Formulation, *in vitro* release and transdermal diffusion of selected retinoids

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Abstract

Acne is a multifactorial skin disease affecting about 80 % of people aged 11 to 30. Several systemic and topical treatments are used to treat existing lesions, prevent scarring and suppress the development of new lesions. Topical therapy is often used as first line treatment for acne, due to the location of the target organ, the pilosebaceous unit, in the skin. Retinoids are widely used as oral or topical treatment for this disease, with tretinoin and adapalene being two of the most used topical retinoids.

The transdermal route offers several challenges to drug delivery, e.g. the excellent resistance of the stratum corneum to diffusion, as well as variable skin properties such as site, age, race and disease. Some additional difficulties are associated with the dermatological delivery of tretinoin and adapalene, which include suboptimal water solubility of the retinoids, isomerisation of tretinoin in the skin, mild to severe skin irritation, as well as oxidation and photo-isomerisation of tretinoin, even before crossing the stratum corneum.

Researchers constantly strive to improve dermatological retinoid formulations in order to combat low dermal flux, skin irritation and instability. The release kinetics of tretinoin varies greatly according to the way in which it is incorporated into the formulation and according to the type of formulation used. Little research has been conducted regarding improved formulations for adapalene.

Pheroid™ technology is a patented delivery system employed in this study in order to improve the dermal delivery of retinoids. Tretinoin and adapalene were separately incorporated into castor oil, vitamin F and Pheroid™ creams. The creams were evaluated in terms of their *in vitro* retinoid release, *in vitro* transdermal diffusion and stability.

Castor oil and Pheroid™ creams were superior in terms of release and dermal delivery of adapalene. Tretinoin was best released and delivered to the dermis by castor oil cream. The castor oil creams were the most stable formulations, whereas the Pheroid™ creams were the most unstable. In terms of release, dermal diffusion and stability, castor oil cream proved to be the most suitable cream for both tretinoin and adapalene.

Keywords: Tretinoin, Adapalene, Dermal delivery, Pheroid™, Castor oil, Vitamin F, Stability

Opsomming

Aknee is ’n velsiekte met veelvoudige oorsake wat ongeveer 80 % van persone tussen die ouderdom van 11 en 30 affekteer. Verskeie sistemiese en topikale behandelings word gebruik om bestaande letsels te behandel, littekens te voorkom en die ontwikkeling van nuwe letsels te onderdruk. Topikale behandeling word dikwels as eerste linie gebruik om aknee te behandel weens die ligging van die teikenorgaan, die trigotalg-eenheid, in die vel. Retinoïede word algemeen as orale of topikale behandeling vir hierdie siekte gebruik. Tretinoïen en adapaleen is twee van die mees gebruikte topikale retinoïede.

Die transdermale weg stel verskeie uitdagings vir die aflewering van geneesmiddels, bv. die uitstekende weerstand wat die stratum corneum teen diffusie bied, asook veranderlike eienskappe van die vel soos plek, ouderdom, ras en siektetoestand. Bykomende probleme word met dermatologiese aflewering van tretinoïen en adapaleen in verband gebring en sluit die volgende in: onvoldoende wateroplosbaarheid van die retinoïede, isomerisasie van tretinoïen in die vel, matige tot erge velirritasie, en oksidering en foto-isomerisasie van tretinoïen, selfs voor dit die stratum corneum oorsteek.

Navorsers poog gedurig om dermatologiese retinoïed-formulerings te verbeter om sodoende lae dermale fluksie, velirritasie en onstabiliteit te bekamp. Die kinetika van vrystelling van tretinoïen wissel baie na gelang van die manier waarin dit in die formulering geïnkorporeer is, asook van die tipe formulering wat gebruik is. Min navorsing aangaande verbeterde formulerings vir adapaleen is al uitgevoer.

Pheroid™-tegnologie is ’n gepatenteerde afleweringsisteem wat in hierdie studie gebruik is om die dermale aflewering van retinoïede te verbeter. Tretinoïen en adapaleen is afsonderlik in kasterolie-, vitamien F- en Pheroid™-rome geïnkorporeer. Die rome is geëvalueer in terme van hul *in vitro* retinoïedvrystelling en transdermale diffusie, asook stabiliteit.

Die kasterolie- en Pheroid™-rome was beduidend beter in terme van vrystelling en dermale aflewering van adapaleen. Tretinoïen is die beste vrygestel en in die dermis afgelewer deur kasterolieroom. Die kasterolierome was die mees stabiele formulerings, terwyl die Pheroid™-rome die onstabielste was. In terme van vrystelling, dermale diffusie en stabiliteit was kasterolieroom die geskikste room vir beide tretinoïn en adapaleen.

Sleutelwoorde: Tretinoïen, Adapaleen, Dermale aflewering, Pheroid™, Kasterolie, Vitamien F, Stabiliteit

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From Him and through Him and for Him are all things. To Him be the glory forever! (Rom. 11:36)

Soli Deo Gloria!

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Chapter 1

Introduction and problem statement

Acne vulgaris is a common, chronic disease of the pilosebaceous unit that may result in physical and psychological scarring (Thiboutot *et al.*, 2009:S4). It is characterised by non-inflammatory comedones and inflammatory papules, pustules or nodules (Strauss *et al.*, 2007:652). These lesions are the result of sebaceous gland hyperproliferation and seborrhoea, hyperkeratinisation, follicular colonisation by *Propionibacterium acnes*, as well as inflammation and immune response (Gollnick *et al.*, 2003:S2).

Almost all cases of acne are curable with existing medication (Gollnick *et al.*, 2003:S35). Several systemic and topical acne treatments are used to resolve existing lesions, prevent scarring and suppress the development of new lesions. The success thereof depends on the use of the right medication as well as patient adherence (Thiboutot *et al.*, 2009:S39-40). Topical treatment is often used as first line therapy, due to the location of the pilosebaceous unit in the skin (Krautheim & Gollnick, 2004:398).

Retinoids have been used for nearly four decades as topical agents to treat acne (Bershad, 2001:154) and are the treatment of choice for comedonal acne, as well as for maintenance therapy (Thiboutot *et al.*, 2009:S7). Tretinoin is the prototype retinoid (Rigopoulos *et al.*, 2004:408) and acts by normalising keratinisation, draining existing comedones and preventing comedone formation (Njar, 2006:433). It is unstable in the presence of UV light and oxygen (Bershad, 2001:156) and frequently causes skin irritation (Czernielewski *et al.*, 2001:6).

Adapalene is a receptor-selective third generation retinoid (Bershad, 2001:157) with increased stability with respect to oxygen and light (Czernielewski *et al.*, 2001:6). Its efficacy is similar to that of other retinoids, but it is better tolerated and, therefore, has an improved therapeutic ratio (Njar *et al.*, 2006:435).

The release kinetics of tretinoin varies greatly according to the way in which it is incorporated into the formulation and according to the type of formulation used (Rebelo & Pina, 1997). Several formulation strategies have recently been developed in order to enhance the delivery of topical tretinoin or adapalene. Researchers aim at increasing the concentration of retinoids at the site of action, diminishing local and systemic side effects, and/or improving the stability of tretinoin in the formulation (Allec *et al*., 1997:S119). Little research has been conducted regarding improved formulations for adapalene. This may be due to adapalene's stability to oxidation and photodegradation, as well as its low irritation profile.

The skin as drug delivery route offers several challenges, e.g. the excellent resistance of the stratum corneum to diffusion (Naik *et al*., 2000:319) and variable skin properties such as site,

age, race and disease (Roberts *et al*., 2002:90; Walters & Roberts, 2002:4). Some additional difficulties are associated with the dermatological delivery of tretinoin and adapalene, which include suboptimal water solubility of the retinoids (Shah *et al.*, 2007:163), isomerisation of tretinoin in the skin (Shroot, 1998:S22; Bershad, 2001:156), mild to severe skin irritation (Sweetman, 2010), as well as oxidation and photo-isomerisation of tretinoin (Bershad, 2001:156), even before crossing the stratum corneum (Elbaum, 1988).

This study made use of Pheroid™ technology in an attempt to optimise the dermal delivery of tretinoin and adapalene. Pheroid™ technology is a novel patented delivery system that has successfully been used to enhance the delivery of active ingredients into the viable epidermis and dermis (Grobler *et al*., 2008:296). The unique and stable submicron and micron-sized structures, called Pheroids™, are typically formulated to have a diameter of between 200 nm and 2 µm. The matrix of essential fatty acids and nitrous oxide is functional in transporting hydrophobic and hydrophilic drugs (Grobler *et al*., 2008:284-285, 288-289).

The ultimate aim of this study was to develop a stable topical formulation for both tretinoin and adapalene with enhanced retinoid release and dermal delivery.

In order to achieve the main objective, the following aims had to be reached:

- Develop and validate HPLC methods to quantitatively determine tretinoin and adapalene concentrations in samples obtained during *in vitro* studies (appendix A);
- Develop and validate HPLC methods to determine the concentration of degradable components in the cream formulations (appendix B);
- Formulate castor oil, vitamin F and Pheroid™ creams with either tretinoin or adapalene as active ingredient (appendix C);
- Evaluate the stability of the cream formulations over a period of six months (appendix D);
- Determine the retinoid release profiles of the cream formulations (appendix E); and
- Determine the rate and extent of diffusion of tretinoin and adapalene into the skin by subjecting the cream formulations to *in vitro* transdermal diffusion studies (appendix E).

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Chapter 2

Dermal delivery of retinoids for the treatment of acne

2.1 Introduction

Acne is an extremely common skin condition which affects nearly 80% of people aged between 11 and 30 (Gollnick *et al.*, 2003:S2) and can result in physical and psychological scarring (Harper, 2004:S36). Several systemic and topical therapies exist for the treatment of acne. Topical treatment, however, is often used as first line therapy, because the target organ, the pilosebaceous unit, is located in the skin (Krautheim & Gollnick, 2004:398).

Retinoids form a class of drugs that have been used for nearly four decades as topical agents to treat acne (Bershad, 2001:154) and are currently used as the therapy of choice to treat mild to moderate acne. It is also preferred as maintainance therapy (Gollnick *et al.*, 2003:S5). In order to limit side effects, researchers have introduced receptor selective retinoids. Existing retinoid formulations have been improved and are still being developed to ensure enhanced dermatological delivery (Bershad, 2001:154).

This chapter will focus on the pilosebaceous unit as well as the pathogenesis and treatment of acne. Topical retinoids, particularly tretinoin and adapalene, will be investigated as drugs to treat acne. Dermatological delivery of tretinoin and adapalene, as well as methods to optimise their delivery, will also be discussed.

2.2 The skin

The human skin is an object of immense fascination and study. It is the largest and perhaps the most complex organ of the human body and contributes to more than 10% of the body's mass (Menon, 2002:S3-4; Walters & Roberts, 2002:1).

Figure 2.1 shows the basic structure of human skin. It is generally divided into two distinct layers, namely the dermis and epidermis (Menon, 2002:S4), although the subcutaneous fat layer and stratum corneum are sometimes regarded as a third and fourth layer (Walters & Roberts, 2002:1).

The dermis is 0.1 to 0.5 cm thick and makes up the largest part of the skin. It is composed of connective tissue elements and contains numerous pilosebaceous units, sweat glands, nerve endings and an extensive vascular network (Menon, 2002:S4; Walters & Roberts, 2002:11).

The epidermis is approximately 100 to 150 μm thick and consists mainly of keratinocytes. It can be subdivided into four layers, namely the stratum basale, stratum spinosum, stratum

granulosum and stratum corneum (Menon, 2002:S4-5). The nonviable stratum corneum as barrier to diffusion will be discussed in more detail in section 2.6.2.

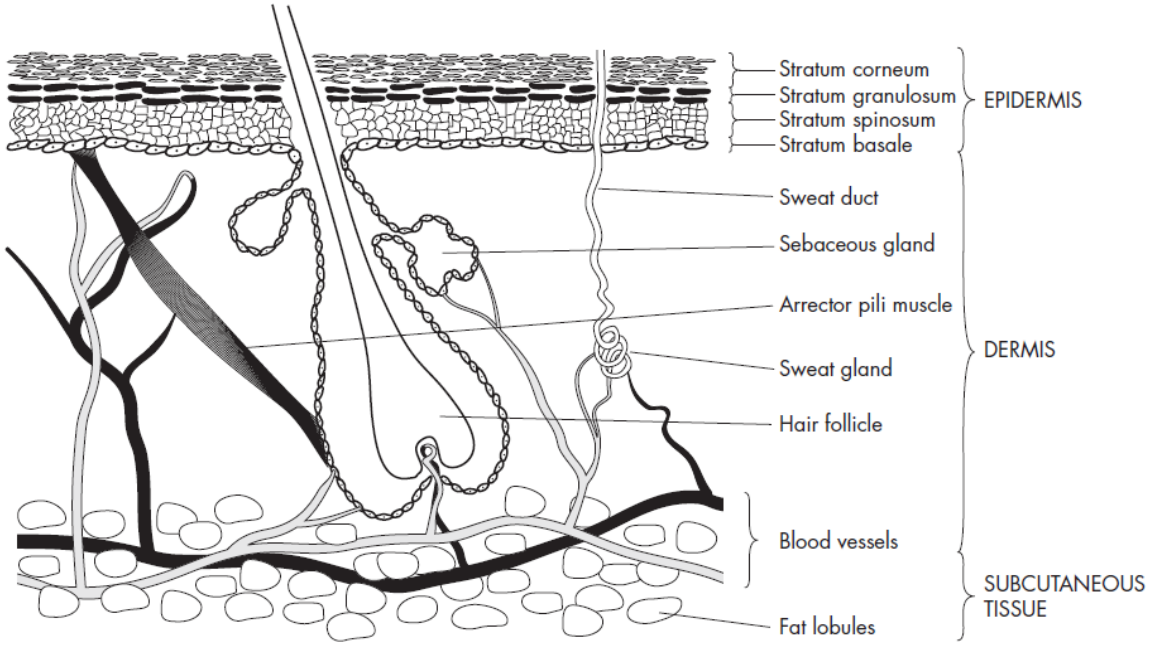


Figure 2.1: The structure of human skin (Williams, 2003:3)

2.3 The pilosebaceous unit

Consisting of a hair follicle, hair shaft, arrector pili muscle and sebaceous gland(s), the pilosebaceous unit (figure 2.2) is an incredibly complex appendage of the human skin (Wosicka & Cal, 2010:85). The pilosebaceous unit has recently sparked renewed interest as it provides important sites for drug targeting, acts as reservoir for localised therapy, plays a role as a transport pathway in systemic drug delivery, and provides opportunities for gene therapy and stem cell research (Wosicka & Cal, 2010:83; Patzelt *et al.*, 2008:e173).

