.2
3	1470.871	102.4
4	1429.057	99.5
5	1463.23	101.9
6	1471.08	102.4
7	1484.447	103.3
8	1480.466	103.1
9	1479.489	103.0
10	1485.335	103.4
11	1492.581	103.9
12	1395.45	97.1
Mean	1458.3	101.5
SD	28.15	1.96
RSD %	1.93	1.93

**

C.4. 3.1.1.5. Vitamin E

Table 20: Ruggedness - Sample stability of vitamin E.

Time (hours)	Peak Area	%
0	1238.958	100.0
1	1239.573	100.0
2	1217.515	98.3
3	1185.595	95.7
4	1190.702	96.1
5	1218.758	98.4
6	1253.209	101.2
7	1245.733	100.5
8	1202.903	97.1
9	1240.256	100.1
10	1253.308	101.2
11	1259.097	101.6
12	1256.163	101.4
Mean	1230.9	99.4
SD	24.27	1.96
RSD %	1.97	1.97

C.4. 3.1.2. DISCUSSION

The % RSD values of the ingredients were as follows:

	% RSD
• Niacinamide	1.80
• Azelaic acid	4.15
• Methyl paraben	1.26
• BHT	1.93
• Vitamin E	1.97

C.4.3.2. System repeatability

Inject a sample or standard six times consecutively in order to test the repeatability of the peak area as well as the retention time.

C.4.3.2.1. RESULTS

C.4. 3.2.1.1. Niacinamide

Table 21: System repeatability of niacinamide.

	Peak area	Retention times (minutes)
	46453	4.190
	47991	4.329
	48445	4.367
	48522	4.355
	48466	4.313
	48957	4.376
Mean	48139	4.322
SD	804.30	0.063
RSD %	1.67	1.451

C.4. 3.2.1.2. Azelaic acid

Table 22: System repeatability of azelaic acid.

	Peak area	Retention times (minutes)
	768	9.250
	762	9.291
	758	9.292
	767	9.281
	780	9.280
	766	9.305
Mean	767	9.283
SD	6.77	0.017
RSD %	0.88	0.183

**

C.4. 3.2.1.3. Methyl paraben

Table 23: System repeatability of methyl paraben.

	Peak area	Retention times (minutes)
	324	9.109
	321	9.150
	321	9.157
	332	9.144
	320	9.139
	320	9.170
Mean	323	9.145
SD	4.33	0.019
RSD %	1.34	0.206

C.4. 3.2.1.4. BHT

Table 24: System repeatability of BHT.

	Peak area	Retention times (minutes)
	1517	12.616
	1521	12.635
	1557	12.636
	1535	12.628
	1553	12.628
	1562	12.647
Mean	1541	12.632
SD	17.54	0.009
RSD %	1.14	0.075

**

C.4. 3.2.1.5. Vitamin E

Table 25: System repeatability of vitamin E.

	Peak area	Retention times (minutes)
	1191	21.490
	1193	21.148
	1172	21.323
	1177	21.329
	1177	21.143
	1181	21.473
Mean	1182	21.318
SD	7.52	0.137
RSD %	0.64	0.645

C.4. 3.2.2. DISCUSSION

The % RSD values for all five ingredients were within the acceptable range of less than 2%. The values obtained were as follows:

	% RSD
• Niacinamide	1.451
• Azelaic acid	0.183
• Methyl paraben	0.206
• BHT	0.075
• Vitamin E	0.645

**

APPENDIX D: STABILITY TESTING OF DIFFERENT FORMULATIONS CONTAINING AZELAIC ACID AND NIACINAMIDE

D.1. INTRODUCTION

The same formulations in Appendix B section B.4.1.2. (cream) and B.4.2.4. (gel), as well as the two Pheroid™ formulations were formulated to conduct stability testing. However, these formulations were upscaled to approximately 600 g per formula.

Approximately 20g of each formulation were placed in small, white, plastic containers with a diameter in the region of 45 mm and a depth of 22 mm.

Stability tests were conducted over a three month period in Labcon Humidity Chambers (Maraisburg, South Africa; see Figure 21 under section D.6) at temperatures 25°C, 30°C and 40°C at humidities of 60%, 60% and 75%, respectively, in accordance with the stability regulations of the Medicines Control Council (2009:5-7).

Stability testing included 6 sub-tests, namely assay, mass variation, viscosity, pH, appearance and confocal laser scanning microscopy (CLSM). These tests are listed as appropriate for stability testing studies (Medicines Control Council, 2009:20-22).

D.2. AIM

Stability testing was performed in order to distinguish if any changes in both physical and chemical characteristics occurred.

D.3. MATERIALS AND METHODS

D.3.1. ASSAY

This sub-test includes preparing standard solutions as mentioned in Appendix C. To prepare samples, 2 g of each formulation at each temperature were weighed in 50 ml volumetric flasks in duplicate. No specific sampling technique was followed. Formulations were randomly sampled from containers. Both the cream and the Pheroid™ cream were made up to volume with water, whereas the gel and Pheroid™ gel were first dissolved in approximately 20 ml of methanol and then made up to volume with water. An Elma Transsonic TS540 sonic bath (Germany) was used to enhance the dissolving process. Each solution was analysed in duplicate with the HPLC. See Table 1 under section D.4. for results.

D.3.2. MASS VARIATION

Mass variation was performed with a Shimadzu AUW120D (Japan) scale (See Figure 22 under section D.6.). Each container of all the formulations at all three temperatures was measured each month. See Table 2 under section D.4. for results.

D.3.3. VISCOSITY

To determine viscosity changes, a 125 ml jar of each formulation was kept at 25 ± 0.5 °C. Each month the viscosity was determined with a Brookfield DV-II+ Programmable Viscometer (USA; see Figure 23 under section D.6.). The temperature controller (waterbath) was set at 25 ± 0.5°C. Cream and Pheroid™ cream viscosity were determined at a speed of 0.3 rpm, whereas the gel and Pheroid™ gel viscosity were determined at a speed of 0.6 rpm. The smallest spindle (S96) was used for all formulations. For each formulation, 32 data points were taken and 30 of the 32 were used to determine an average viscosity. The time interval between each reading was 10 seconds. See Table 2 under section D.4. for results.

D.3.4. pH

The pH of each formulation at all three temperatures was determined every month with a Mettler Toledo pH meter (China; see Figure 24 under section D.6.). For month 0, the pH of only one of each formulation was determined, as all formulations had the same pH at month 0, due to the fact that it was all formulated at the same time. See Table 2 under section D.4. for results.

D.3.5. APPEARANCE

Determining appearance was based on the colour changes of each formulation due to time, temperature and humidity. This was conducted by taking a picture of every formulation at each temperature every month with a Samsung S1060 camera (USA). Colour charts were used in order to establish whether there was any change in colour. Figures 1 - 4 under section D.4. depict photos of different formulations at different temperatures. Tables 3 - 6 under section D.4. show colours from colour charts and Figures 9 - 20 under section D.6. depict colour charts.

D.3.6. CONFOCAL LASER SCANNING MICROSCOPY

This part of stability testing was conducted with the Nikon Digital Eclipse C1 si Confocal Laser Scanning Microscope, or CLSM (Japan; see figure 25 under section D.6.), with violet diode laser 400-405 nm, a 543 nm He/Ne laser and an Argon ion laser 457-514 nm. It facilitates image capture in 3D.

Firstly, the samples were prepared by weighing 0,1 g of each formula at each temperature in an Eppendorf vial. As with the assay, no specific sampling technique was followed. 1000 µl of distilled water was added and dissolved thoroughly with a Stuart Scientific Autovortex SA 6 mixer (UK). 2 µl Nile Red was then added. Nile Red is a phenoxazine dye which highlights lipids. Samples with Nile Red were then left in a dark area for approximately 15 minutes. 20 µl of each sample was put on separate 76 x 26 x 1 mm microscope slides and each slide was covered with a 24 x 60 mm microscope cover glass. A drop of A nd = 1.515 (23 °C) immersion oil (Japan) was placed on each microscope slide in order to be viewed with the CLSM. The homogeneousness of each sample was determined by its micrograph. See Figures 5-8 under section D.4. for micrographs taken with the CLSM.

D.4. RESULTS

The following tables and figures show the results obtained during and after stability testing.

Table 1: Assay - Recovery of the four formulations at three temperatures over three months as determined by HPLC.

		Cream			Pheroid™ cream			Gel			Pheroid™ gel		
		25 °C	30 °C	40 °C	25 °C	30 °C	40 °C	25 °C	30 °C	40 °C	25 °C	30 °C	40 °C
Month 0	Niacinamide	129.7	129.7	129.7	110.8	110.8	110.8	110.4	110.4	110.4	109.1	109.1	109.1
	Methyl paraben	109.3	109.3	109.3	108.2	108.2	108.2	106.8	106.8	106.8	121.1	121.1	121.1
	Azelaic acid	106.3	106.3	106.3	104.2	104.2	104.2	119.8	119.8	119.8	104.2	104.2	104.2
	BHT	-	-	-	126.7	126.7	126.7	-	-	-	141.3	141.3	141.3
	Vitamin E	-	-	-	101.1	101.1	101.1	-	-	-	111.0	111.0	111.0
Month 1	Niacinamide	112.0	127.1	109.7	103.2	108.6	110.1	105.5	108.6	107.8	105.6	103.8	104.7
	Methyl paraben	96.8	94.7	93.6	101.7	100.6	99.4	100.1	100.6	99.5	119.2	117.9	118.3
	Azelaic acid	102.2	101.4	100.4	98.4	102.1	103.5	99.9	105.7	84.5	78.0	84.4	63.8
	BHT	-	-	-	102.8	107.8	101.8	-	-	-	102.8	95.2	93.2
	Vitamin E	-	-	-	97.4	85.9	78.0	-	-	-	83.1	80.9	70.9
Month 2	Niacinamide	106.3	109.9	109.5	102.9	104.1	101.9	103.3	102.9	96.0	103.8	103.2	90.7
	Methyl paraben	94.5	91.0	88.2	100.6	97.2	95.1	90.0	88.9	89.2	105.7	109.3	103.7
	Azelaic acid	92.1	92.2	91.1	96.9	98.4	96.4	99.7	82.2	73.0	76.6	62.5	63.0
	BHT	-	-	-	94.2	96.4	87.3	-	-	-	69.5	51.2	44.5
	Vitamin E	-	-	-	70.4	63.0	51.4	-	-	-	52.9	44.7	40.0
Month 3	Niacinamide	102.0	103.3	81.8	102.7	80.4	81.0	83.8	73.7	74.1	84.2	75.2	74.3
	Methyl paraben	88.6	84.8	82.9	96.9	89.8	88.6	63.1	64.4	64.5	74.8	75.8	70.3
	Azelaic acid	89.8	81.6	78.1	89.7	82.2	81.1	77.8	73.7	72.6	54.3	53.8	34.2
	BHT	-	-	-	67.5	45.2	15.3	-	-	-	39.6	25.7	7.8
	Vitamin E	-	-	-	48.9	35.0	21.7	-	-	-	10.4	4.9	3.1

Table 2: Results for viscosity, pH and mass variation of the four formulations at various temperatures over three months.