The hair follicle not only manufactures hair, but has sensory, protective, excretory and psychosocial communicative functions as well. Furthermore, it has the unique ability to regenerate itself cyclically (Krause & Foitzik, 2006:2). The superficial infundibulum and isthmus form the permanent part of the hair follicle, whereas the inferior portion extends from the bulge region to the hair bulb to form the transient cycling component. Stem cells and skin mast cell precursors are abundant in the bulge region (Patzelt *et al.*, 2008:e173). The infundibulum plays a major role in drug penetration and acts as a reservoir and interface for interactions after topical application of compounds (Knorr *et al.*, 2009:174).

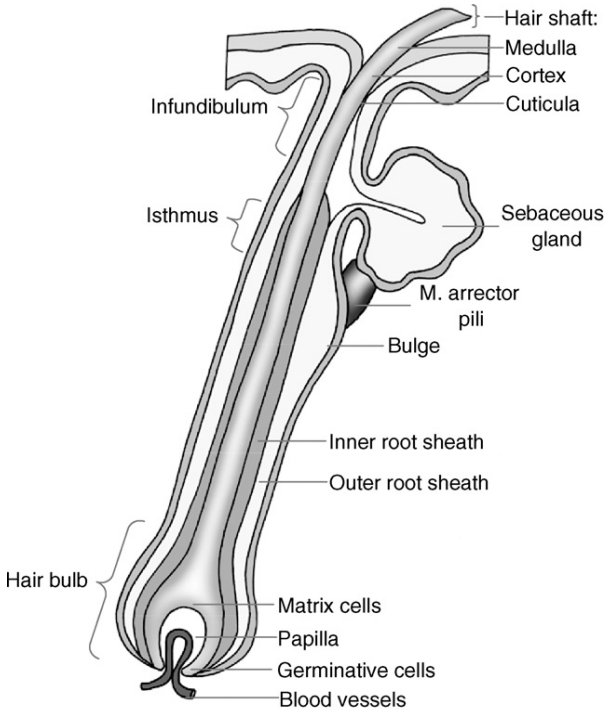


Figure 2.2: Structure of the pilosebaceous unit with the major subdivisions of the hair follicle (Patzelt *et al.*, 2008:e174)

Hair follicles are enveloped by three layers, namely an inner root sheath, an outer root sheath and an outermost acellular membrane (Meidan *et al.*, 2005:2). During a period of rapid growth (anagen), cell division takes place in the hair papilla with some cells differentiating to form the inner root sheath (Krause & Foitzik, 2006:3) which ends about halfway up the hair follicle (Meiden *et al.*, 2005:2). The outer root sheath is continuous with the epidermis and is composed of keratinised cells (keratinocytes) derived from epithelial stem cells in the bulge area (Meidan *et al*., 2005:2; Krause & Foitzik, 2006:3). Keratinocytes are normally shed as single cells into the lumen from where it is finally excreted (Gollnick *et al.*, 2003:S3).

Each hair follicle has one or more associated sebaceous gland which releases sebum into the infundibulum part of the follicular canal (Patzelt *et al.*, 2008:e174). Sebum is fungistatic and bacteriostatic (Petzelt *et al.*, 2008:e174). It consists of squalene, wax esters and triglycerides which are metabolised to free fatty acids by cutaneous bacterial lipases (Cunliffe *et al.*, 2004:367). Sebaceous lipids have several functions such as maintaining the integrity of the skin barrier, organising skin surface lipids in a three-dimensional structure, transporting antioxidants to the skin surface, and maintaining a skin surface pH of approximately 5 (Zouboulis, 2004:362; Walters & Roberts, 2002:12).

Sebocytes are cells of the sebaceous glands with three major functions, namely (1) differentiation, (2) proliferation and (3) sebum synthesis (sebogenesis) (Zouboulis, 2004:361). The functions of these cells are influenced by several factors and substances.

Hormones are the main regulatory compounds and act through complex endocrinologic mechanisms. Androgens stimulate cell proliferation and sebum production, whereas oestrogens inhibit sebaceous gland activity (Schneider & Paus, 2010:183). The response to androgens varies in different regions of the human body with facial sebocytes being more androgen sensitive (Zouboulis, 2004:362). Other factors that influence sebum production include circadian rhythm, skin hydration, pathology, drugs, age and, possibly, temperature (Meidan *et al.*, 2005:4).

2.4 Acne

Acne, also called acne vulgaris, is a multifactorial disease of the pilosebaceous unit that could result in inflammatory and non-inflammatory lesions (Dessinioti & Katsambas, 2010:2). It primarily affects the face, chest and back, because these areas contain the highest concentration pilosebaceous glands (Gollnick *et al.*, 2003:S2). According to Thiboutot *et al.* (2009:S4), acne should be recognised and investigated as a chronic disease with a psychological impact.

2.4.1 Pathophysiology

Four primary pathogenic factors interact to produce acne (figure 2.3). These factors are:

- Enlargement of the sebaceous gland together with increased sebum production (seborrhoea);
- Excessive growth and altered differentiation of follicular keratinocytes (hyperkeratinisation);
- Colonisation of the follicle by *Propionibacterium acnes*; and
- Inflammation and immune response (Gollnick *et al.*, 2003:S2; Thiboutot *et al.*, 2009:S5).

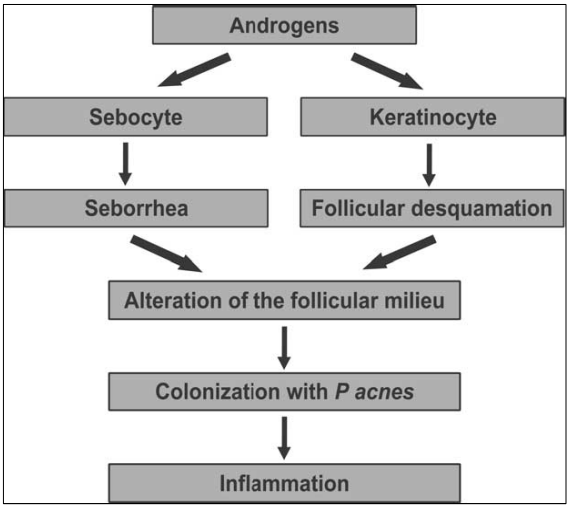


Figure 2.3: Pathogenic factors involved in the development of acne (Gollnick *et al.*, 2003:S2)

Changes in sebocytes are mainly influenced by androgens. It has been suggested that affected sebaceous glands might be hyper-responsive to androgens. This could explain the presence of acne in the majority of patients presenting with acne, as most of these patients do not show significant endocrine abnormalities. Keratinocyte hyperproliferation and differentiation may be influenced by sebum lipid composition, androgens and local cytokines (Gollnick *et al.*, 2003:S2-4).

When sebum and desquamated keratinocytes obstruct the pilosebaceous follicle, the primary acne lesion, known as the microcomedo, is formed (Krautheim & Gollnick, 2004:398). A microcomedo, which is invisible to the naked eye, may develop into an open or closed non-inflammatory comedo or into an inflamed acne lesion (Gollnick *et al.*, 2003:S2-3). In an open comedo, or "blackhead", the comedo's black tip is visible (figure 2.4 (a)). The black tip is composed of oxidised sebum and melanin. A closed comedo, also called a "whitehead", is flesh-coloured or white, and has no visible pore (Webster, 2001:15; McCoy, 2008). It is possible for non-inflammatory comedones to resolve without treatment (Gollnick *et al.*, 2003:S4).

a) **b)**

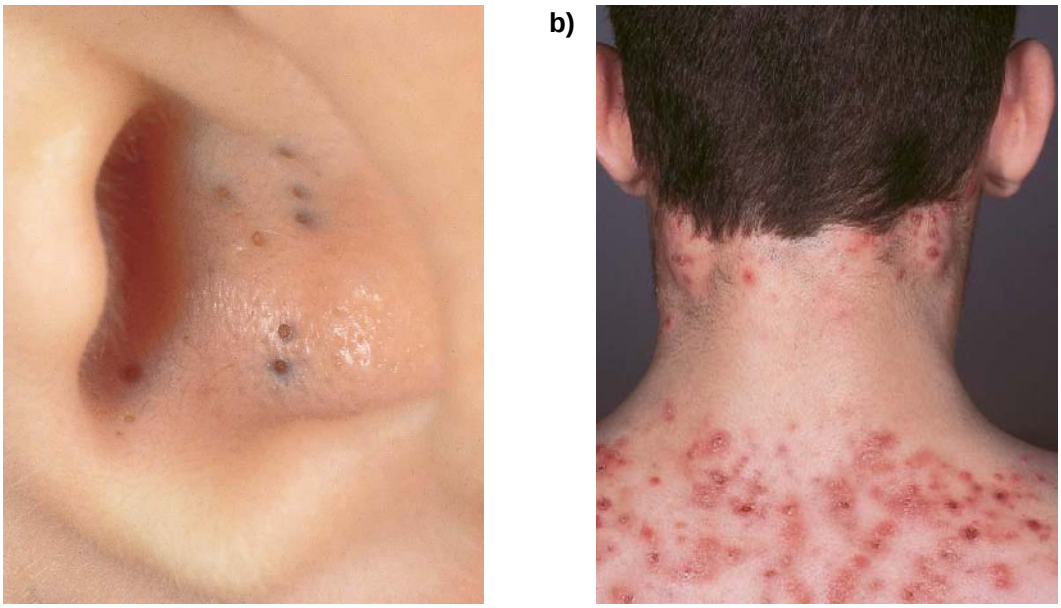


Figure 2.4: Different types of acne lesions: **(a)** non-inflammatory open comedones inside the ear; and **(b)** severe inflammatory acne on the neck and upper back (Shaw & Kennedy, 2007:386)

Inflammatory acne (figure 2.4 (b)) can present in the form of papules, pustules, nodules or cysts. Different types of acne lesions may be present simultaneously. Papules and pustules are formed when the follicular epithelium becomes damaged and neutrophils and lymphocytes accumulate. The epithelium raptures and triggers an acute inflammatory reaction in the dermis (McCoy, 2008).

Pustules are superficial lesions, whereas papules are formed by relatively deep inflammation. Nodules are larger and deeper than papules and have a more solid structure. Cysts, on the other hand, are purulent nodules and may develop into abscesses or scars (McCoy, 2008).

Acne conglobata is acne in its most severe form and affects men more than women. Patients with this form of acne have abscesses, draining sinuses and scars. It affects the back, chest and even the arms, abdomen, buttocks and scalp (McCoy, 2008).

Propionibacterium acnes (*P. acnes*) was first suggested as the cause of acne in 1896 (Dessinioti & Katsambas, 2010:2). However, its precise mechanism in the pathogenesis of acne remains controversial. *P. acnes* is an anaerobic gram-positive bacterium resident on healthy human skin (Long, 2002:49). Lipase produced by this bacterium metabolises triglycerides in the infundibulum to free fatty acids which irritates the follicular wall (Gollnick *et al.*, 2003:S6). Colonisation of the pilosebaceous follicle by *P. acnes* leads to disruption of the follicular epithelium with resulting inflammation (Dessinioti & Katsambas, 2010:3). Recent research has suggested multiple mechanisms of action by which *P. acnes* might trigger inflammation (Dessinioti & Katsambas, 2010:3-5). Evidence has shown that inflammation might occur even before hyperkeratinisation (Thiboutot *et al.*, 2009:S5-6). It is important to note that acne is currently regarded as an inflammatory disorder of the pilosebaceous unit, rather than a hyperproliferative and keratinocyte disease (Dessinioti & Katsambas, 2010:5).

2.4.2 Treatments

Almost all cases of acne are curable with existing medication (Gollnick *et al.*, 2003:S35). Acne treatment aims to resolve existing lesions, prevent scarring and suppress the development of new lesions. The success thereof depends on the use of the right medication as well as patient adherence (Thiboutot *et al.*, 2009:S39-40).

When treating acne, it is important to target several pathogenic factors simultaneously. Combination therapy is thus recommended for both inflammatory and non-inflammatory acne (Thiboutot *et al.*, 2009:S6). Figure 2.5 illustrates the way in which different anti-acne agents affect the four major acne causing factors. These agents normalise hyperkeratinisation, inhibit excessive seborrhoea, suppress *P. acnes* and relieve inflammation.

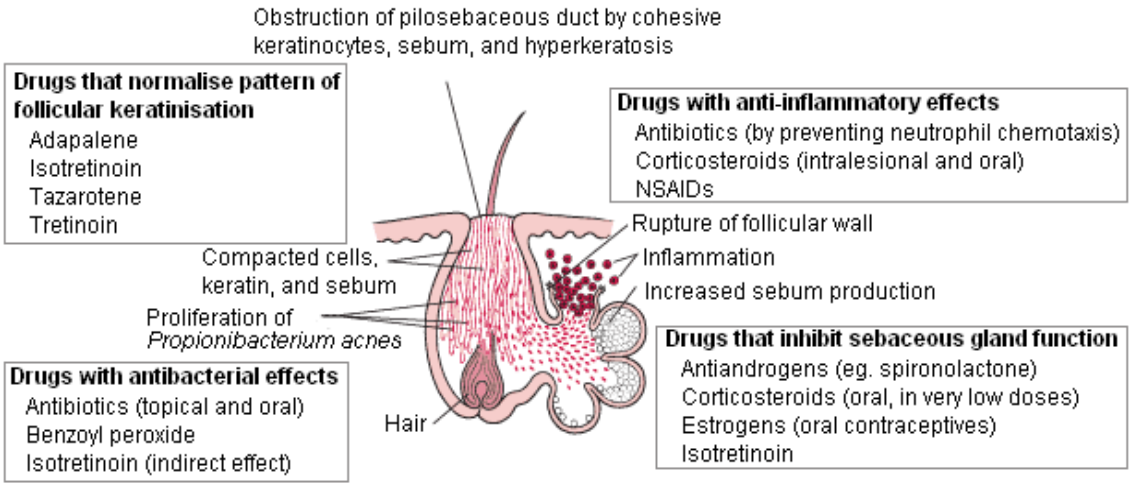


Figure 2.5: Mechanisms of action of different drugs used for the treatment of acne (McCoy, 2008)

Table 2.1 presents guidelines for treating acne. Note that maintenance therapy is important in acne management and is thus included in the guidelines. The different systemic and topical treatment options will be discussed in sections 2.4.2.1 and 2.4.2.2.

Table 2.1: Acne treatment algorithm suggested by the Global Alliance to Improve Outcomes in Acne (Thiboutot *et al.*, 2009:S7) (BPO = Benzoyl peroxide)

Acne Severity	MILD		MODERATE		SEVERE
	Comedonal	Mixed and Papular/pustular	Mixed and Papular/pustular	Nodular (2)	Nodular/Conglobate
1st Choice	Topical Retinoid	Topical Retinoid + Topical Antimicrobial	Oral Antibiotic + Topical Retinoid +/- BPO	Oral Antibiotic + Topical Retinoid + BPO	Oral Isotretinoin (3)
Alternatives (1)	Alt. Topical Retinoid or Azelaic acid or Salicylic acid	Alt. Topical Retinoid Antimicrobial Agent + Alt. Topical Retinoid or Azelaic acid	Alt. Oral Antibiotic + Alt. Topical Retinoid +/- BPO	Oral Isotretinoin or Alt. Oral Antibiotic +Alt. Topical Retinoid +/- BPO/Azelaic acid	High dose Oral Antibiotic + Topical Retinoid + BPO
Alternatives for females (1,4)	See 1st Choice	See 1st Choice	Oral Antiandrogen (3) +Topical Retinoid/ Azelaic acid +/- Topical Antimicrobial	Oral Antiandrogen (3) + Topical Retinoid/ +/- Oral Antibiotic +/- Alt. Antimicrobial	High Dose Oral Antiandrogen (3) + Topical Retinoid +/- Alt. Topical Antimicrobial
Maintenance Therapy	Topical Retinoid			Topical Retinoid +/- BPO	

1. Consider physical removal of comedones. 2. With small nodules (<0.5 cm). 3. Second course in case of relapse. 4. For pregnancy, options are limited.

2.4.2.1 Topical treatments

- **Retinoids**

Topical retinoids are anticomedogenic and comedolytic, thereby reducing the formation of microcomedones and comedones. They have some direct and indirect anti-inflammatory effects. The penetration of other topical compounds may be enhanced, because retinoids normalise desquamation (Thiboutot *et al.*, 2009:S11).

As shown in table 2.1, topical retinoids are the treatment of choice for comedonal acne as well as for maintenance therapy. Topical tretinoin, isotretinoin and adapalene are currently available in South Africa. In some countries, topical tazarotene, motretinide, retinaldehyde and β-retinoyl glucuronide are used to treat acne (Gollnick *et al.*, 2003:S6). This study focused on tretinoin and adapalene. Therefore, these two topical retinoids are discussed in more detail in sections 2.5.4 and 2.5.5

- **Antibiotics**

Clindamycin and erythromycin are the topical antibiotics most frequently used for treating inflammatory acne. They not only suppress *P. acnes*, but also have an indirect effect on comedones and are anti-inflammatory (Gollnick *et al.*, 2003:S16).

Topical antibiotics act slower and are generally less effective than oral antibiotics. Due to the possibility of bacterial resistance, they should not be used as monotherapy (Gollnick *et al.*, 2003:S16). They may be used in mild to moderate acne when combined with benzoyl peroxide or a topical retinoid (Thiboutot *et al.*, 2009: S7).

- **Benzoyl peroxide**

Gollnick *et al.* (2003:S16-17) described benzoyl peroxide as a safe, effective and powerful antimicrobial that could destroy bacterial organisms and yeasts. When compared to topical antibiotics, it produces a more significant and faster effect in suppressing *P. acnes*. It has an indirect effect on comedogenesis by reducing the number of *P. acnes*. When used in combination with an antibiotic, it reduces the possibility of bacterial resistance. To date, no evidence of microbial resistance to benzoyl peroxide has been found. It is preferably used in conjunction with a topical antibiotic or topical retinoid for treating mild to moderate acne (Gollnick *et al.*, 2003:S17) and with a topical retinoid for maintenance therapy, if additional antimicrobial action is needed (Thiboutot *et al.*, 2009:S26).

- **Azelaic acid**

Azelaic acid has an effect on *P. acnes*, although the degree thereof is a matter of dispute. It is mildly comedolytic and anti-inflammatory. It is indicated for mild comedonal and papulopustular acne. Post-inflammatory hyperpigmentation caused by acne may be reduced by topical application of azelaic acid (Gollnick *et al.*, 2003:S15-16).

2.4.2.2 Systemic treatments

- **Oral isotretinoin**

The Global Alliance to Improve Outcomes in Acne regards oral isotretinoin as the mainstay for treating severe acne. This retinoid is also used to treat moderate to severe acne unresponsive to topical therapy. It targets all pathogenic factors by decreasing the size and secretion of sebaceous glands, normalising follicular keratinisation, preventing the formation of new comedones, altering the follicular milieu to indirectly inhibit *P. acnes* growth, and by exerting an anti-inflammatory effect. Isotretinoin is normally used as monotherapy (Gollnick *et al.*, 2003:S26).

Patient counselling is critical due to the possibility of significant side effects. Dryness of the skin and mucous membranes is a common side effect. Other possible side effects include muscle aches, headaches, nosebleeds, skin fragility and psychiatric events, e.g. mood changes and depression. Isotretinoin is a potent teratogen (Gollnick *et al.*, 2003:S26-28).

- **Oral antibiotics**

As shown in figure 2.5, systemic antibiotics reduce the number of *P. acnes* and have anti-inflammatory actions. Their indirect effect on comedogenesis is increased when antibiotics are combined with zinc or benzoyl peroxide. Oral antibiotics are primarily indicated for moderate to moderately severe acne. Preferred antibiotics are tetracyclines and derivatives, macrolides, co-trimoxazole, and trimethoprim (Gollnick *et al.*, 2003:S15-16).

The frequency and duration of antibiotic use must be limited in order to prevent antibiotic resistance. It must not be used as monotherapy for acute treatment or maintenance therapy, but must be combined with either a topical retinoid or benzoyl peroxide. Simultaneous use of topical and systemic antibiotics should be avoided (Thiboutot *et al.*, 2009:S7-9).

- **Hormonal therapy**

Hormonal therapy counters the effects of androgens on sebaceous glands and probably in follicular keratinocytes as well. It is an excellent option for women, especially when oral contraception is desirable. Hormonal therapy is sometimes used as alternative to repeated courses of isotretinoin. According to the regimen suggested in table 2.1, hormonal therapy is indicated for moderate to severe acne. Hormonal agents used include estrogens, anti-androgens, oral contraceptives, glucocorticoids, gonadotropin-releasing hormone agonists and 5α-reductase inhibitors. The different hormonal agents decrease sebum production by suppressing ovarian or adrenal androgen production, blocking androgen receptors, or inhibiting androgen metabolism (Gollnick *et al.*, 2003:S18-24).

2.4.2.3 Adjunctive drug treatments

Several drugs may provide relief from acne, although these drugs are not part of the treatment algorithm shown in table 2.1. These agents are used as alternative or complementary treatment.

Zinc taken orally has shown effectiveness against non-inflammatory acne lesions, but does not affect comedones. It may be used as alternative to tetracyclines (Gollnick *et al.*, 2003:S30).

A short course of oral corticosteroids will immediately reduce the amount of inflammatory lesions. Intralesional injections may be helpful for large inflammatory lesions, but may cause atrophic scars. Topical corticosteroids may be used for a short time period (Gollnick *et al.*, 2003:S32).

2.4.2.4 Non-drug treatments

- **Acne surgery**

Acne surgery provides immediate improvement and can be used to manage comedonal acne. Techniques used to remove the comedo include extraction, light cautery and laser puncture. Macrocomedos not responding to oral or topical retinoids may benefit from acne surgery (Gollnick *et al.*, 2003:S31-32).

- **Chemical peels**

Light chemical peels may be used after acne is brought under control. Peeling agents used include alpha-hydroxy acids, salicylic acid and trichloroacetic acid (Gollnick *et al.*, 2003:S32).

- **Photodynamic therapy**

Light-based treatments aim at reducing *P. acnes* levels and disrupting sebaceous gland function. Narrowband light therapies primarily target inflammatory lesions. Although evidence is limited, laser and light therapies are promising, whether alone or in combination with photosensitisers (Thiboutot *et al.*, 2009:S18, 24).

2.4.3 Patient education

Acne is surrounded by several myths that may influence a patient's perception of the disease. It is, therefore, important to inform and educate the patient. Patients should know that acne is not infectious, nor related to diet or a result of poor hygiene. They should know how to care for their skin and how to use their medication. It is important to inform the patient that therapy requires time, usually four to six weeks. Patients need to be reassured that, if their acne appears to worsen in the early weeks, it is due to the medication working on previously unseen lesions (Gollnick *et al.*, 2003:S30, S35-36).

2.5 Retinoids

2.5.1 Background

In 1925 the importance of vitamin A in normal skin was first recognised. Since then, research on vitamin A and its analogues has grown immensely and is still expanding. Vitamin A taken orally as treatment for acne was studied for the first time in 1943. During the 1960's, research was conducted on the use of oral retinol (vitamin A) and topical all-*trans*-retinoic acid (tretinoin) for the treatment of hyperproliferative disorders like psoriasis, ichthyoses and epithelial tumours (Bershad, 2001:154).

Topical tretinoin became commercially available for treating acne in the early 1970's. During this time, it was, falsely, believed that retinoids must cause cutaneous irritation in order to be effective. The rest of the 1970's and the 1980's saw the discovery of numerous naturally occurring and synthetic vitamin A analogues, although tretinoin remained the only FDA approved retinoid for topical use during that time. Since topical tretinoin was first utilised to treat acne, thousands of retinoids have been discovered. Tretinoin served, and still serves, as parent compound to new molecules (Bershad, 2001:154-155).

Retinoids are divided into three generations. Tretinoin and its *cis*-isomers, e.g. isotretinoin (13-*cis*-retinoic acid) and alitretinoin (9-*cis*-retinoic acid), are part of the first generation. The second generation retinoids are monoaromatic isomers of tretinoin and include etretinate and acetretin. Members of the third generation are polyaromatic isomers, also called arotinoids, and include adapalene and tazarotene (Bershad, 2001:155).

2.5.2 *In vivo* functions

There are at least a dozen endogenous retinoids, e.g. retinol, tretinoin, isotretinoin and alitretinoin. Carotene pigments of plant origin and retinyl esters obtained from animals are converted *in vivo* to retinol. Retinol (vitamin A) is then metabolised to retinaldehyde (vitamin A aldehyde) or retinoic acid (vitamin A acid) (Bershad, 2001:154).

Retinoids are nonsteroidal small-molecule hormones with essential functions in vision, embryonic development, brain function, reproduction and epidermal integrity. They act by regulating organogenesis, organ homeostasis, cell growth, differentiation and apoptosis (Njar *et al.*, 2006:431; Bershad, 2001:154).

2.5.3 Mechanism of action

Retinoids may bind to two types of receptors (figure 2.6). The first is the cellular retinoic acid binding receptor (CRABR or CRABP), a binding protein present in the cytoplasm. Binding to this receptor may facilitate regulation of the amount of intracellular retinoic acid or may function as part of the retinoic acid feedback mechanism. It is, however, not necessary for a retinoid to bind

to CRABR in order to produce a biological retinoid effect such keratinocyte differentiation (Rigopoulos *et al.*, 2004:408).