	Month 0			Month 1			Month 2			Month 3		
	25 °C	**30 °C**	**40 °C**	**25 °C**	**30 °C**	**40 °C**	**25 °C**	**30 °C**	**40 °C**	**25 °C**	**30 °C**	**40 °C**
Viscosity (cP)												
Cream	2331099.815	-	-	1896366.189	-	-	1054358.356	-	-	1623091.167	-	-
Pheroid™ Cream	1987180.000	-	-	1281289.100	-	-	1932608.417	-	-	2179847.337	-	-
Gel	1107576.167	-	-	625179.100	-	-	544258.867	-	-	590551.072	-	-
Pheroid™ gel	824094.980	-	-	654235.400	-	-	652256.656	-	-	532646.761	-	-
pH												
Cream	4.000	4.000	4.000	4.605	4.768	4.445	4.892	4.862	4.564	4.488	4.442	4.333
Pheroid™ Cream	4.737	4.737	4.737	4.717	4.812	4.776	4.728	4.967	4.679	4.680	4.678	4.612
Gel	4.347	4.347	4.347	4.963	4.923	4.595	4.688	4.565	4.536	4.517	4.498	4.466
Pheroid™ gel	4.604	4.604	4.604	4.865	4.731	4.750	4.990	4.641	4.724	4.999	4.601	4.569
Mass variation (g)												
Cream	26.10	26.08	25.90	26.10	26.06	25.39	26.09	26.04	25.35	26.09	26.03	25.35
Pheroid™ Cream	25.99	25.90	25.95	25.89	25.85	25.60	25.82	25.80	25.54	25.76	25.76	25.29
Gel	25.81	25.69	25.66	26.14	25.96	25.95	26.14	25.95	25.94	26.13	25.93	25.95
Pheroid™ gel	25.67	25.62	25.60	25.77	25.88	25.89	25.68	25.87	25.87	25.63	25.86	25.86

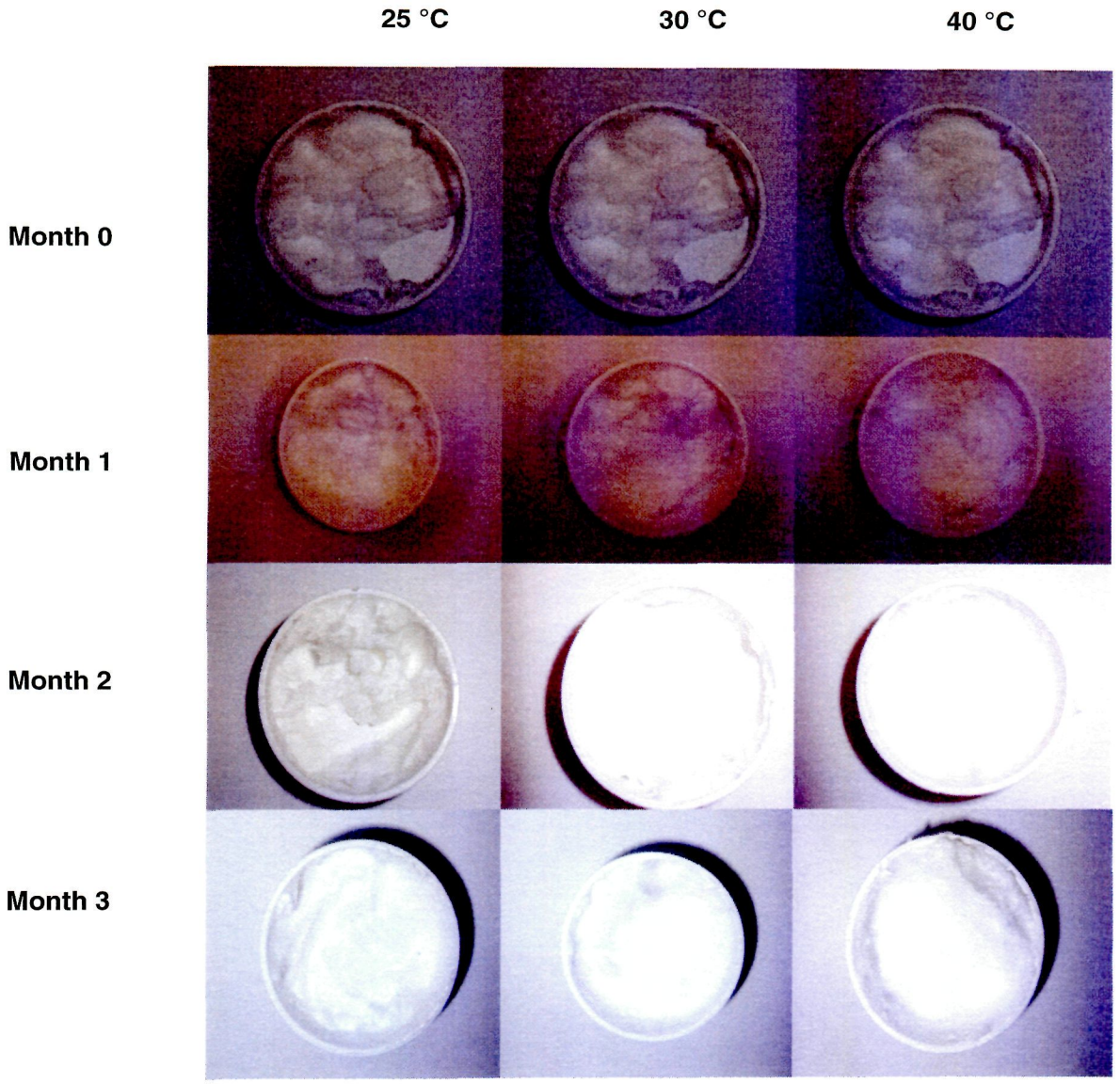

Figure 1: Cream appearance at three temperatures over three months.

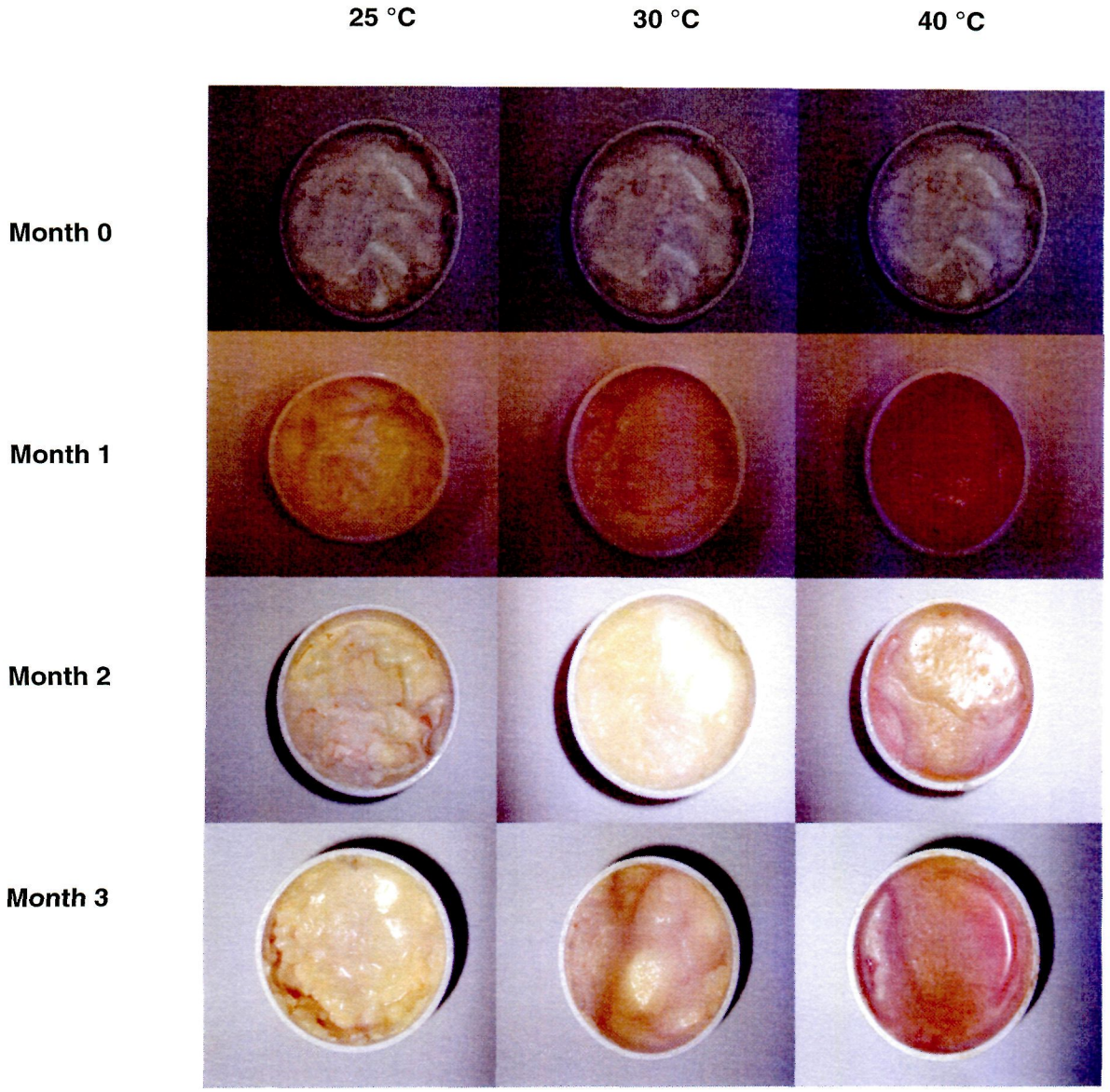

Figure 2: Pheroid™ cream appearance at three temperatures over three months.

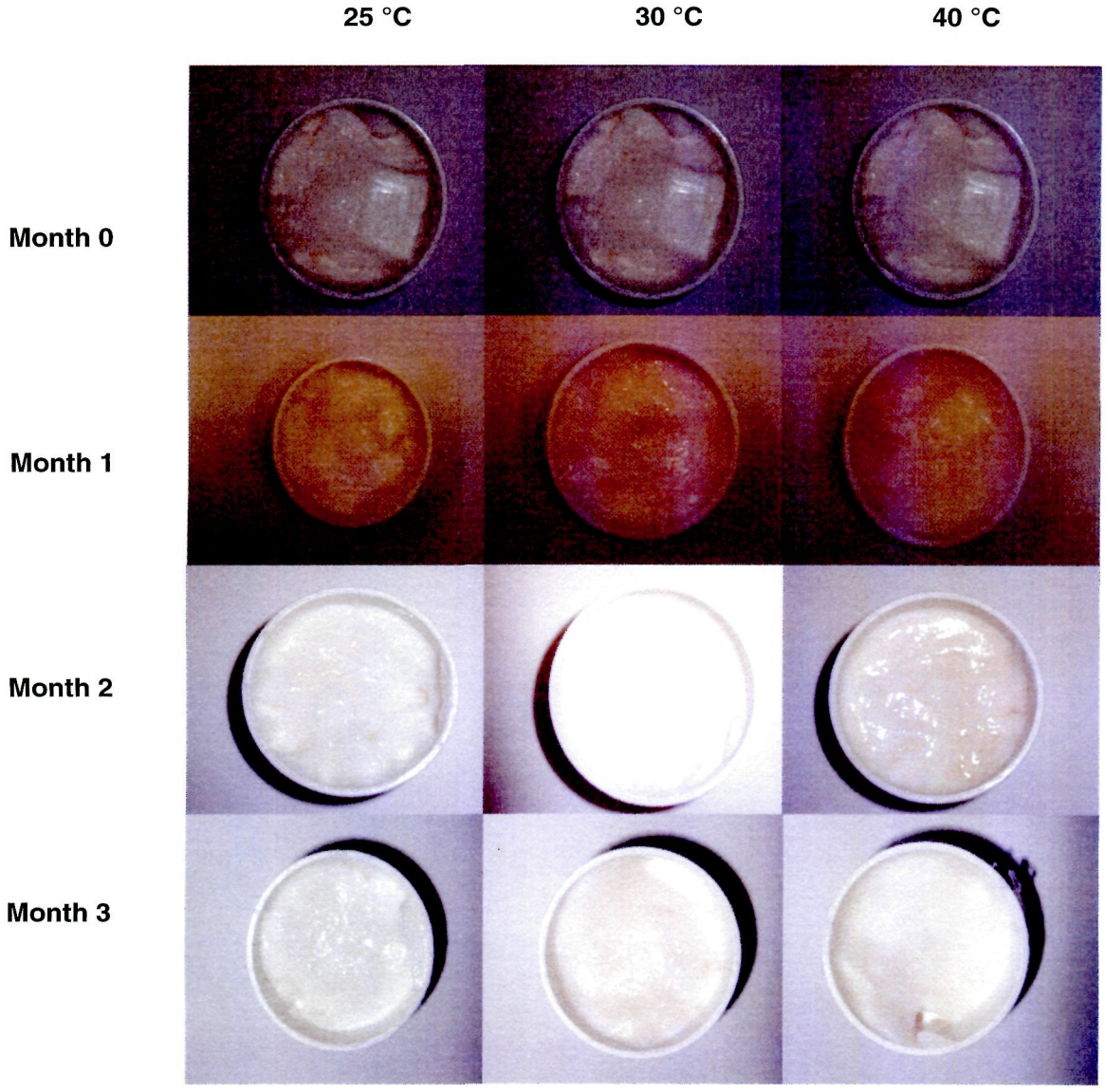

Figure 3: Gel appearance at three temperatures over three months.

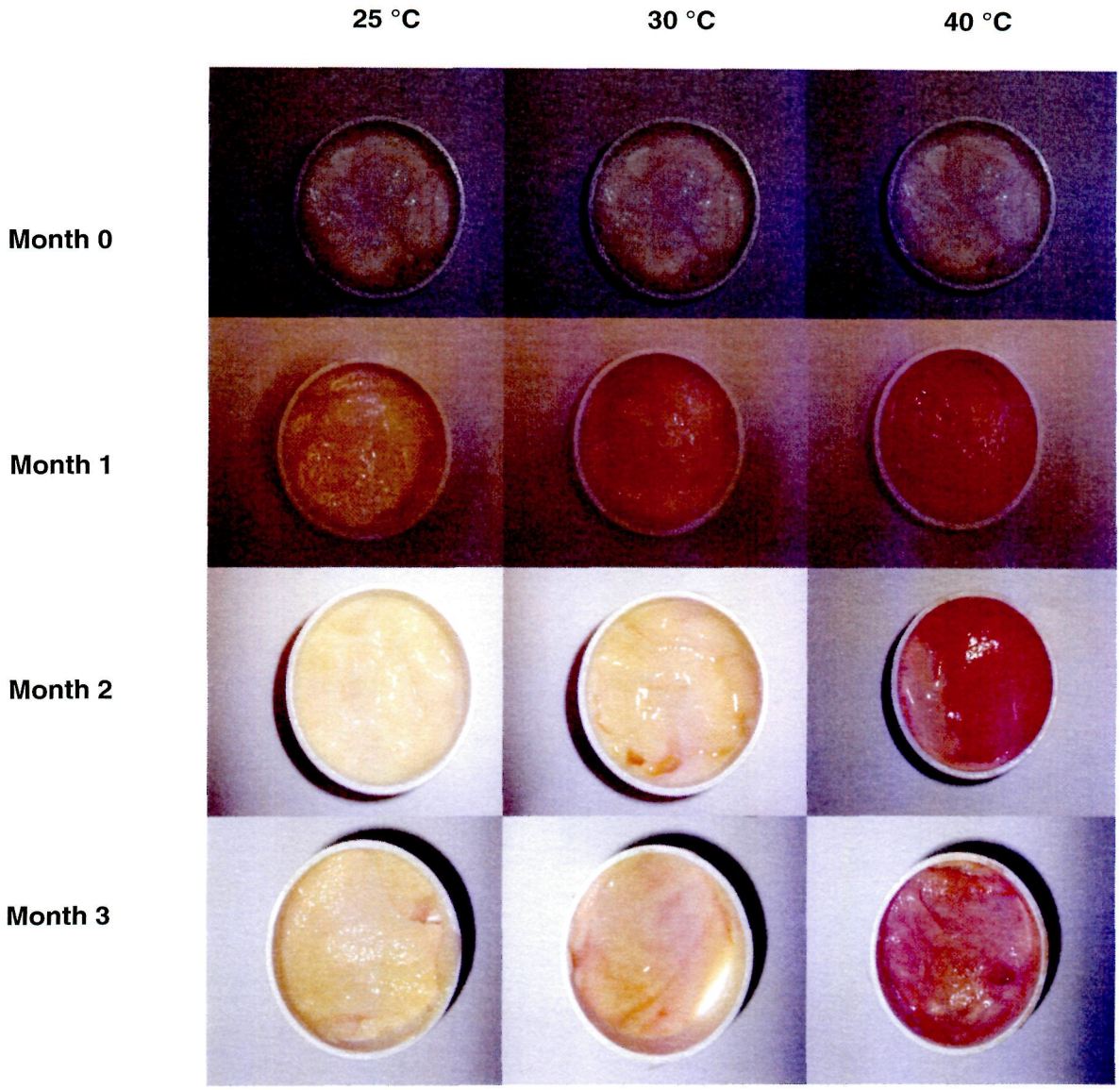

Figure 4: Pheroid™ gel appearance at three temperatures over three months.

Table 3: Cream appearance at three temperatures over three months according to colour charts.

	Temperature	Colour	Colour Chart
Month 0	25 °C	Light Reflection	Figure 20, 3rd from left
	30 °C	Light Reflection	Figure 20, 3rd from left
	40 °C	Light Reflection	Figure 20, 3rd from left
Month 1	25 °C	Light Reflection	Figure 20, 3rd from left
	30 °C	Light Reflection	Figure 20, 3rd from left
	40 °C	Light Reflection	Figure 20, 3rd from left
Month 2	25 °C	Light Reflection	Figure 20, 3rd from left
	30 °C	Light Reflection	Figure 20, 3rd from left
	40 °C	Light Reflection	Figure 20, 3rd from left
Month 3	25 °C	Light Reflection	Figure 20, 3rd from left
	30 °C	Light Reflection	Figure 20, 3rd from left
	40 °C	Light Reflection	Figure 20, 3rd from left

Table 4: Pheroid™ cream appearance at three temperatures over three months according to colour charts.

	Temperature	Colour	Colour Chart
Month 0	**25 °C**	Light Reflection	Figure 20, 3rd from left
	30 °C	Light Reflection	Figure 20, 3rd from left
	40 °C	Light Reflection	Figure 20, 3rd from left
Month 1	**25 °C**	Mesa Light	Figure 9, left
	30 °C	Banana Dream 5	Figure 11, 2nd from right
	40 °C	Butterscotch Royal	Figure 13, 4th from right
Month 2	**25 °C**	Lemon Yogurt	Figure 9, 3rd from left
	30 °C	Lemon Dew	Figure 15, 3rd from left
	40 °C	Au Gratin	Figure 17, 3rd from right
Month 3	**25 °C**	Lemon Dew	Figure 15, 3rd from left
	30 °C	Butterscotch Royal	Figure 13, 4th from right
	40 °C	Imperial Crown	Figure 17, 2nd from right

Table 5: Gel appearance at three temperatures over three months according to colour charts.