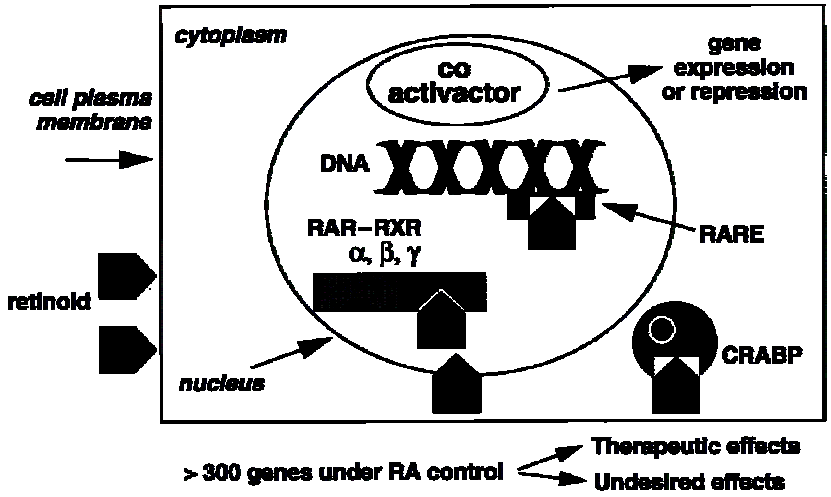


Figure 2.6: Simplified illustration of the interaction of retinoids with receptors to regulate gene transcription. RA = retinoic acid (Shroot, 1998:S23)

The second type of retinoid receptors consists of two structurally distinct families of nuclear receptors, namely the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Bershad, 2001:155). Both families are subdivided into α, β and γ subtypes, with each subtype having a different affinity for retinoids and a distinctive distribution in tissue. RAR-α and RAR-γ are located mainly in the epidermis and RAR-β in the dermis (Rigopoulos *et al*., 2004:360). Tretinoin and alitretinoin are the natural ligands for RARs and RXRs, respectively, although alitretinoin demonstrates a high affinity for RARs as well (Njar *et al.*, 2006:431).

Nuclear receptors in keratinocytes always exist as dimers. RARs are at all times linked with RXRs (RAR-RXR). RXRs may exist as homodimers (RXR-RXR) or may form heterodimers with other small-hormone receptors, including receptors for thyroid hormone, corticosteroids, oestradiol and vitamin D3 (Bershad, 2001:155-156). The RAR site of RAR-RXR heterodimers acts as the binding site for retinoids, whereas its RXR partner allosterically increases the potency of the RAR unit (Njar *et al.*, 2006:431).

In the absence of a retinoid, RAR-RXR heteromers bind to retinoic acid response elements (RAREs) which are DNA regions in the promoter area of target genes (Njar *et al.*, 2006:431). When a retinoid binds to the RARE-bound receptor dimer, it activates the receptor, leading to a direct induction of gene transcription. This process, called RAR-RXR transactivation, results in the generation of protein products necessary for keratinocyte differentiation (Bershad, 2001:156). As indicated in figure 2.6, more than 300 genes are activated by retinoic acid (tretinoin) which may lead not only to therapeutic effects, but also to unwanted side effects.

Retinoids have additional indirect effects on gene transcription that may explain their role in suppressing inflammation and modulating cellular proliferation (Bershad, 2001:156).

2.5.4 Tretinoin

Tretinoin, the prototype retinoid, is an active metabolic product of vitamin A (Rigopoulos *et al.*, 2004:408). It has a biological activity hundreds of times greater than that of vitamin A (Bershad, 2001:155).

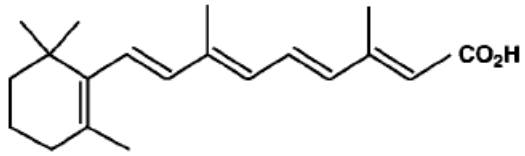
2.5.4.1 Physicochemical properties

Tretinoin consists structurally of three distinct parts (table 2.2), namely (1) a cyclic end group, (2) a polyene side chain and (3) a polar end group (Bershad, 2001:155). The polyene side chain is conformationally flexible due to its array of double bonds. This property allows tretinoin to be an agonist of all three RAR subtypes. A disadvantage of the flexible side chain is its susceptibility to photoisomerisation by UV light, as well as to oxidation. Tretinoin's instability in light makes it unsuitable for use during the day (Bershad, 2001:156). When used in conjunction with benzoyl peroxide, inactivation due to oxidation can be prevented by applying benzoyl peroxide in the morning and tretinoin in the evening (Gollnick *et al.*, 2003:S17).

Special precautions must be applied when working with tretinoin. All actions involving tretinoin should be carried out as rapidly as possible while avoiding actinic light. Low-actinic glassware should be used and solutions should be freshly prepared. Tretinoin should be stored in an airtight container at a temperature less than 25 °C and protected from light. After opening a container, the tretinoin must be used as soon as possible and the remaining part stored under an inert gas (British Pharmacopoeia, 2010; US Pharmaopoeial Convention, 2010).

A synopsis of the physical and chemical properties of tretinoin is provided in table 2.2.

Table 2.2: Physicochemical properties of tretinoin (Sweetman, 2010; British Pharmacopoeia, 2010; Shroot, 1998:S18; Syracuse Research Corporation, 2010; Brisaert *et al.*, 2001:913)

Synonyms	Retinoic acid; vitamin A acid; NSC-122758
Chemical names	All-*trans*-retinoic acid; 15-apo-β-caroten-15-oic acid; 3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)-nona-2,4,6,8-*all-trans*-tetraenoic acid
Molecular formula	$C_{20}H_{28}O_2$
Molecular structure	
Molecular weight	300.4 g/mol
CAS number	302-79-4
Appearance	Yellow or light orange crystalline powder
Solubility	Practically insoluble in water (0.126 mg/L); soluble in methylene chloride; slightly soluble in ethanol
Octanol-water partition coefficient (log P)	6.30
Dissociation constant (pK_a)	6
Melting point	About 182 °C (with decomposition)
Stability	Sensitive to air, heat and light, especially in solution; Unstable in the presence of benzoyl peroxide

2.5.4.2 Pharmacokinetics

Oral tretinoin is well absorbed from the gastrointestinal tract with a bioavailability of approximately 50 %. Peak plasma concentrations are obtained after a period of one to two hours. The terminal elimination half-life is half an hour to two hours. When metabolised in the liver by the cytochrome P450 isoenzyme system, metabolites such as isotretinoin, 4-oxo-*trans*-retinoic acid and 4-oxo-*cis*-retinoic acid are formed. Topical tretinoin results in minimal systemic absorption (Sweetman, 2010) and it may be isomerised in the skin to isotretinoin (Bershad, 2001:156).

2.5.4.3 Indications

Apart from being used to treat acne, topical tretinoin is currently used to treat psoriasis, ichthyosis and photodamaged skin (Njar *et al.*, 2006:433). Paquette *et al.* (2001:382) reported the healing properties of topical tretinoin on full-thickness skin wounds and chronic ulcers.

Tretinoin taken orally is used to treat acute promyelocytic leukaemia with more than 90% of patients achieving complete remission with this treatment (Njar *et al.*, 2006:433).

2.5.4.4 Action against acne

Tretinoin is used as a topical anti-acne drug to treat non-inflammatory comedones (as monotherapy) and mild, moderate and severe inflammatory acne (in combination with topical or systemic agents). It is furthermore used as maintenance therapy (alone or in combination with benzoyl peroxide). Tretinoin normalises keratinisation by increasing differentiation of follicular epithelial cells and by quickening the shedding of keratinocytes. This leads to drainage of comedones and prevents the formation of new comedones (Njar *et al.*, 2006:433).

2.5.4.5 Adverse effects

Topical tretinoin may lead to skin irritation characterised by stinging, erythema, dryness and peeling. In sensitive patients it may cause oedema, blistering and crusting (Sweetman, 2010). Other adverse reactions include photosensitivity and aggravation of acne after two to four weeks, also called a pustular flare (Rigopoulos *et al.*, 2004:361).

Systemic tretinoin may have cardiovascular effects, for example arrhythmias, flushing, hypotention or hypertension, and heart failure. Serious adverse effects include teratogenicity and the potentially life-threatening retinoic acid syndrome (RAS) (Sweetman, 2010).

2.5.5 Adapalene

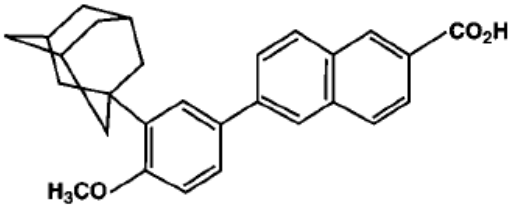
Adapalene is a receptor-selective third generation retinoid derived from naphtoic acid (Njar *et al.*, 2006:434; Bershad, 2001:157).

2.5.5.1 Physicochemical properties

As can be observed in table 2.3, adapalene is structurally very different from tretinoin. During the search for a molecule more stable than tretinoin, but with similar therapeutic effects, some major changes were brought about in tretinoin's structure. The unstable side chain of tretinoin was replaced by aromatic naphtoic acid rings, which rendered it more stable to oxygen and light. A phenoxy-adamantyl group was added which gave the molecule a higher melting point, lower solubility, good lipophilicity and low skin flux, thereby enhancing its safety profile (Shroot & Michel, 1997:S98; Czernielewski *et al.*, 2001:6).

Table 2.3 provides a summary of the physical and chemical properties of adapalene.

Table 2.3: Physicochemical properties of adapalene (Sweetman, 2010; US Pharmacopoeial Convention, 1998:26; Shroot, 1998:S18; Trichard *et al*, 2008:435; Valiveti *et al*., 2008:14)

Synonym	CD-271
Chemical name	6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid
Molecular formula	$C_{28}H_{28}O_3$
Molecular structure	
Molecular weight	412.5 g/mol
CAS number	106685-40-9
Appearance	White to off-white crystalline powder
Solubility	Insoluble in water (4.01 ng/L); slightly soluble in ethanol
Octanol-water partition coefficient (log P)	8.04
Dissociation constant (pK_a)	4.23
Melting point	325-327 °C

2.5.5.2 Pharmacokinetics

Adapalene has very low percutaneous absorption after penetrating the stratum corneum. This results in adapalene becoming trapped in the epidermis and the hair follicle, which is its target area (Rigopoulos *et al.*, 2004:409).

2.5.5.3 Indications

Topical adapalene is used to treat acne and psoriasis (Njar *et al.*, 2006:433).

2.5.5.4 Action against acne

Adapalene normalises proliferation and differentiation by interacting selectively with RAR-β and RAR-γ. Its efficacy is similar to that of other retinoids, but it is better tolerated and, therefore, has an improved therapeutic ratio (Njar *et al.*, 2006:435). Adapalene has additional anti-inflammatory activity due to the oxidative metabolism of arachidonic acid and inhibition of lipoxygenase activity (Rigopoulos *et al.*, 2004:409).

2.5.5.5 Adverse effects

Adapalene is well tolerated with a significantly lower irritation potential than tretinoin (Czernielewski *et al.*, 2001:9). Possible adverse skin reactions include dryness, peeling, erythema, burning and itching (Rigopoulos *et al.*, 2004:410). Although there is no evidence of teratogenicity after topical application of adapalene, its use by pregnant women is not recommended (US Pharmacopoeial Convention, 1998:26).

2.6 Dermatological and transdermal drug delivery

Aside from being of utmost importance to human life, the skin offers an opportunity and unique means for drug delivery due to its large surface area of approximately two square metres (Block, 2006:871). Multiple drug targets reside in the skin, thereby providing possibilities of treating skin disorders with topical compounds while avoiding systemic side effects (Long, 2002:41). The skin, however, also challenges the delivery of drugs by acting as a tough barrier (Menon, 2002:S4).

Dermatological biopharmaceutics aims to deliver drugs with selective penetrability to their active sites at a controlled rate and concentration, and for the necessary time. This is done by incorporating the drug into a vehicle or device that delivers it to the target site (Barry, 2002:507).

2.6.1 Drug targets in skin

A topically applied therapeutic compound may remain on the skin for a topical effect. Alternatively, it may cross the stratum corneum to reach deeper targets. This includes delivery to the following:

1) dermis, epidermis or skin appendages (dermatological delivery);
2) deeper tissues, e.g. muscles or joints (local or regional delivery); and
3) systemic circulation (transdermal delivery) (Walters & Brain, 2002:319-320; Surber & Davis, 2002:403).

Due tot the stratum corneum barrier (discussed in section 2.6.2), it may be difficult for a drug to penetrate into the viable epidermis and dermis. Once the drug has crossed the epidermis, continued diffusion into the dermis can lead to diffusion of the drug into the dermal vasculature and, hence, into the systemic circulation. However, drug delivery systems can be formulated to provide ample dermatological delivery without obtaining high systemic concentrations (Block, 2006:872).

2.6.2 The stratum corneum

The excellent barrier function of the skin is mainly provided by the 10 to 20 µm thick outermost layer, namely the stratum corneum (Walters & Roberts, 2002:4). Not only does this layer limit water loss through the skin, but it also protects the body by preventing exogenous compounds from entering (Roberts *et al.*, 2002:89).

Cells of the stratum corneum originate in the basal layer of the viable epidermis (figure 2.7). During their migration towards the stratum corneum, these cells undergo many morphological changes (Walters & Roberts, 2002:6). The basal layer (stratum basale) of the epidermis consists of a single layer of basal keratinocytes. These cells become filled with keratin filaments and keratohyalin granules during their travel through the spinous layer (stratum spinosum) and granular layer (stratum granulosum). Upon reaching the stratum corneum, the keratinocytes have been differentiated into nonviable flattened cells known as corneocytes (Menon, 2002:S5, S7). The stratum corneum, also called the horny layer, typically consists of 15 to 25 cell layers (Walters & Roberts, 2002:4-5). On average, one layer of corneocytes are shed daily and replaced by keratinocytes from the stratum granulosum (Wickett & Visscher, 2006:S101).