	Temperature	Colour	Colour Chart
Month 0	**25 °C**	Light Reflection	Figure 20, 3rd from left
	30 °C	Light Reflection	Figure 20, 3rd from left
	40 °C	Light Reflection	Figure 20, 3rd from left
Month 1	**25 °C**	Lemony Mist	Figure 10, right
	30 °C	Lemony Mist	Figure 10, right
	40 °C	Lemony Mist	Figure 10, right
Month 2	**25 °C**	Lemony Mist	Figure 10, right
	30 °C	Lemony Mist	Figure 10, right
	40 °C	Lemony Mist	Figure 10, right
Month 3	**25 °C**	Lemony Mist	Figure 10, right
	30 °C	Lemony Mist	Figure 10, right
	40 °C	Lemony Mist	Figure 10, right

Table 6: Pheroid™ gel appearance at three temperatures over three months according to colour charts.

	Temperature	Colour	Colour Chart
Month 0	**25 °C**	Lemony Mist	Figure 10, right
	30 °C	Lemony Mist	Figure 10, right
	40 °C	Lemony Mist	Figure 10, right
Month 1	**25 °C**	Morning Sun	Figure 9, 2nd from left
	30 °C	Blazing Sun	Figure 12, right
	40 °C	Texas Sun	Figure 13, 3rd from right
Month 2	**25 °C**	Spring Breeze 4	Figure 14, 3rd from right
	30 °C	Crocus Yellow	Figure 16, 4th from right
	40 °C	Mustard Flower	Figure 13, right
Month 3	**25 °C**	Buttercup Fool 4	Figure 18, 3rd from right
	30 °C	Lemon Rind	Figure 12, 2nd from right
	40 °C	Bumble Bee	Figure 19, right

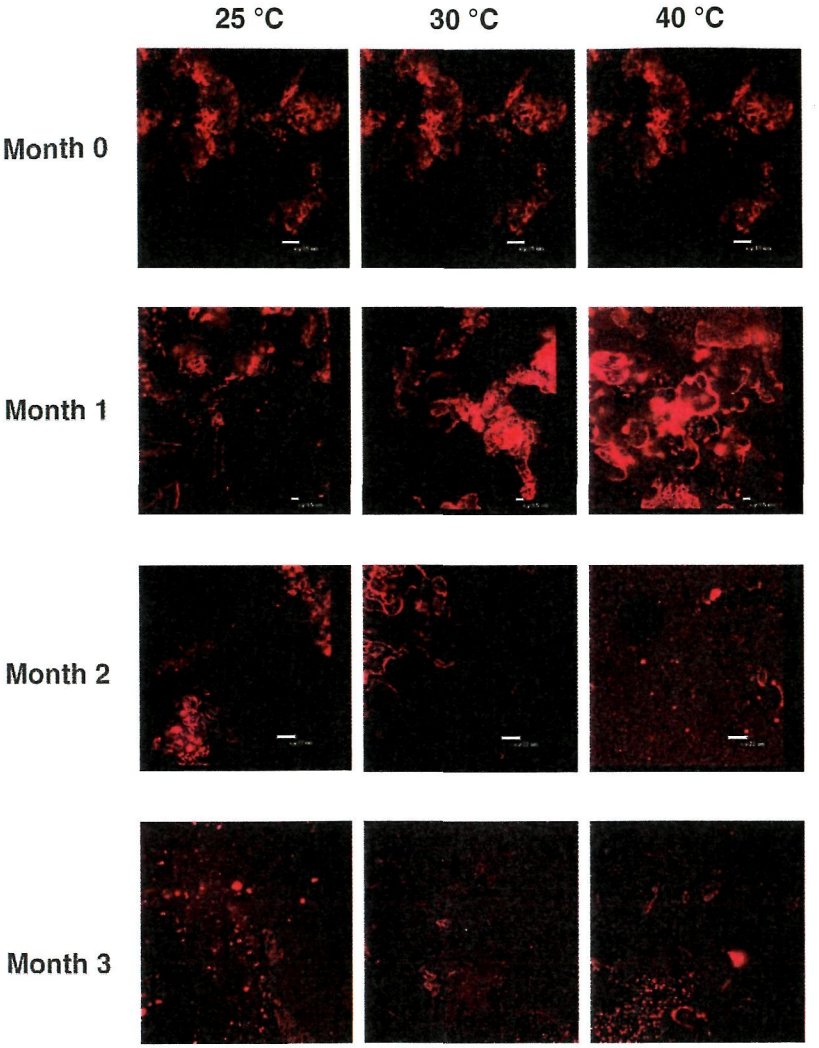

Figure 5: Micrographs of the cream CLSM.

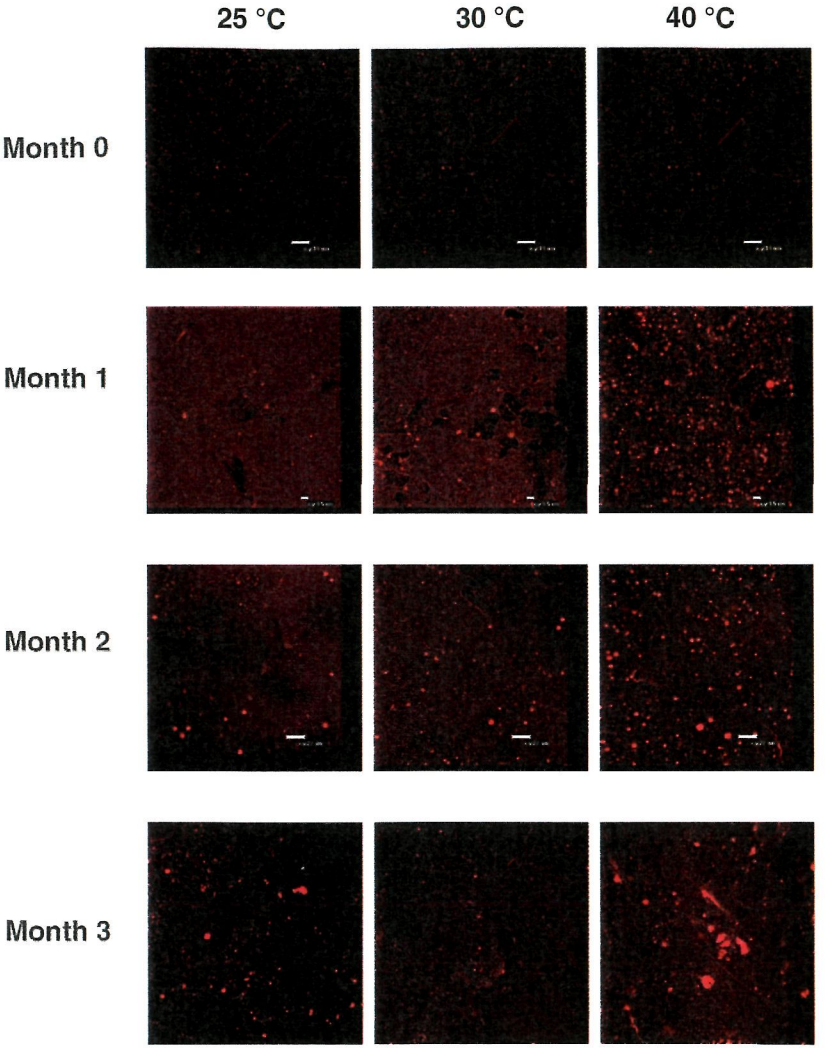

Figure 6: Micrographs of the Pheroid™ cream CLSM.

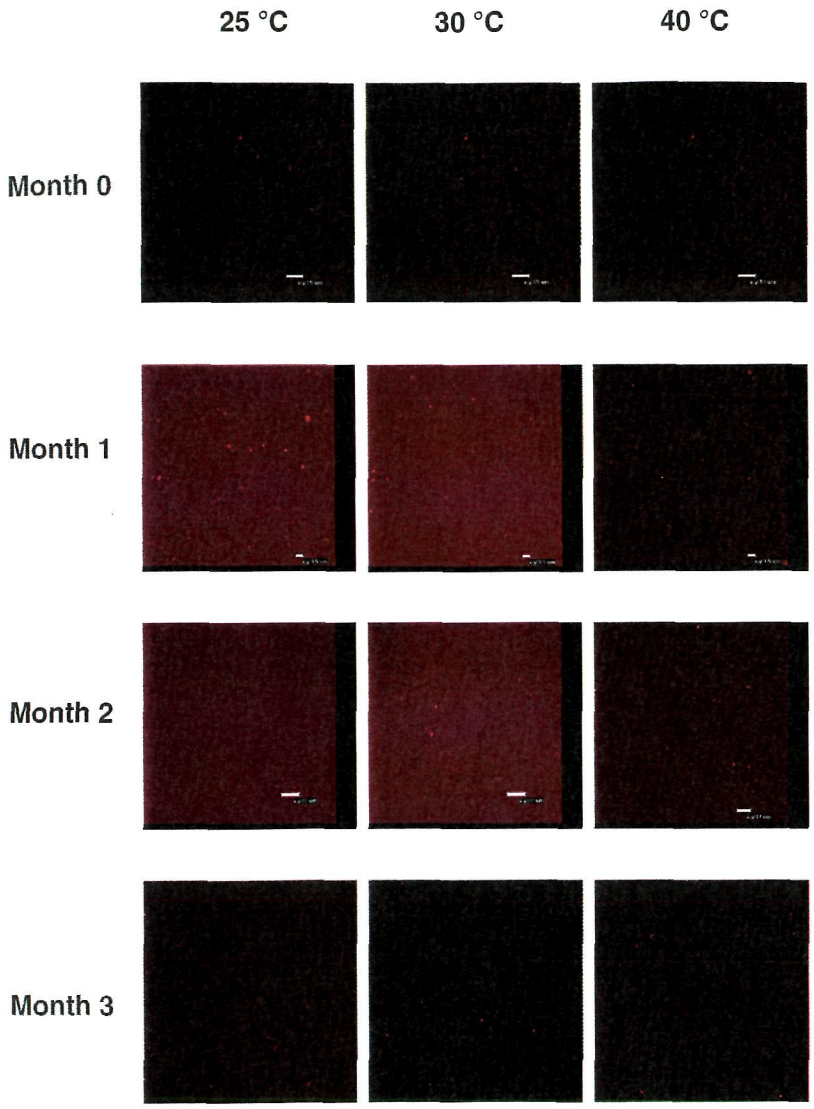

Figure 7: Micrographs of the gel CLSM.