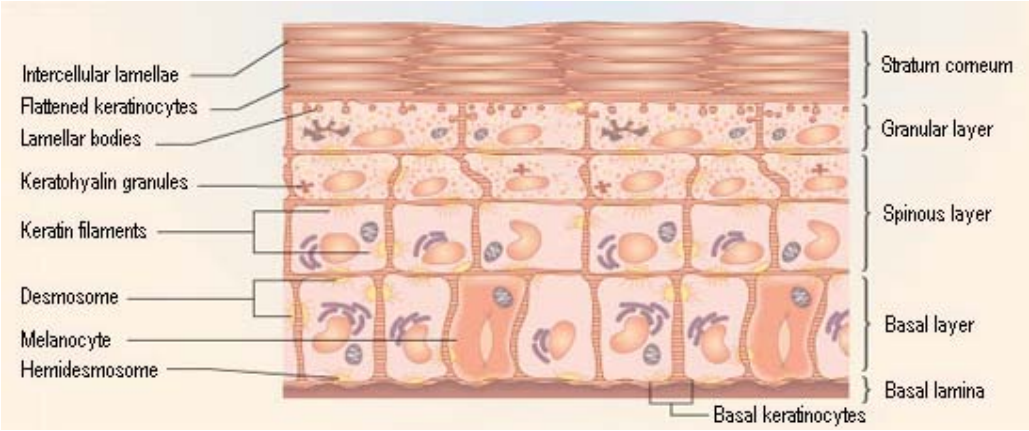


Figure 2.7: The epidermal layers showing the various stages of keratinocyte differentiation (In Vivo Health, 2009)

The bricks-and-mortar model describes the structure of the stratum corneum as corneocytes embedded in a matrix of intercellular lipid layers. Corneocytes (the “bricks”) are surrounded by resistant envelopes of cornified lipids which render it highly impermeable. The hydrophilic corneocytes are furthermore strengthened by microfibrils which limit swelling of the stratum corneum. The lipid “mortar” is composed of mainly ceramides, cholesterol and free fatty acids released by keratinocytes during their differentiation into corneocytes. This lipophilic matrix is arranged in lamellar structures between hydrophilic layers (Wickett & Visscher, 2006:S99-102; Block, 2006:873). The corneocytes, together with the non-polar, water-tight lipids, provide a tortuous route to water and xenobiotics wanting to cross the stratum corneum (Menon, 2002:S7;

Hadgraft, 2004:291), with the intercellular lipids providing the main barrier (Roberts *et al*., 2002:96; Wickett & Visscher, 2006:S99).

2.6.3 Penetration pathways

Molecules may cross the stratum corneum by means of three possible pathways (figure 2.8), namely by intracellular, intercellular or transappendageal (shunt) diffusion (Roberts *et al.,* 2002:94).

A molecule following the intracellular (or transcellular) route passes repeatedly through the corneocytes and lipid matrix of the stratum corneum. Diffusion through only the intercellular lipids is considered the predominant pathway of diffusion (Roberts *et al.*, 2002:96-97). This intercellular (or paracellular) pathway is approximately 500 µm in length and, therefore, much longer than the thickness of the stratum corneum (Hadgraft, 2004:292).

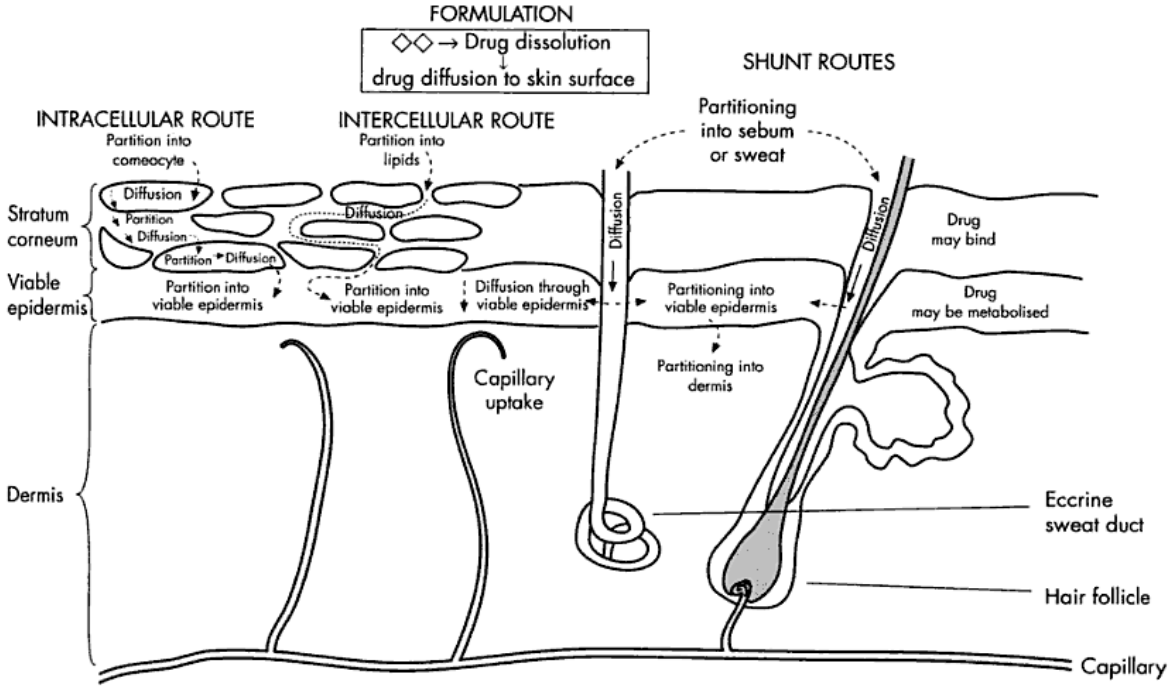


Figure 2.8: A representation of the skin showing the principal mechanisms and pathways by which topically applied compounds may cross the stratum corneum (Williams, 2003:29)

Transappendageal penetration includes diffusion through the hair follicles and eccrine glands (sweat glands). These pathways, also called shunt routes, were long considered insignificant. Research done by various independent groups during the last decade suggested that the follicular route might be especially relevant for hydrophilic compounds, molecules with a high molecular weight, and particle-based delivery systems. Hair follicles represent invaginations which extend deep into the dermis and increase the actual surface area for penetration significantly. Follicular delivery may maximise the delivery of drugs, especially anti-acne agents,

to the pilosebaceous unit (Knorr *et al.*, 2009:173, 175). An extensive dermal capillary network supplies blood to the superior part of the hair follicle and the sebaceous gland(s), whereas the inferior follicle part receives blood from the deep dermis and subcutaneous tissues (Wosicka & Cal, 2010:85; Meidan *et al.*, 2005:5). This rich supply of blood enables systemic drug delivery via the transfollicular route (Wosicka & Cal, 2010:85).

2.6.4 Factors influencing skin penetration

Molecules are transported across the skin by means of passive diffusion (Watkinson & Brain, 2002:62) with the skin acting as a passive, but not inert, membrane (Block, 2006:873). Effective therapy of a topically applied drug is affected by properties of the drug, vehicle and skin. As cutaneous delivery is a complicated, dynamic process, a change in one variable usually causes several effects on drug flux (Barry, 2002:509). Some biological and physicochemical factors that influence permeation will be discussed in the following paragraphs.

2.6.4.1 Biological factors

Factors relating to the physiology of the skin have an influence on cutaneous permeation. The main factors include skin condition, skin age, blood flow to the skin, anatomical site used for permeation, metabolism inside the skin, as well as lipids on the skin's surface.

- **Skin condition**

Damage of the skin's barrier may be caused by cuts, abrasions, dermatitis and many solvents, resulting in enhanced penetration. Diseases causing inflammation, loss of stratum corneum or altered keratinisation increase permeability, whereas diseases causing thickening of the skin decrease its permeability (Barry, 2002:509-510).

- **Skin age**

Children have a greater surface area per unit body weight which makes them more susceptible to toxic effects of a topically applied drug (Barry, 2002:510). Skin permeability is better in premature or newborn infants (Surber & Davis, 2002:436). Some premature infants are born without a stratum corneum (Barry, 2002:510).

- **Blood flow**

Increased peripheral blood flow may reduce the amount of time a drug remains in the dermis or deeper parts of the skin, thereby increasing the concentration gradient across the skin (Barry, 2002:510; Surber & Davis, 2002:433).

- **Anatomical site**

Permeation is dependant on the thickness and nature of the stratum corneum, as well as the density of skin appendages at the site of application. Facial skin is generally more permeable

than other body sites. Absorption varies between different individuals, even if the skin site is identical (Barry, 2002:510).

- **Skin metabolism**

The therapeutic efficacy of topically applied drugs may be influenced by metabolism inside the skin. It is estimated that the skin can metabolise approximately 5% of topical drugs (Barry, 2002:510). Metabolism in the skin includes oxidative, reductive, hydrolytic and conjunctive reactions (Surber & Davis, 2002:435).

- **Skin surface lipids**

Lipids on the skin's surface may dissolve the drug, decrease its thermodynamic activity and, hence, diminish drug permeation (Surber & Davis, 2002:433).

2.6.4.2 Physicochemical factors

Physicochemical factors have pronounced effects on drug permeation into and through the skin. These include properties of the skin and properties of the vehicle and drug.

- **Skin hydration**

Hydration of the stratum corneum is one of the most important factors resulting in an increase in a drug's penetration rate. When the skin is saturated with water, it swells, softens and wrinkles, leading to a marked increase in permeability. Skin hydration may be caused by water diffusing from underlying epidermal layers, or perspiration accumulating between the skin and an occlusive vehicle or dressing. Different vehicles used to formulate drug products have diverse effects on skin hydration (Barry, 2002:511).

- **Skin temperature**

Changes in the skin's temperature are associated with other physiological reactions, e.g. increased blood flow or an increased moisture content of the stratum corneum, both of which can lead to increased absorption. An increase in temperature results in increased drug solubility in the vehicle, as well as in the stratum corneum. This may lead to increased diffusivity and transdermal absorption (Surber & Davis, 2002:433). As the skin's temperature decreases, the diffusion coefficient decreases correspondingly (Barry, 2002:511).

- **pH and ionisation**

The degree of ionisation of a molecule influences its solubility and, therefore, its permeation into the skin (Hadgraft, 2004:292). The pH of the vehicle, as well as the molecule's pK_a or pK_b, determines the proportion of unionised drug which, in turn, mainly determines the effective membrane gradient (Surber and Davis, 2002:433; Barry, 2002:511). According to the simple form of the pH-partition hypothesis, only unionised molecules readily cross lipid membranes. However, ionised molecules cross the stratum corneum to a limited extent. Due to their much

higher aqueous solubility, these ionised molecules contribute significantly to the total flux when in saturated or near-saturated solutions (Barry, 2002:511-512). Ionisation of a weak electrolyte substantially reduces its permeability (Block, 2006:873).

- **Diffusion coefficient**

The diffusion coefficient measures the penetration rate of a molecule under specified conditions. At a constant temperature, the diffusion coefficient of a drug in a topical vehicle or in the skin depends on the properties of the drug, the properties of the diffusion medium, and the interaction between them. Drug molecules binding to the stratum corneum become immobilised and alter the diffusion coefficient (Barry, 2002:506).

- **Drug concentration**

The flux of a solute is proportional to the concentration gradient across the entire barrier phase. For maximal flux in a thermodynamically stable situation, the donor solution should be saturated. By controlling the solvent composition of the vehicle, the solubility of a drug can be optimised to obtain a saturated solution (Barry, 2002:512).

- **Partition coefficient**

The partition coefficient is essential in establishing the flux of a drug through the stratum corneum. When the membrane offers the only or main resistance, the partition coefficient is very important, as it may differ tremendously between different drugs or different vehicles (Barry, 2002:512). If the partition coefficient is too low, the molecules are too water soluble to partition into the stratum corneum well. Molecules with a high partition coefficient may be too lipid soluble to diffuse from the stratum corneum into the water-rich viable tissue (Barry, 2002:512; Naik *et al*., 2000:319). Therefore, molecules with good solubility in both oils and water will permeate the stratum corneum well (Hadgraft, 2004:292).

- **Particle size**

Particles with a diameter larger than 10 µm generally remain on the surface of the skin, whereas particles between 3 and 10 µm penetrate the follicular duct. When smaller than 3 µm, particles penetrate both the hair follicles and the stratum corneum (Barry, 2001a:970; Allec *et al.*, 1997, S119).

2.6.4.3 Ideal molecular properties

Barry (2002:513) and Naik *et al*. (2000:319) described the ideal physicochemical properties of a drug molecule able to successfully pass through the stratum corneum. These properties include:

- Low molecular mass, preferably less than 600 g/mol. This will increase the diffusion coefficient;

- Adequate solubility in oil and water in order to increase the concentration gradient in the membrane. The aqueous solubility should be higher than 1 mg/ml;
- An oil-water partition coefficient (log P) between one and three;
- A low melting point (less than 200 °C), correlating with good ideal solubility; and
- A pH of between five and nine for a saturated aqueous solution.

2.6.5 Penetration enhancement

Due to resistance of the stratum corneum, most drugs penetrate human skin poorly. Several strategies have been developed to circumvent the stratum corneum in order to improve drug penetration. Barry (2001b:103) arranged these strategies into five groups, namely (1) drug and vehicle interactions, (2) vesicles and particles, (3) stratum corneum modification, (4) stratum corneum bypassing or removal and (5) electrically assisted methods.

2.6.5.1 Drug and vehicle interactions

Drug and vehicle interactions can be modified in a number of ways. Penetration of a drug without the correct physicochemical properties (usually with a partition coefficient that is too low) may be improved by designing a prodrug with an optimal partition coefficient. After absorption, the prodrug is activated by enzymes (Barry, 2001b:102).

By increasing the chemical potential of a drug inside the vehicle, its thermodynamic activity is increased as well. Chemical potential could be increased by using supersaturated solutions (Barry, 2002:521).

To improve penetration of a charged molecule, an oppositely charged species may be added to form a lipophilic ion pair. After reaching the viable epidermis, the ion pair dissociates into its charged species (Barry, 2001b:103).

2.6.5.2 Vesicles and particles

Liposomes are colloidal particles and consist typically of phospholipids and cholesterol. These lipid molecules may entrap drugs in their concentric bimolecular layers and deliver them to and through the skin. The vesicles usually accumulate in the stratum corneum or other upper skin layers (Barry, 2001b:104).

Transferosomes are deformable liposomes with incorporated surfactant edge activators such as sodium chelate (Touitou & Godin, 2006:263). It is claimed that these ultradeformable vesicles can squeeze through pores in the stratum corneum (Barry, 2001b:104-105). Ethosomes are soft lipid vesicles in a hydroalcoholic milieu that enhance delivery to deep skin layers or to the systemic circulation (Touitou & Godin, 2006:264). Niosome vesicles are formed by using non-ionic surfactants and offer several advantages over liposomes, e.g. higher chemical stability,

lower costs, as well as intrinsic skin penetration enhancing properties (Manconi *et al.*, 2002:238). Particle-based delivery can be used to target specific regions of the follicular duct. Penetration of nano and microparticles in the follicle has been widely investigated recently. The penetration depth apparently depends on the size of the particles (Wosicka & Cal, 2010:86).

2.6.5.3 Stratum corneum modification

Hydration of the stratum corneum is one of the most important factors in increasing the penetration rate of most substances as it opens up compact stratum corneum layers (Barry, 2002:521). Water has been described as the ultimate penetration enhancer due to its safety and efficacy (Block, 2006:875-876).

Chemical penetration enhancers are substances that enhance drug flux (Barry, 2001b:106). These penetration enhancers act on the stratum corneum by either increasing its degree of hydration or disrupting its lipid matrix (Block, 2006:875). Examples of penetration enhancers include alcohols, polyalcohols, pyrrolidones, amines, amides, fatty acids, esters, alkanes, surfactants and phospholipids (Naik *et al*., 2000:321). The ideal penetration enhancer is required to be, i.a., non-toxic, non-irritating, non-allergenic, pharmacologically inert, and compatible with all drugs and excipients (Barry, 2002:522).

2.6.5.4 Stratum corneum bypassed or removed

The stratum corneum may be bypassed using microneedles that penetrate the horny layer without affecting underlying pain receptors or blood vessels (Birchall, 2006:339).

Chemical peels, microdermabrasion or laser abrasion may be used to remove the stratum corneum. Adhesive tape may also be used to remove the horny layer prior to application of a drug (Barry, 2001b:107-108; Birchall, 2006:346).

By using the follicular route of penetration, the intact stratum corneum may be bypassed, as discussed in section 2.6.3.

2.6.5.5 Electrically assisted methods

Some physical strategies increase penetration by using electricity. Methods used include ultrasound, iontophoresis, electroporation, magnetophoresis and photomechanical waves (Naik *et al.*, 2000:322-324; Barry, 2002:521-522).

Solid particles can be delivered by firing them through the stratum corneum with a supersonic shockwave of helium gas. This is called the PowderJect® system (Barry, 2002:524).

2.6.6 Dermatological delivery of tretinoin and adapalene

In order to be therapeutically effective, sufficient amounts of tretinoin or adapalene must reach the hair follicles and sebaceous glands. When the physicochemical properties of tretinoin and adapalene are compared to the properties necessary to successfully penetrate the stratum corneum (section 2.6.4.3), both retinoids fail in at least two criteria. The oil-water partition coefficients of both tretinoin and adapalene are too high, and both are too poorly water soluble. The low water solubility may limit their incorporation into a suitable vehicle (Shah *et al.*, 2007:163). The melting point of adapalene is higher than required of an ideal molecule, making its solubility suboptimal.

Topical tretinoin and adapalene have, nonetheless, proved to be effective for the treatment of dermatological disorders (Manconi *et al.*, 2002:238). However, several drawbacks limit their topical use. Advantages and limitations of delivering topical tretinoin and adapalene for a dermatological effect are discussed in section 2.6.6.1, followed by a discussion on their distribution in the skin (section 2.6.6.2) and strategies to enhance their stability and penetration (section 2.6.6.3).

2.6.6.1 Advantages and difficulties

Dermatological delivery of tretinoin and adapalene to the pilosebaceous unit offers various advantages, as well as several difficulties. Advantages include the following:

- By avoiding the systemic circulation, systemic side effects may be limited (Cziernielewski *et al*., 2001:10);
- The skin offers a relatively large and readily accessible surface area for absorption (Naik *et al*., 2000:319);
- As tretinoin is susceptible to hepatic metabolism (Sweetman, 2010), this first-pass metabolism can be avoided with topical therapy (Walters & Roberts, 2002:4);
- Problems associated with the gastrointestinal tract, e.g. stomach emptying, pH dependant degradation, enzyme deactivation, and presence of food, may be excluded (Walters & Roberts, 2002:4; Roberts *et al*., 2002:90);
- Patient compliance may be improved due to the non-invasive method of delivery (Naik *et al*., 2000:319); and
- The retention of tretinoin by the skin after topical application is desirable (Contreras *et al*., 2005:135);

Difficulties associated with dermatological delivery of tretinoin and adapalene include the following:

- The stratum corneum offers excellent resistance to diffusion (Naik *et al*., 2000:319);

- The rate and extend of absorption may vary between individuals. Other factors influencing absorption include site, age, race, and skin disease (Walters & Roberts, 2002:4; Roberts *et al*., 2002:90);
- Due to metabolic enzymes located in the skin, drugs may be affected by cutaneous "first-pass" metabolism (Walters & Roberts, 2002:4; Roberts *et al*., 2002:91). Tretinoin in the skin may isomerise to form isotretinoin (Bershad, 2001:156) and other isomers (Shroot, 1998:S22);
- Topically applied tretinoin and adapalene are known to cause mild to severe skin irritation (Sweetman, 2010);
- Diffusion is influenced by a number of physicochemical parameters (Roberts *et al*., 2002:91), as discussed in section 2.6.4.2;
- Tretinoin may undergo oxidation and photoisomerisation (Bershad, 2001:156) even before crossing the stratum corneum (Elbaum, 1988); and
- Adapalene is known to have a low skin flux (Sweetman, 2010).

2.6.6.2 Skin distribution profiles

An adapalene gel developed by Allec *et al.* (1997:S119-120) rapidly penetrated the pilosebaceous unit. The epidermis and dermis contained significant quantities of adapalene, whereas only 0.01 % diffused across the skin. At the end of the diffusion study, an excess of 59.7 % of the applied dose was recovered from the skin surface. Research done by Rigopoulos *et al.* (2004:409) confirmed the significantly low transdermal absorption of adapalene. Adapalene was trapped in the epidermis and its target, the hair follicle.

In another study, adapalene and tretinoin both showed relatively high concentrations in the epidermis and small concentrations in the dermis after topical application. Adapalene in the epidermis was virtually unchanged, whereas, in the case of tretinoin, a mixture of isomers and other unidentified materials were present (Shroot, 1998:S21-22).

Approximately 2 % of tretinoin in commercial cream vehicles has been found to penetrate into the systemic circulation (Bershad, 2001:157).

2.6.6.3 Formulation strategies for improved stability and penetration

Rebelo and Pina (1997) suggested that the release kinetics of tretinoin varied strongly according to the way in which it was incorporated into the formulation and according to the type of formulation used. Several formulation strategies have been developed in order to enhance the delivery of topical tretinoin or adapalene. Researchers aim at (1) increasing the concentration of retinoid at the site of action, (2) diminishing local and systemic side effects and/or (3) improving the stability of tretinoin in the formulation (Allec *et al*., 1997:S119). Some

recent developments in formulation strategies for tretinoin and adapalene are discussed in the following paragraphs.

- **Tretinoin containing formulations**

Trotta *et al*. (2003:319) employed ion pairing with amino esters to increase tretinoin flux. They concluded that this method did not improve drug targeting without increasing systemic side effects as well. When micro-emulsion systems were tested, the *in vitro* release rate of tretinoin was lower than from an aqueous solution. Oil-in-water micro-emulsions containing amino esters, however, enhanced tretinoin accumulation in the skin.

Tretinoin incorporated into a liposomal gel induced the formation of a drug reservoir, leading to a prolonged action of tretinoin in the epidermis and dermis, and an improved effect in the skin (Contreras *et al*., 2005:143).

Due to tretinoin's instability, several research efforts have endeavoured to protect tretinoin from photodegradation in order to limit side effects. Ioele *et al*. (2005:254) explored the photostability of tretinoin in an ethanol solution and suggested the importance of new pharmaceutical formulations to minimise isomerisation caused by photodegradation. Inclusion of tretinoin in liposomes resulted in an increased stability to light.

Degradation of tretinoin has been shown to be considerably higher in cream formulations than in ethanol. It has been suggested that side effects of topical tretinoin therapy could be due to photodegradation, even after its penetration into the skin. Improved formulations and UVA sunscreens may limit photodegradation and, therefore, side effects (Tashtoush *et al.,* 2008:127).

Research was conducted by Brisaert and Plaizier-Vercammen (2000:55-56) to protect a tretinoin lotion from degradation during exposure to light. They concluded that surfactants, cyclodextrins and UV-filters failed to protect this retinoid. Yellow colourants, however, slowed down photodegradation.

Manconi *et al.* (2002:272) reduced photodegradation of tretinoin by incorporating it in niosomes and liposomes. They concluded that stability was affected by the composition and structure of the vesicles.

Photodegradation of tretinoin trapped inside solid lipid nanoparticles (SLNs) was strongly reduced. SLN based tretinoin gel depicted an improved skin tolerability compared to marketed tretinoin cream (Shah *et al*., 2007:167-169).

- **Adapalene containing formulations**

Little research has been conducted regarding improved formulations for adapalene. This may be due to adapalene's stability to oxidation and photodegradation, as well as its low irritation profile.

Allec *et al.* (1997:S119-120) developed a gel containing 0.1 % adapalene microcrystals which rapidly penetrated the pilosebaceous unit. This formulation was based on the knowledge that particles less than 10 µm in diameter selectively penetrate follicular ducts.

2.6.7 Pheroid™ technology

Pheroid™ technology is a novel patented delivery system used to enhance absorption and efficacy of dermatological, biological and oral pharmacological compounds. It is a colloidal system consisting of lipid-based structures, called Pheroids™, dispersed uniformly in a continuous phase (Grobler *et al*., 2008:283-284). This section describes the basic characteristics of Pheroid™ technology and its advantages, with the focus of last part on the possibility of incorporating Pheroids™ into a dermatological delivery system for tretinoin and adapalene.

2.6.7.1 Characteristics of Pheroids™

The colloidal design of Pheroid™ technology is based on features of other colloidal dosage forms, e.g. liposomes, emulsions, nano and micro-emulsions, and polymeric and macromolecular microspheres (Grobler *et al*., 2008:287-288).

Pheroids™ are unique and stable submicron and micron-sized structures, typically formulated to have a diameter of between 200 nm and 2 µm. They have a lipid bilayer consisting of ethylated and pegylated polyunsaturated fatty acids, which includes omega-3 and omega-6 fatty acids, but excludes arachidonic acid and cholesterol (Grobler *et al*., 2008:284-285). Nitrous oxide (N_2O) is a unique component of Pheroids™ and is associated with the fatty acid dispersed phase. The resulting matrix of essential fatty acids and nitrous oxide is functional in transporting hydrophobic and hydrophilic drugs (Grobler *et al*., 2008:288-289). All topical Pheroid™-based formulations contain tocopherol or a tocopherol derivative as antioxidant and emulsion stabiliser (Grobler *et al*., 2008:293).

Numerous Pheroid™ types can be formulated by manipulating their size, structure, charge, morphology, entrapment volume and function. The choice of type and diameter are influenced by the administration route and the desired rate of delivery (Grobler *et al*., 2008:283-285).

The amount of molecules of an active compound entrapped within one Pheroid ™ depends on the compound's size, charge and solubility. A simple Pheroid™ formulation contains on average 150 Pheroids™/µl (Grobler *et al*., 2008:294-295).

2.6.7.2 Advantages of Pheroid™ technology

Advantages of the Pheroid™ system include the following:

- Its components are pharmaceutically safe;
- Pheroid™ technology is effective, versatile and inexpensive;
- Dosing frequency may be reduced without a decrease in potency. Alternatively, higher doses can be given to increase therapeutic impact;
- Immunologic reactions can be avoided, because fatty acids are inherent components of the human skin;
- A larger variety of structures can be formulated with Pheroid™ technology than with other colloidal systems;
- Pheroids™, unlike phospholipids or cholesterol, are extremely elastic and have the ability to deform in order to cross densely packed cohesive capillary walls without fracturing;
- Drugs with different solubilities, as well as insoluble drugs, can be entrapped; and
- Pheroids™ cause no cytotoxicity, but help maintain the cell membrane (Grobler *et al*., 2008:285-309).

2.6.7.3 Pheroids™ for enhanced dermatologic delivery of tretinoin and adapalene

The Pheroid™ delivery system can be used to enhance the absorption of active ingredients into the viable epidermis and dermis. Pheroids™ have proved to cross the stratum corneum rapidly with their entrapped compound (Grobler *et al*., 2008:296). Faster penetration of tretinoin and adapalene could possibly reduce their associated skin irritation. Several studies have shown an increase in the percentage of active compound delivered to the skin when using Pheroids™. The fluidity of the Pheroid™ membrane probably contributes to efficient (trans)dermal delivery (Grobler *et al*., 2008:297).

Permeation by a Pheroid™ formulation is influenced by various factors, which include the following:

- Size and morphology of the Pheroids™;
- Molecular geometry, concentration and ratios of the fatty acids;
- Hydration medium;
- pH of the preparation;
- Presence of charge-changing molecules;
- Presence of molecules influencing the electrostatic environment;
- Character and concentration of the active compound; and
- State of the Pheroid™ (e.g. gel or fluid state) (Grobler *et al*., 2008:297-299).

Essential fatty acids have inherent therapeutic effects. Apart from normalising the water barrier of the skin, they suppress epidermal hyperproliferation and have an anti-inflammatory action by normalising the physiological micro-environment (Grobler *et al*., 2008:309). These properties could enhance the anti-acne effects of retinoids.