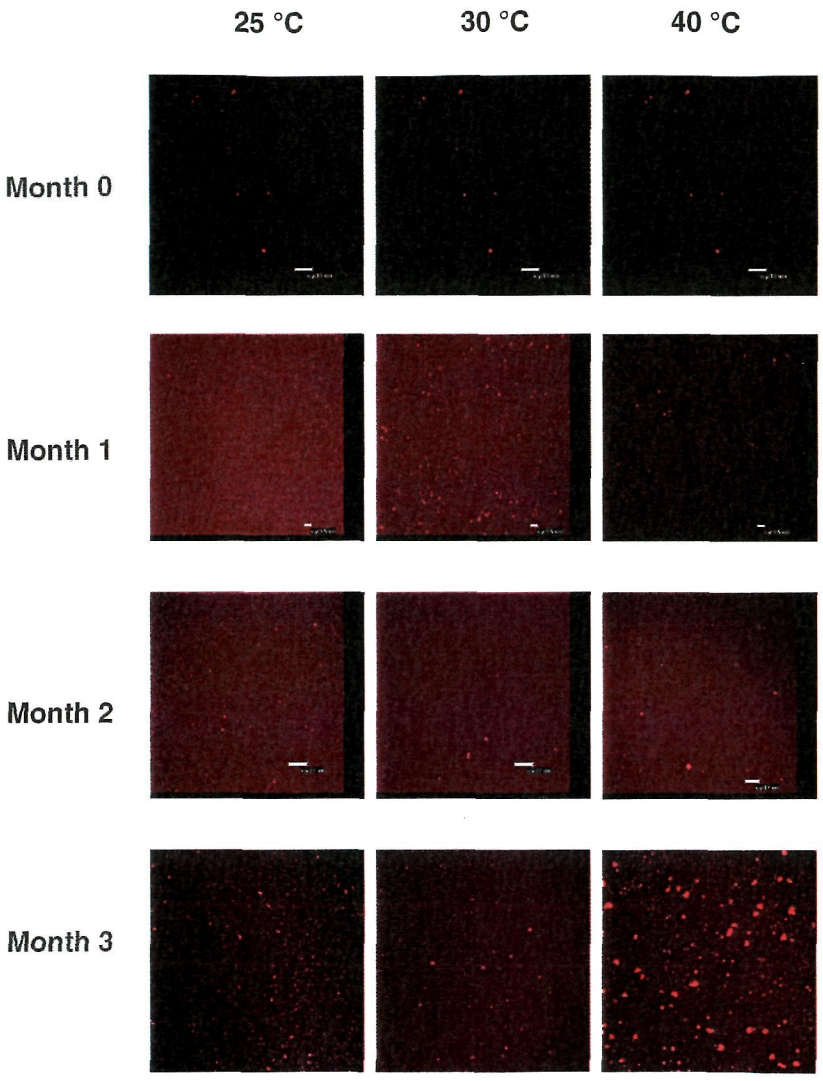

Figure 8: Micrographs of the Pheroid™ gel CLSM.

D.5. DISCUSSION

D.5.1. ASSAY

Overall the recovery of the formulations showed a decrease in all of the ingredients. The most significant reductions appeared to be azelaic acid, which is unstable with excess heat (Material Safety Data Sheet), BHT, which decomposes due to its antioxidant properties (Chemicalland21) and Vitamin E, which is known to be unstable (Bolomey, 1947:327-328). The Pheroid™ formulations depicted more significant decreases than the non-Pheroid™ formulations.

D.5.2. MASS VARIATION

Table 7: Mass variation of the four formulas at the different storage conditions.

Cream 25 °C	Approximately 0.04% reduction
Cream 30 °C	Approximately 0.19% reduction
Cream 40 °C	Approximately 2.12% reduction
Pheroid™ cream 25 °C	Approximately 0.88% reduction
Pheroid™ cream 30 °C	Approximately 0.54% reduction
Pheroid™ cream 40 °C	Approximately 2.54% reduction
Gel 25 °C	Approximately 1.28% increase
Gel 30 °C	Approximately 1.05% increase
Gel 40 °C	Approximately 1.13% increase
Pheroid™ gel 25 °C	Approximately 0.39% increase
Pheroid™ gel 30 °C	Approximately 1.01% increase
Pheroid™ gel 40 °C	Approximately 1.13% increase

It could be established that both creams showed a reduction in weight. The weight decreased as the temperature increased. Both the gels showed a slight increase initially, however, after a period of approximately 2 months it almost stabilized or slightly reduced. This initial slight increase can be attributed to xanthan gum which was not fully hydrated during the formulation process, and only hydrated during storage.

D.5.3. VISCOSITY

Table 8: Viscosity changes of the four formulas at different storage conditions.

Cream	Month 0-1:	Approximately 18.62% reduction
	Month 0-2:	Approximately 54.77% reduction
	Month 0-3:	Approximately 30.37% reduction
Pheroid™ cream	Month 0-1:	Approximately 35.52% reduction
	Month 0-2:	Approximately 2.75% reduction
	Month 0-3:	Approximately 9.70% increase
Gel	Month 0-1:	Approximately 43.55% reduction
	Month 0-2:	Approximately 50.86% reduction
	Month 0-3:	Approximately 46.68% reduction
Pheroid™ gel	Month 0-1:	Approximately 20.61% reduction
	Month 0-2:	Approximately 20.85% reduction
	Month 0-3:	Approximately 35.37% reduction

Overall a reduction in viscosity was established. In the Pheroid™ formulations, this reduction can be due to the decomposition of the Pheroid™ which caused oil to form on the surface of the formulations.

D.5.4. pH

Table 9: pH changes of the four formulations at the different storage conditions

Cream 25 °C	Month 0-1:	Approximately 15.13% increase
	Month 0-2:	Approximately 22.3% increase
	Month 0-3:	Approximately 12.2% increase
Pheroid™ cream 25 °C	Month 0-1:	Approximately 0.42% reduction
	Month 0-2:	Approximately 0.19% reduction
	Month 0-3:	Approximately 1.2% reduction
Gel 25 °C	Month 0-1:	Approximately 14.17% increase
	Month 0-2:	Approximately 7.84% increase
	Month 0-3:	Approximately 3.91% increase
Pheroid™ gel 25 °C	Month 0-1:	Approximately 5.67% increase
	Month 0-2:	Approximately 8.38% increase

	Month 0-3:	Approximately 8.58% increase
Cream 30 °C	Month 0-1:	Approximately 19.2% increase
	Month 0-2:	Approximately 21.55% increase
	Month 0-3:	Approximately 11.05% increase
Pheroid™ cream 30 °C	Month 0-1:	Approximately 1.58% increase
	Month 0-2:	Approximately 4.86% increase
	Month 0-3:	Approximately 1.25% reduction
Gel 30 °C	Month 0-1:	Approximately 13.25% increase
	Month 0-2:	Approximately 5.01% increase
	Month 0-3:	Approximately 3.47% increase
Pheroid™ gel 30 °C	Month 0-1:	Approximately 2.76% increase
	Month 0-2:	Approximately 0.8% increase
	Month 0-3:	Approximately 0.07% reduction
Cream 40 °C	Month 0-1:	Approximately 11.13% increase
	Month 0-2:	Approximately 14.1% increase
	Month 0-3:	Approximately 8.33% increase
Pheroid™ cream 40 °C	Month 0-1:	Approximately 0.82% increase
	Month 0-2:	Approximately 1.22% reduction
	Month 0-3:	Approximately 2.64% reduction
Gel 40 °C	Month 0-1:	Approximately 5.71% increase
	Month 0-2:	Approximately 4.35% increase
	Month 0-3:	Approximately 2.74% increase
Pheroid™ gel 40 °C	Month 0-1:	Approximately 3.17% increase
	Month 0-2:	Approximately 2.61% increase
	Month 0-3:	Approximately 0.76% reduction

No specific pattern could be detected with the pH changes, however, it could be depicted that a reduction in pH only occurred at the Pheroid™ formulations, whilst the cream and gel depicted increased pH values.

D.5.5. APPEARANCE

No change in colour could be detected for the cream formulation throughout the three month period. However, the Pheroid™ cream underwent dramatic colour changes with time. Colour

change increased with an increase in temperature. This could also be seen with the Pheroid™ gel, whereas the gel slightly changed colour where after it stayed unchanged. The colour changes may be ascribed to the fact that incompatibilities occurred between the ingredients of the formulations and those of the Pheroid™.

D.5.6. CONFOCAL LASER SCANNING MICROSCOPY

The micrographs of the cream (figure 5) highlight waxes present in the formulation. This could be due to the cetyl alcohol that was not spread homogeneously throughout the formulation. The small red dots on the micrograph of the Pheroid™ cream depicted the Pheroids™. Little or no waxes is visible, therefore it can be said that the Pheroid™ cream was more homogeneous than the cream. The gel appeared to be very homogeneous, no crystals could be seen (figure 7). Pheroids™ were depicted by the micrograph of the Pheroid™ gel (figure 8). The 40 °C sample of month three in this figure also depicted larger red dots, which indicates oil drops that formed with time at such high temperatures.