2.7 Summary

Acne is a very common disease of the pilosebaceous unit that may result in physical and psychological scarring. It is caused by four primary factors, namely abnormal follicular desquamation, excessive sebum production, bacterial overgrowth and inflammation. Several systemic and topical treatment options are available. Treatment must, however, target multiple factors simultaneously.

Topical retinoids are regarded as the mainstay of acne therapy and maintenance therapy, whether alone or in combination with other anti-acne agents. They inhibit the formation of the microcomedo by normalising keratinocyte differentiation and sebum production. Some retinoids have additional anti-inflammatory effects.

Tretinoin is the prototype retinoid against which all other retinoids are measured. Due to severe systemic effects, it is exclusively used as a topical agent, except in acute promyelocytic leukaemia, where systemic therapy is indicated. Topical application of tretinoin may result in skin irritation and photosensitivity. It is furthermore extremely prone to oxidation and photo-isomerisation.

Adapalene, on the other hand, is a third generation retinoid with an improved stability profile. It is stable in the presence of oxidising agents and light. Although adapalene is better tolerated than tretinoin, skin irritation may occur after topical application.

The stratum corneum offers excellent resistance against ingress of molecules and egress of water. Three pathways of stratum corneum penetration have been identified, namely transcellular diffusion, intercellular diffusion and transappendageal diffusion. The latter route may be important for the delivery of retinoids to the sebaceous gland and superior part of the hair follicle.

Numerous strategies have been developed to enhance the delivery of therapeutic compounds in or across the skin. Some of these strategies have been applied to tretinoin and adapalene in order to increase their concentration in the pilosebaceous unit, to reduce side effects or to increase tretinoin's stability. Pheroid™ technology is a novel delivery system that has successfully been used to enhance delivery of active compounds into and across the skin. This technology has the potential to improve delivery of tretinoin and adapalene to the pilosebaceous unit. It may also accelerate the penetration of retinoids, which may lead to reduced skin irritation.

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Chapter 3

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Formulation, *in vitro* release and transdermal diffusion of selected retinoids

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Abstract

Acne is a skin condition affecting about 80% of people aged 11 to 30. Retinoids are used widely as oral or topical treatment for this disease, with tretinoin and adapalene being two of the most used topical retinoids. Researchers constantly strive to improve dermatological retinoid formulations in order to combat low dermal flux, skin irritation and instability. This study incorporated tretinoin and adapalene separately into castor oil, vitamin F and Pheroid™ creams and evaluated these creams in terms of their *in vitro* retinoid release, *in vitro* transdermal diffusion and stability. Castor oil and Pheroid™ creams were found to be superior in terms of release and dermal delivery of adapalene. Tretinoin was best released and delivered to the dermis by castor oil cream. The castor oil creams were found to be the most stable formulations, while the Pheroid™ creams were the most unstable. In terms of release, dermal diffusion and stability, castor oil cream proved to be the most suitable cream for both tretinoin and adapalene.

Keywords: Tretinoin, Adapalene, Dermal delivery, Pheroid™, Castor oil, Stability

1 Introduction

Acne vulgaris is a multifactorial disease of the pilosebaceous unit (Dessinioti and Katsambas, 2010) which affects nearly 80% of people aged between 11 and 30 (Gollnick et al., 2003). It primarily affects areas with the highest concentration pilosebaceous glands like the face, chest and back. Four primary pathogenic factors interact to produce acne, namely (1) sebaceous gland hyper-proliferation and seborrhoea, (2) hyper-keratinisation, (3) follicular colonisation by *Propionibacterium acnes*, and (4) inflammation and immune response (Gollnick et al., 2003). Thiboutot et al. (2009) described acne as a chronic disease with a psychological impact. Several systemic and topical acne treatments are used to resolve existing lesions, prevent scarring and suppress the development of new lesions. The success thereof depends on the use of the right medication as well as patient adherence (Thiboutot et al., 2009). Topical treatment is often used as first line treatment for acne, due to the location of the target organ, the pilosebaceous unit, in the skin (Krautheim and Gollnick, 2004).

Topical retinoids have been used for nearly four decades as topical agents to treat acne (Bershad, 2001) and are the treatment of choice for comedonal acne as well as for maintenance

therapy (Thiboutot et al., 2009). These vitamin A derivatives are anticomedogenic, comedolytic and have some direct and indirect anti-inflammatory effects (Thiboutot et al., 2009). The focus of this paper falls on two retinoids, namely tretinoin and adapalene. These two retinoids have proved to be effective for the treatment of dermatological disorders after topical administration (Manconi et al., 2002).

The transdermal drug delivery route offers several challenges, e.g. the excellent resistance of the stratum corneum to diffusion (Naik et al., 2000) and variable skin properties such as site, age, race and disease (Roberts et al., 2002; Walters and Roberts, 2002). Some additional difficulties are associated with the dermatological delivery of tretinoin and adapalene and include suboptimal water solubility of the retinoids (Shah et al., 2007), isomerisation of tretinoin in the skin (Shroot, 1998; Bershad, 2001), mild to severe skin irritation (Sweetman, 2010), and oxidation and photo-isomerisation of tretinoin (Bershad, 2001), even before crossing the stratum corneum (Elbaum, 1988).

Rebelo and Pina (1997) suggested that the release kinetics of tretinoin varied strongly according to the way in which it was incorporated into the formulation and according to the type of formulation used. Several formulation strategies have been developed in order to enhance the delivery of topical tretinoin or adapalene. Researchers aim at (1) increasing the concentration of retinoid at the site of action, (2) diminishing local and systemic side effects and/or (3) improving the stability of tretinoin in the formulation (Allec et al., 1997). Recent developments in formulation strategies for tretinoin include ion pairing with amino esters for enhanced skin flux (Trotta et al., 2003), liposomal gel with prolonged action (Contreras et al., 2005), and niosomes and liposomes protecting tretinoin from photo-degradation (Manconi et al., 2002; Ioele et al., 2005). Little research has been conducted regarding improved formulations for adapalene. This may be due to adapalene's stability to oxidation and photo-degradation, as well as its low irritation profile.

Pheroid™ technology is a novel patented delivery system that has successfully been used to enhance the delivery of active ingredients into the viable epidermis and dermis (Grobler et al., 2008). The unique and stable submicron and micron-sized structures, called Pheroids™, are typically formulated to have a diameter of between 200 nm and 2 µm. They have a lipid bilayer

consisting of ethylated and pegylated polyunsaturated fatty acids, including omega-3 and omega-6 fatty acids. Nitrous oxide (N_2O) is a unique component of Pheroid™ and is associated with the fatty acid dispersed phase. The resulting matrix of essential fatty acids and nitrous oxide is functional in transporting hydrophobic and hydrophilic drugs (Grobler et al., 2008). This study incorporated Pheroid™ technology into topical creams containing either one of the selected retinoids. The stability, *in vitro* drug release profile and transdermal diffusion of the Pheroid™ creams were compared to non-Pheroid™ retinoid creams.

2 Materials and methods

2.1 Materials

Tretinoin and adapalene were obtained from Iffect Chemphar (Hong Kong, China). Cetyl alcohol, stearic acid, Tween®-80 and polyethylene glycol 400 were used for the formulation of creams and were supplied by Merck Laboratory Supplies (Midrand, South Africa). Span®-60 (Fluka Analytical, Buchs, Germany), vitamin F ethyl ester (Chemisches Laboratorium Dr. Kurt Richter, Berlin, Germany), veegum® HV (R.T. Vanderbilt Company, Norwalk, USA) and dl-α-tocopherol (Chempure, Pretoria, South Africa) were adjuvants used in the creams. The rest of the ingredients for the creams, namely xanthan® gum, castor oil, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), methylparaben and propylparaben, were obtained from Warren Chem Specialities (Johannesburg, South Africa). Potassium dihydrogen orthophosphate (KH_2PO_4) and sodium hydroxide (NaOH) used for the preparation of phosphate buffered solutions (PBS) were provide by Merck Laboratory Supplies (Midrand, South Africa). Ethanol and tetrahydrofuran (THF) used as solvents were obtained from Merck Laboratory Supplies (Midrand, South Africa) and Sigma-Aldrich Corporation (Johannesburg, South Africa), respectively. Reagents used during high performance liquid chromatography (HPLC) include gradient grade acetonitrile, octane-1-sulphonic acid sodium salt, gradient grade methanol and acetic acid (CH_3COOH), and were supplied by Merck Laboratory Supplies (Midrand, South Africa). Deionised HPLC grade water was used throughout this study and was prepared using a Milli-Q water purification system (Millipore, Milford, USA).

2.2 Methods

2.2.1 Precautions

In order to prevent photo-isomerisation, all actions involving tretinoin were performed under subdued lighting using amber glassware. Only freshly prepared retinoid samples and standards were used. Fresh receptor phase was prepared before each drug release study or transdermal diffusion study, due to the occurrence of crystallisation when it was stored at 4 °C. Degradation of tretinoin was prevented during HPLC analysis by setting the thermostat at 5 °C.

2.2.2 Formulation of creams

Three oil-in-water creams were formulated for both tretinoin and adapalene. The three cream formulations included a different component in the oil phase, namely castor oil, vitamin F or Pheroid™ vesicles.

The water phase of the creams consisted of distilled water, Veegum® and Xanthan® gum. The oil phase consisted of cetyl alcohol, stearic acid, Span® 60, Tween® 80, as well as either castor oil, vitamin F or Pheroid™. Methylparaben and propylparaben were used as antimicrobial preservatives, and BHA and BHT were the antioxidants. Tretinoin was dissolved in PEG 400 before it was added to the oil phase. The water and oil phases were added together at 70 °C and homogenised at 13 500 rpm.

2.2.3 Stability testing

The six creams were subjected to three conditions, namely 25 °C and 60 % relative humidity (RH); 30 °C and 60 % RH; as well as 40 °C and 75 % RH. Stability tests were conducted before the creams were stored in the climate chambers (at time = 0) and again after one, two, three and six months. The creams were tested in terms of concentration assay, pH, rheology and mass variation.

2.2.3.1 Concentration assays

Gradient HPLC methods were developed and validated to accurately determine the concentration of degradable components (i.e. parabens, antioxidants, retinoid and tocopherol) in the cream formulations.

2.2.3.1.1 Preparation of standards

Tretinoin containing standard solutions were prepared by weighing 5 mg tretinoin, 40 mg methyl paraben, 8 mg propyl paraben, 4 mg BHA, 40 mg BHT and 40 mg dl-α-tocopherol in a 50 ml volumetric flask. This was made up to volume with THF. The final standard solution was obtained by diluting 5 ml of the solution to 50 ml with THF. Standard solutions for adapalene were prepared as described above for tretinoin, with tretinoin replaced by 20 mg adapalene.

2.2.3.1.2 Preparation of samples

A sample solution was prepared by adding 2 g cream to a 100 ml volumetric flask and making it up to volume with THF. The solution was filtered using a 0.45 µm pre-filter. For each cream formulation at each storage condition, two sample solutions were prepared. All samples were analysed in duplicate.

2.2.3.1.3 Chromatographic conditions

An Agilent® 1200 series HPLC system (Agilent Technologies, Palo Alto, CA) was equipped with an Agilent® 1200 quaternary pump, degasser, thermostat, auto sampler injection mechanism, UV detector and ChemStation software (Rev. A.10.02) for the analysis. A reversed phase Phenomenex® Luna C18 (2) column (250 x 4.60 mm) with a particle size of 5 µm was used. The mobile phase consisted of 1 g octane-1-sulphonic acid sodium salt in 1000 ml HPLC water with pH set to 3.5 using phosphoric acid (solvent A) and methanol (solvent B). The initial ratio of the solvents was 1:1. After 1 min, a linear gradient to 100 % of solvent B was applied for 7 min, followed by isocratic elution of solvent B for 17 min. At the end of each run, the gradient was reversed to its initial conditions and the column re-equilibrated for 5 min. The flow rate was 1.0 ml/min and the injection volume was 20 µl. Adapalene containing samples were analysed using UV detection at 220 nm. Detection of tretinoin containing samples required a change in wavelengths, namely 220 nm (0 to 13 min), 349 nm (13 to 16 min) and 220 nm (16 to 25 min). The retention times of the analytes were as follows: methylparaben eluted at 7.0 min, propylparaben at 9.3 min, BHA at 10.3 min, BHT at 12.6 min, tretinoin at 13.8 min, adapalene at 16 min, and tocopherol at 22.7 min.

2.2.3.2 pH

A Mettler Toledo MultiSeven pH meter and Mettler Tolodo Inlab® 410 probe were used to determine the pH of the creams. Before use, the pH meter was calibrated at pH 4.01, 7.00 and 10.01 using Mettler Toledo pH buffer solutions.

2.2.3.3 Rheology

Only creams stored at 25 °C and 60 % RH conditions were subjected to viscosity testing. A Brookfield DV-II+ viscometer, together with a Brookfield helipath and water bath (Brookfield Engineering Laboratories, Inc., Middleboro, U.S.A.), were used to measure each cream formulation's resistance to flow. A temperature of 25 °C was maintained in the water bath. Readings were taken every 10 seconds while the helipath descended and ascended, allowing the T-bar spindle (spindle T-E) to cut through the cream. A total of 30 readings were used to calculate the average viscosity.

2.2.3.4 Mass variation

A Shimadzu AUW 120D balance (Shimadzu, Japan) was used to determine the mass of each cream formulation.