D.6. FIGURES

Figure 9: Colour chart 1

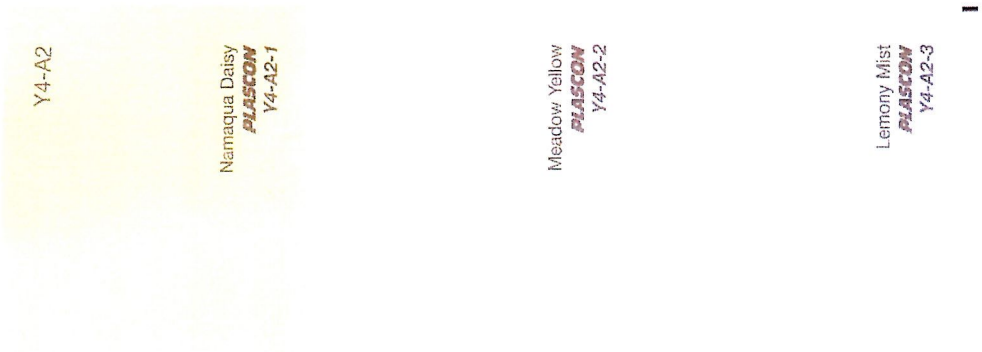

Figure 10: Colour chart 2

Figure 11: Colour chart 3

Figure 12: Colour chart 4

Figure 13: Colour chart 5

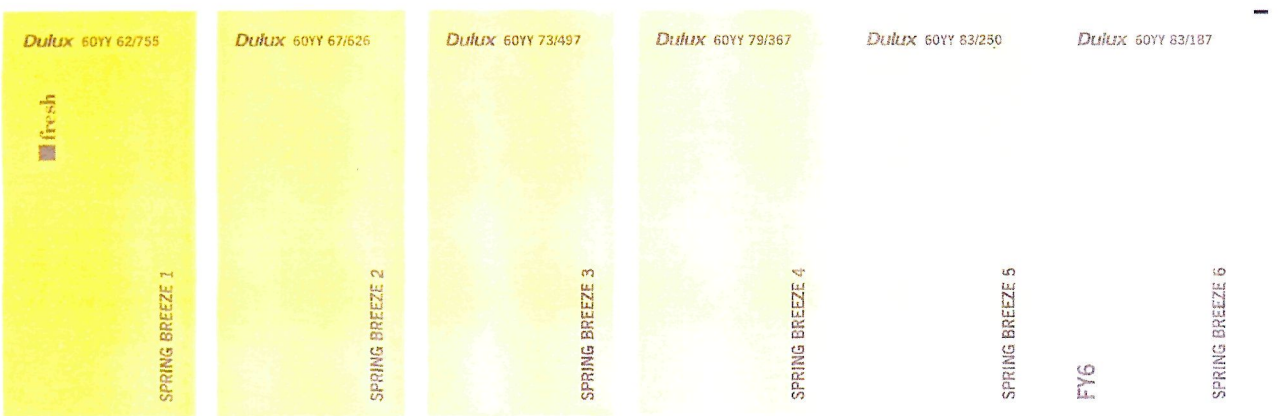

Figure 14: Colour chart 6

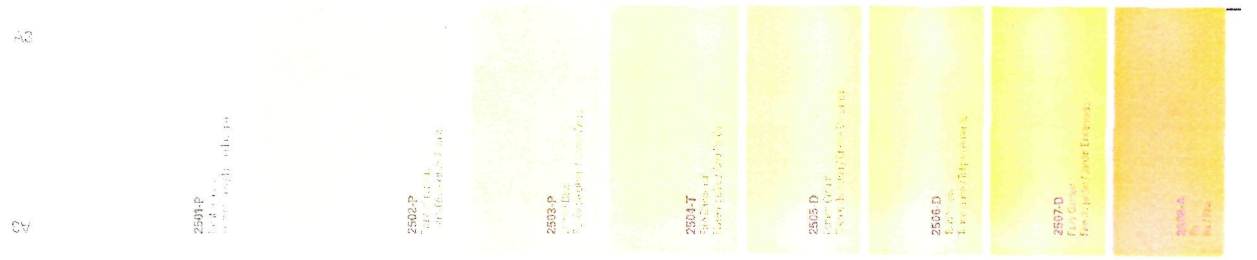

Figure 15: Colour chart 7

Figure 16: Colour chart 8

Figure 17: Colour chart 9

Figure 18: Colour chart 10

Figure 19: Colour chart 11

Figure 20: Colour chart 12

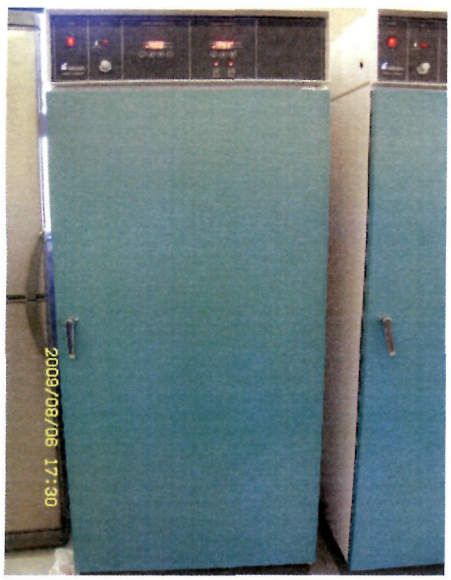

Figure 21: Labcon Humidity Chamber

Figure 22: Shimadzu AUW120D balance

Figure 23: Brookfield DV-II+ Programmable Viscometer

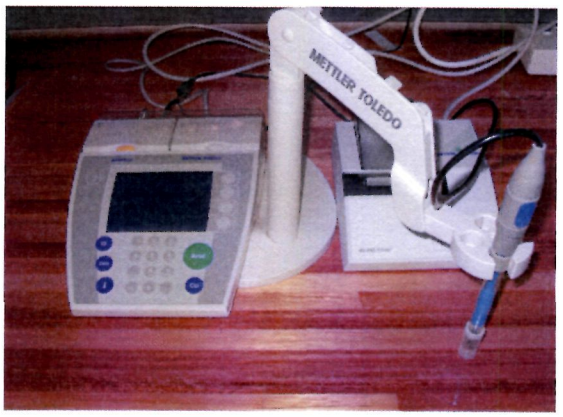

Figure 24: Mettler Toledo pH meter

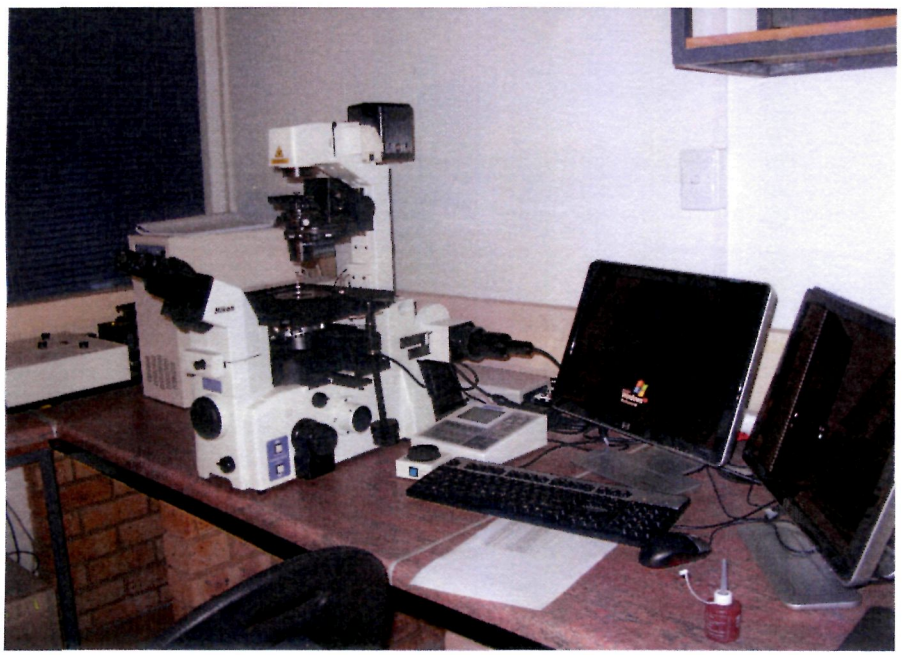

Figure 25: Nikon Digital Eclipse C1 si Confocal Laser Scanning Microscope

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APPENDIX E: TRANSDERMAL DIFFUSION STUDIES OF FORMULATIONS CONTAINING AZELAIC ACID AND NIACINAMIDE

E.1. INTRODUCTION

Diffusion studies (12 hours long in total) with vertical Franz cells were conducted with Caucasian female skin obtained after abdominoplastic surgery. Tape-stripping followed in order to establish the epidermis and dermis concentrations of azelaic acid and niacinamide.

E.2. MATERIALS AND METHODS

E..2.1. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: LC/MS - Agilent 1100 series HPLC with a binary gradient pump, auto sampler and vacuum degasser attached to an Applied Biosystems API 2000 triple quadruple mass spectrometer.

Column: Restek Ultra IBD 5 µm, 150 x 2.1 mm

Mobile phase: 0.1 % Formic acid/Acetonitrile

Gradient:

Time (min)	A (%)	B (%)
0.00	90	10
1.00	90	10
2.00	15	85
7.00	15	85
7.20	90	10
12.00	90	10

Where: A = 0.1% Formic acid

B = Acetonitrile (100%)

Flow rate: 250 µl/min

Injection volume:	5 µl
Retention time:	Niacinamide – 3.002 min
	Azelaic acid – 9.002 min
Runtime:	12.004 min
Solvent:	Acetonitrile (100%), gradient grade for HPLC.
Mass Spec.:	Niacinamide : +MRM (122.993)
	Azelaic acid : - MRM (186.960)

E.2.2. DIFFUSION STUDY AND TAPE STRIPPING

See page 50, from section 2.2.3.

E.3. RESULTS

See page 55, from section 3.2.

In the following box charts, the average percentages diffused, 12 h cumulative concentrations, epidermis concentrations and dermis concentrations of both niacinamide and azelaic acid are depicted.

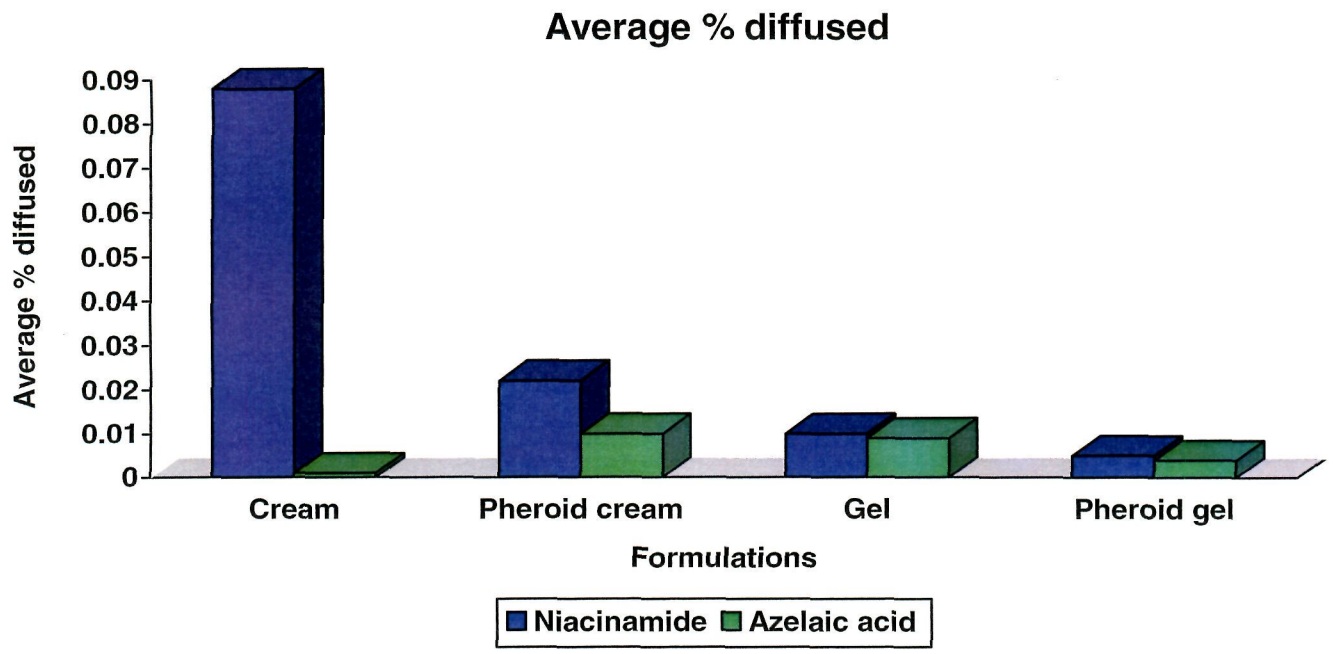

Figure 1: Box chart depicting average % diffused of niacinamide and azelaic acid from the four formulations.

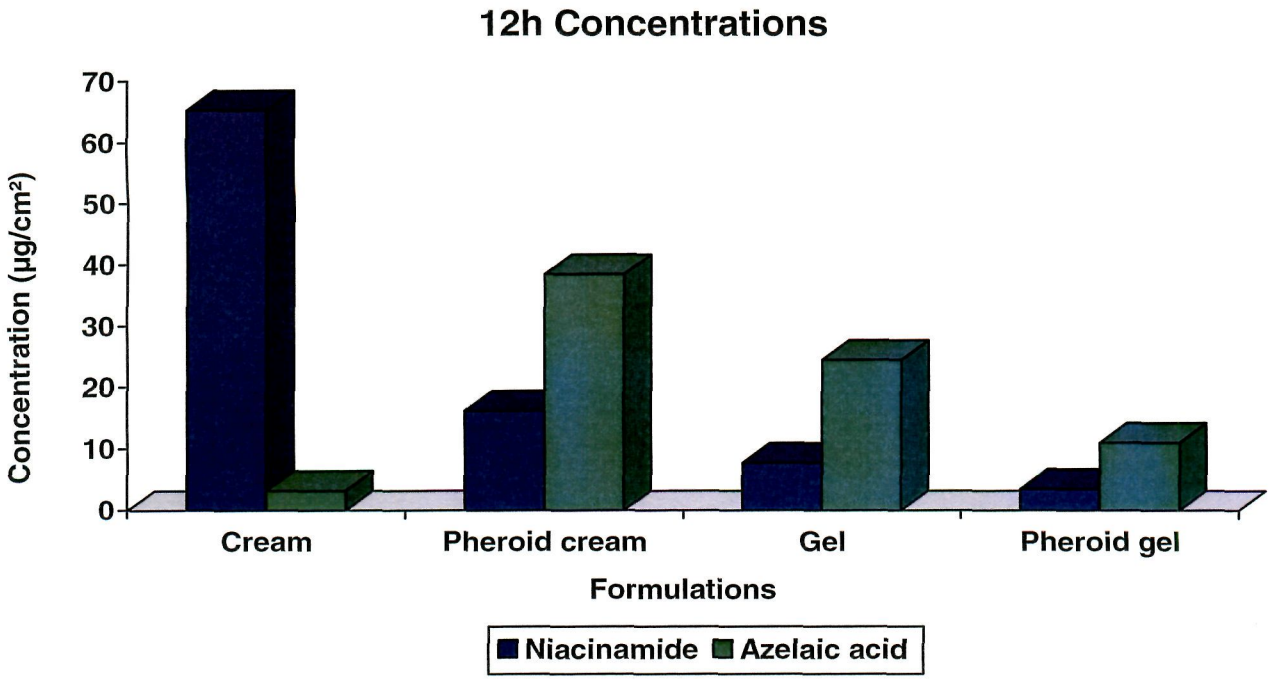

Figure 2: Box chart depicting 12h concentrations of niacinamide and azelaic acid in the receptor compartment of the Franz cell.

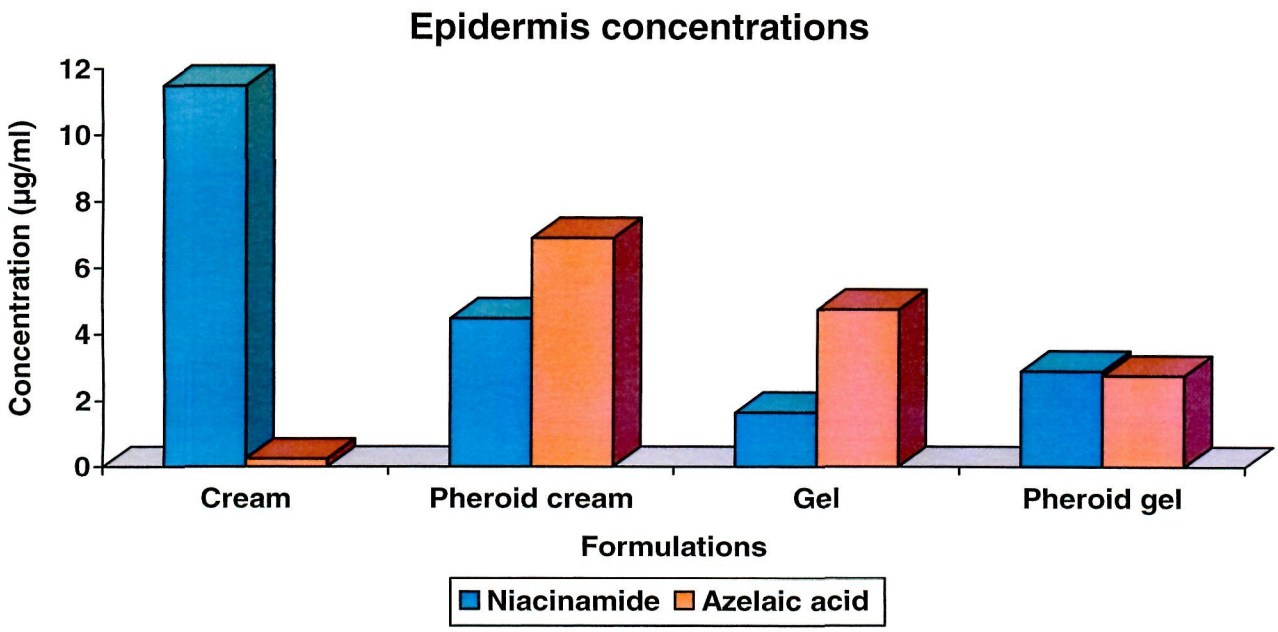

Figure 3: Box chart depicting epidermis concentrations of niacinamide and azelaic acid

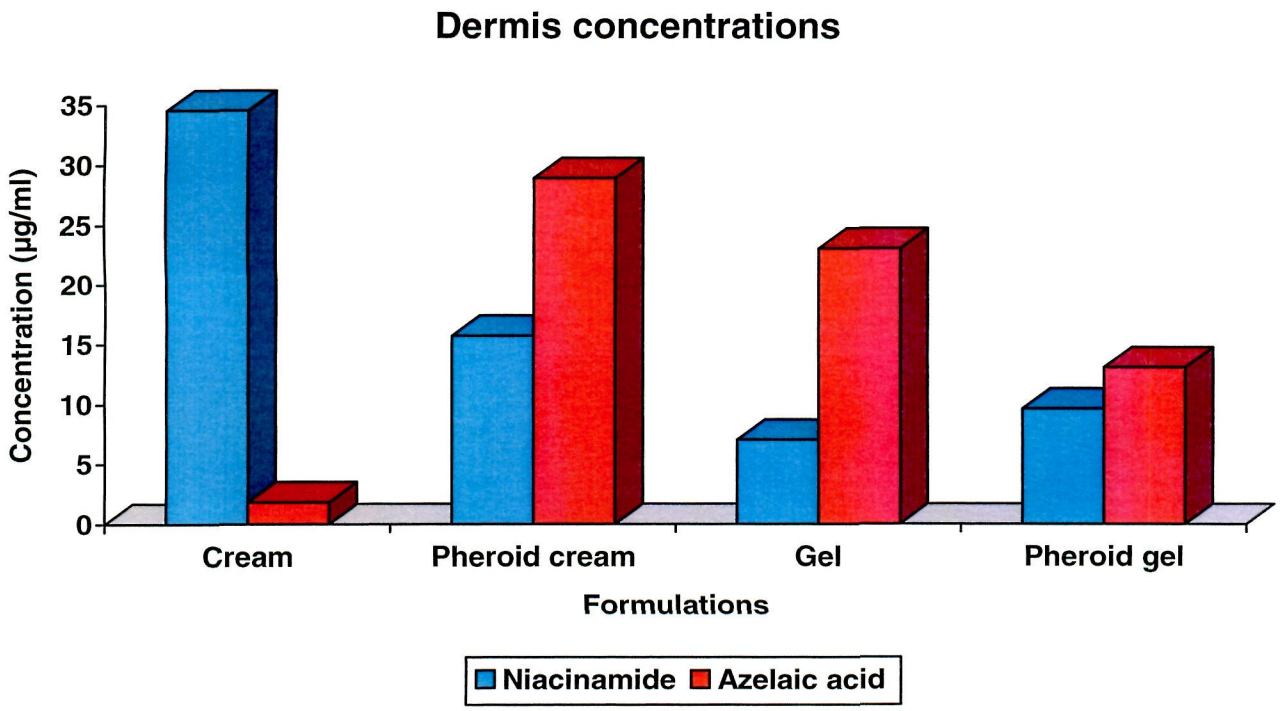

Figure 4: Box chart depicting dermis concentrations of niacinamide and azelaic acid

In the following box-plots, the median values of the epidermis and dermis are illustrated.

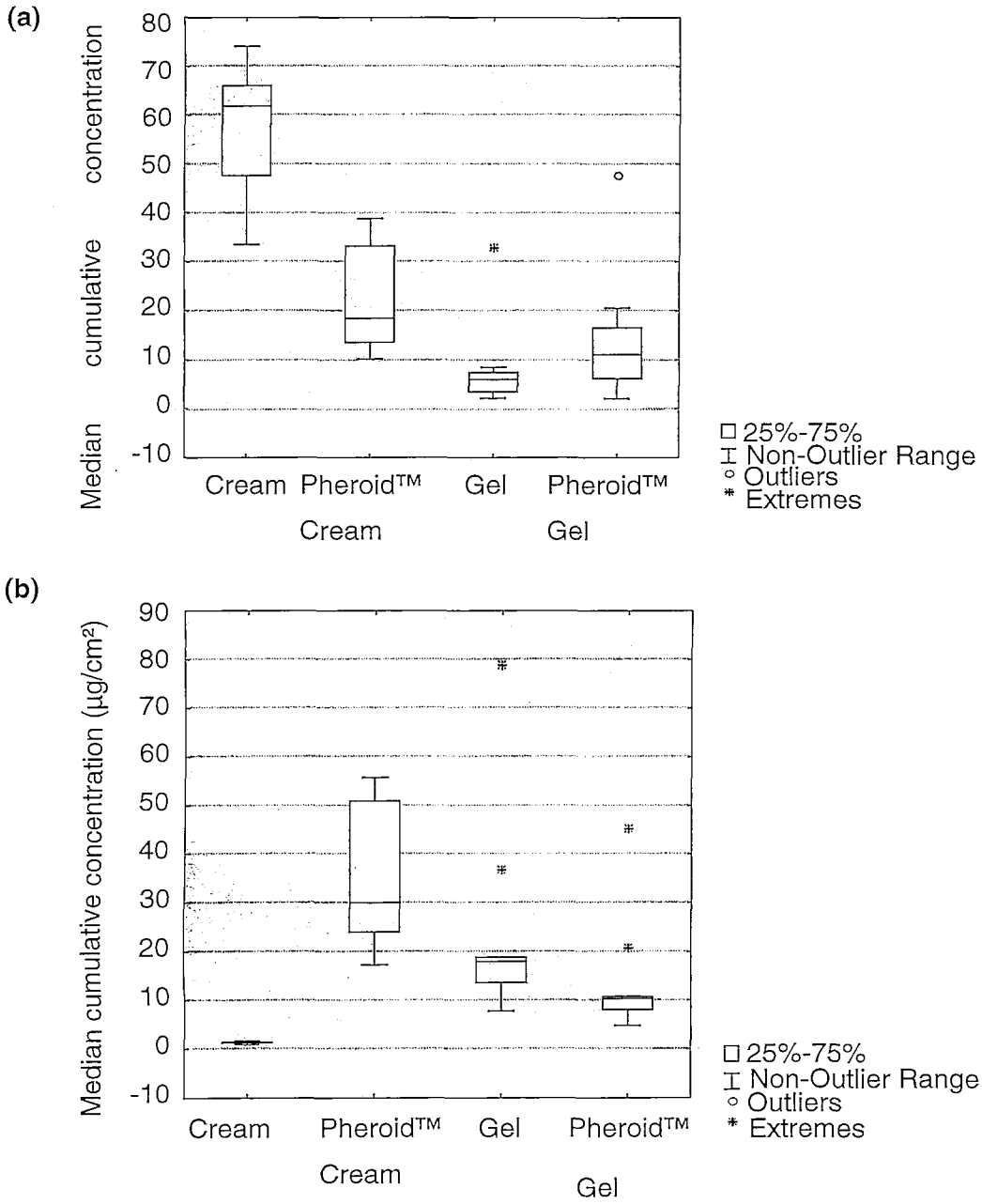

Figure 5: *Box-plots indicating median cumulative concentration (µg/cm²) of* **(a)** *niacinamide and* **(b)** azelaic acid present in the epidermis

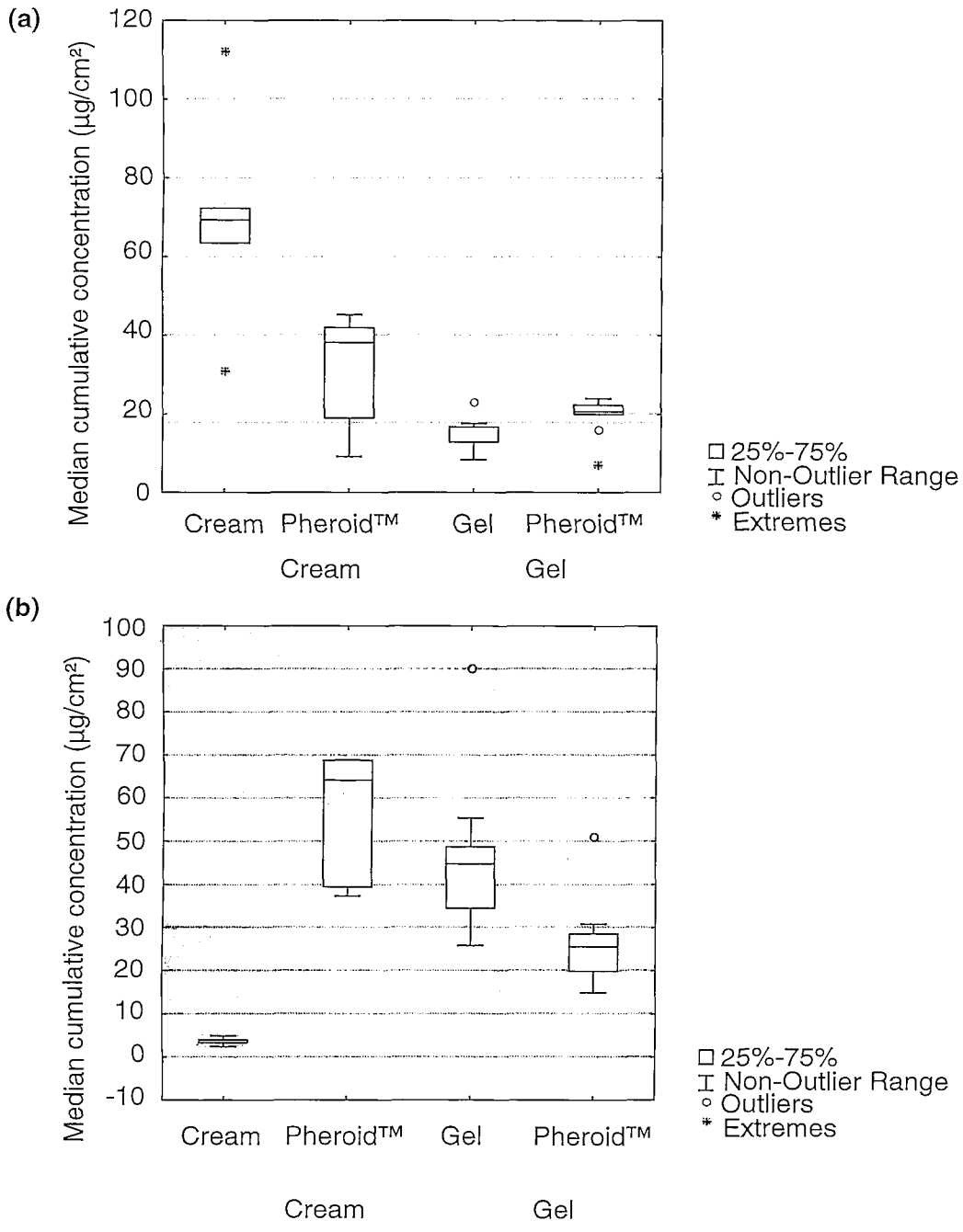

Figure 6: Box-plots indicating median cumulative concentration (µg/cm^2) of **(a)** niacinamide and **(b)** azelaic acid present in the dermis

E.4. FIGURES

The following figures show the equipment used during the diffusion studies.

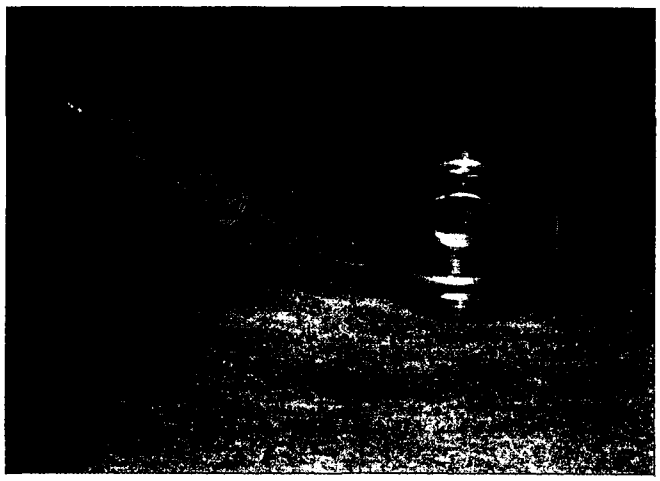

Figure 7: Donor- and Receptor compartment of vertical Franz diffusion cell

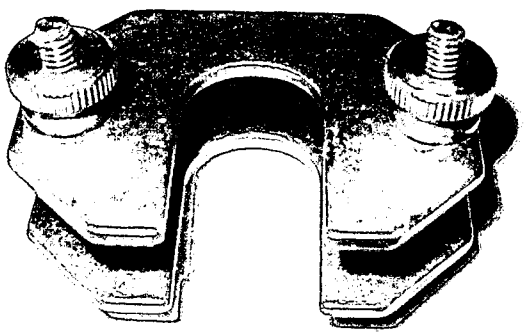

Figure 8: Horseshoe clamp

Figure 9: Grant water bath with Variomag® magnetic stirring plate and assembled Franz diffusion cells

Figure 10: Syringes used to withdraw PBS from receptor compartments and HPLC vials wherein samples were placed for analysis