2.2.4 Drug release studies

Synthetic membranes were used to determine retinoid release from the cream formulations. Studies (8 h) were performed using vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and a diffusion area of 1.075 cm^2. Ten Franz cells were used for each release study. The receptor phase consisted of a mixture of PBS with a pH of 7.4 (British Pharmacopoeia, 2009), ethanol and THF in the ratio 9:9:2. Care was taken to prevent entrapment of air bubbles underneath the membranes. The Franz cells were placed in a water bath with a maintained temperature of 37 °C. The receptor phase was continuously stirred using a Variomag® magnetic stirrer plate inside the water bath and a small stirring magnet inside each receptor compartment. A sample of cream (1 ml) was placed in contact with the whole diffusion area of the membrane in the donor compartment. In order to prevent evaporation, the donor compartment was covered with Parafilm®. The entire receptor volume of each cell was withdrawn after 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 h. After withdrawal, the receptor compartment was

immediately refilled with 2 ml receptor phase mixture at 37 °C to maintain sink conditions. Samples obtained from the receptor compartments were analysed by HPLC for

2.2.5 Transdermal diffusion studies

2.2.5.1 Skin preparation

Abdominal skin was obtained from Caucasian female patients after cosmetic abdominoplastic surgery and frozen at -20 °C. The Research Ethics Committee of the North-West University provided ethical approval (reference number 04D08) to acquire and utilise the skin. Informed consent was obtained from all donors beforehand. The skin was thawed at room temperature and the adipose and connective tissue layers were removed with a scalpel, keeping the dermal and epidermal skin layers intact. Circles with a diameter of approximately 15 mm were punched into the skin, placed between Whatman® filter papers, wrapped with aluminium foil and frozen at -20 °C. Before conducting diffusion studies, the skin circles were thawed at room temperature and visually examined for any defects.

2.2.5.2 Skin diffusion

Transdermal diffusion studies were carried out using the method described for drug release studies in section 2.2.3, and employing full thickness skin instead of silicone membranes. Each skin circle was placed between the two compartments with the stratum corneum facing towards the donor compartment. The Franz cells were placed in a water bath with a maintained temperature of 37 °C, thereby ensuring a skin surface temperature of 32 °C. A sample of cream (1 ml) in each donor compartment made contact with the whole diffusion area of the skin. The receptor phase consisted of a mixture of equal parts of PBS (pH 7.4) and ethanol. The entire receptor volume was withdrawn after 20, 40, 60, 80, 100 and 120 min and after 4, 6, 8, 10 and 12 h. After withdrawal, the receptor compartment was immediately refilled with 2 ml receptor phase mixture at 37 °C to maintain sink conditions. Samples obtained from receptor compartments were immediately analysed by HPLC for retinoid content.

2.2.5.3 Tape stripping

After the 12 h samples have been withdrawn, the Franz cells were removed from the water bath and disassembled. Parafilm® was stapled to hardboard and the skin circles pinned onto the Parafilm® with the stratum corneum facing upwards. The diffusion area of each skin circle,

clearly marked by an indent made by the two cell compartments, was carefully patted clean with tissue paper. Tape stripping was done according to the method described by Pellet et al. (1997). 3M Scotch® Magic™ tape was cut into pieces large enough to cover only the diffusion area. Sixteen strips of tape were used for each skin circle. Due to the probability of a cream residue on the surface of the skin, the first tape strip was discarded. The 15 remaining tape strips were used to separate the stratum corneum-epidermis from the epidermis-dermis. After discarding the non-diffusion area of the skin, the diffusion area was cut into small pieces. In order to extract the retinoids, the skin cuttings (epidermis-dermis) and tape strips (stratum corneum-epidermis) were placed in separate vials and soaked in a mixture of ethanol and THF in the ratio 9:1. These vials were stored at 4 °C for approximately 8 h. The skin cuttings and tape samples were centrifuged and the supernatants analysed by HPLC.

2.2.6 Standard preparation

For the analysis of sample solutions obtained from drug release studies, standards were prepared by dissolving 2 mg of the appropriate retinoid in 5 ml THF and adding that solution to 45 ml of a PBS and ethanol mixture with a 1:1 ratio.

Sample solutions withdrawn from receptor compartments during transdermal diffusion studies were compared to standards that were prepared by dissolving 2 mg of the appropriate retinoid in 5 ml THF and adding that solution to 45 ml receptor phase.

Standards used for samples obtained from skin cuttings and tape strips were prepared by dissolving 2 mg of the appropriate retinoid in 5 ml THF, where after it was added to 45 ml ethanol.

2.2.7 HPLC analysis

HPLC methods for the analysis of tretinoin and adapalene were developed and validated with the same equipment used in section 2.2.3.1.3. A Phenomenex® Luna C18 (2) column (150 x 4.60 mm) with a particle size of 5 µm was used (Phenomenex, Torrance, CA). The UV-detector was set at 349 nm for the detection of tretinoin, and at 241 nm for adapalene. The mobile phase consisted of a mixture of acetonitrile and 1% CH_3COOH in the ratio 9:1 with an operating flow rate of 1.5 ml/min. The total run time for tretinoin samples was 7.0 min with tretinoin eluting at 4.0 min. Adapalene samples had a total run time of 11.0 min with a retention time of 7.8 min for

adapalene. Standard solutions were used to draw a standard curve before analysing samples and were reanalysed after every 20 sample runs for control purposes. Injection volumes of 100 µl were used for sample solutions and 50 µl for standard solutions.

retinoid content.

2.2.8 Statistical data analysis

The average (mean) cumulative concentration of tretinoin or adapalene that diffused into the receptor phase and the average percentage released from each cream matrix were calculated. In order to determine the average flux of the retinoids, the average cumulative amount of retinoid that diffused through each cm^2 of membrane was plotted against time. The slope of the linear portion of the obtained curve was interpreted as the average flux. It was observed to extend from 2 to 8 h.

SAS software (SAS Institute, 2005) was used to calculate the median flux by means of regression coefficients. The median is the preferred method to determine flux (Gerber et al., 2008) in the case of asymmetrical (skewed) distributions or when outliers are present, because it is, unlike the mean, not affected by these distributions (Feinstein, 2002:32). Kruskal-Wallis tests were used to compare the medians of the three tretinoin creams and the medians of the three adapalene creams using Statistica software (Statsoft, 2008). If the result of the test is statistically significant, i.e. the obtained p-value is less than 0.05, at least one of the samples is different from the other samples (Corder & Foreman, 2009:100). In the case of significant differences indicated by Kruskal-Wallis testing, samples that were different from the others were identified using multiple comparison tests.

3 Results and discussion

3.1 Formulated creams

Three creams for both tretinoin (0.025 %) and adapalene (0.1 %) were formulated, namely castor oil cream, vitamin F cream and Pheroid™ cream, separately. All the creams were fine and homogeneous. It could be applied easily to the skin without being oily or tacky. The tretinoin containing castor oil and vitamin F creams had a pale yellow colour, while the Pheroid™ cream was slightly deeper yellow. The castor oil and vitamin F creams with adapalene were very light beige, whereas the Pheroid™ cream presented with a very light yellow colour.

3.2 Stability studies

3.2.1 Concentration assays

All the cream formulations showed evidence of degradation of parabens, antioxidants, retinoids and, in the case of the Pheroid™ creams, tocopherol. The castor oil creams proved to be the most stable formulations, whereas the Pheroid™ creams were the most unstable. Adapalene was more stable than tretinoin in similar formulations. The tocopherol in the Pheroid™ creams was completely degraded at 40 °C and 75 % RH after six months, possibly due to a more significant antioxidant effect than BHA and BHT. The possibility of a pro-oxidant effect of tocopherol, leading to accelerated oxidation of other components, might have contributed to the Pheroid™ creams being the least stable formulations.

3.2.2 pH

No significant change in pH was observed for any cream formulation during six months.

3.2.3 Rheology

All the creams demonstrated a significant increase in viscosity, with the percentage increase ranging from 12.5 to 48.4 %. The viscosity of the vitamin F cream with adapalene was the most stable, while castor oil cream with adapalene had the highest increase in viscosity. The increase in viscosity was due to hydration of the gums during the stability testing period.

3.2.4 Mass variation

All the creams showed an insignificant and acceptable amount of mass loss. The highest mass loss after six months (1.194 %) was observed in adapalene and vitamin F cream at 40 °C and 75 % RH.

3.2 Drug release studies and statistical data analysis

Table 1: Results obtained from release studies of over a period of 8 h

The castor oil formulation showed the highest average tretinoin flux (0.448 μg/cm^2.h), followed by the vitamin F cream (0.199 μg/cm^2.h) and Pheroid™ cream (0.079 μg/cm^2.h). Castor oil cream, therefore, presented with the best tretinoin release. Table 1 summarises tretinoin and adapalene release from the cream formulations.

Box-plots representing the average tretinoin fluxes of the three cream formulations can be seen in Figure 1a. Median fluxes are illustrated by the thick horizontal black lines and the average

fluxes by the vertical grey lines. Castor oil cream had a nearly symmetrical distribution with approximately the same average and median flux values. The Pheroid™ cream had a smaller and slightly asymmetrical distribution; its average and median flux values did not differ significantly. Vitamin F cream showed a wide distribution that was skewed to the right, resulting in a significant difference between the average flux (0.119 ± 0.108 μg/cm^2.h) and median flux (0.083 μg/cm^2.h). In this case the median was a better indicator of flux than the average, because the skewed distribution did not affect the median (Gerber et al., 2008).

A p-value of 0.001 was calculated by means of a Kruskal-Wallis test, indicating at least one significant difference between the median fluxes of the three tretinoin creams. Multiple comparison tests indicated no significant difference between the median fluxes of the Pheroid™ and vitamin F creams (p = 1.0000). The median flux of castor oil cream differed significantly from those of the Pheroid™ and vitamin F creams with respective p-values of 0.0006 and 0.0003.

Tretinoin metabolites were detected in samples withdrawn from receptor compartments. Receptor phase samples of vitamin F cream and castor oil cream respectively contained 0.018% and 0.014 % metabolites. Pheroid™ cream could have protected tretinoin from degradation as no metabolites resulted from this formulation.

Figure 1: Box-plots of the flux values (μg/cm^2.h) for **a)** tretinoin and **b)** adapalene in castor oil cream, Pheroid™ cream and vitamin F cream, respectively

Adapalene was best released from castor oil cream with an average flux of 1.5106 μg/cm^2.h. The average flux values for the Pheroid™ and vitamin F creams were 1.289 μg/cm^2.h and 0.902 μg/cm^2.h, respectively.

Box-plots of the flux values of adapalene are shown in Figure 1b. Castor oil cream had a slightly asymmetrical distribution. Its median flux and average flux was the same. The distribution of data points of the Pheroid™ cream was slightly skewed to the left. The average flux was higher than the median flux due to the presence of an outlier. The low minimum values of the vitamin F cream rendered its average flux slightly lower than its median flux.

A Kruskal-Wallis test indicated at least one significant difference between the median fluxes of the three adapalene creams (p = 0.000). Multiple comparison tests gave p-values of 0.000018

and 0.019715 when vitamin F cream was compared to castor oil cream and Pheroid™ cream respectively, indicating that the median flux of vitamin F cream was significantly different from the fluxes of the other two creams. A p-value of 0.213975 resulted when castor oil cream was compared to Pheroid™ cream. The median fluxes of these two creams were, therefore, not significantly different.

3.3 Transdermal diffusion studies

No retinoids were detected in any receptor compartments, indicating that neither tretinoin nor adapalene diffused across the skin. This could be attributed to the high lipophilicity of the retinoids, as indicated by their high log P (octanol-water partition coefficient) values (6.30 (tretinoin) and 8.04 (adapalene)) (Valiveti et al., 2008; Syracuse Research Corporation, 2010).

3.4 Tape stripping

The stratum corneum-epidermal samples (tape strips) also contained no retinoids, possibly due to the significantly low drug release of the creams which led to low concentrations of retinoids available for diffusion.

The castor oil cream was the only formulation with tretinoin present in the epidermis-dermis (skin cuttings) (0.0076 %). Very small amounts of adapalene were present in the epidermis-dermis. The percentage of the applied dose of adapalene that reached the dermis was identical for the castor oil and Pheroid™ creams (0.109 %). The vitamin F formulation was inferior with only 0.053 % adapalene in the epidermis-dermis. Therefore, adapalene from all three its formulations and tretinoin from only castor oil cream reached the target site.

Due to the small amounts of tretinoin and adapalene applied to the skin (250 µg and 1000 µg, respectively), their concentration gradients across the skin were possibly suboptimal. It must be kept in mind that the applied concentration of adapalene (0.1 %) was four times higher than that of tretinoin (0.025 %). This higher concentration could have contributed to the significantly better penetration of adapalene.

As had been observed in the drug release studies, the composition of the cream matrices hindered adequate release of the retinoids which might have led to insufficient concentrations in the skin. The small amounts of tretinoin and adapalene released by the creams influenced the

concentration of retinoids available for diffusion and, therefore, lowered the concentration gradient.

When compared to results of the release studies of the tretinoin creams, it was observed that the castor oil cream showed both a significantly higher release and higher skin penetration than the other two creams. There was a correlation between the percentage of adapalene released and the percentage that penetrated the epidermis-dermis. The castor oil and Pheroid™ creams were the two adapalene creams with the highest release, as well as the highest skin penetration.

4 Conclusion

Cream formulations were developed and incorporated tretinoin or adapalene in a castor oil, vitamin F or Pheroid™ based oil phase. *In vitro* release studies identified castor oil cream as superior in tretinoin release, with the most adapalene released by the castor oil and Pheroid™ creams. During *in vitro* diffusion studies, castor oil cream was superior in delivering tretinoin to the dermis. Both the castor oil and Pheroid™ creams delivered the most adapalene to the epidermis-dermis. The castor oil creams were the most stable creams during a period of six months, while the Pheroid™ creams were the most unstable, possibly due to oxidation reactions involving tocopherol. It can be concluded that castor oil cream was the most suitable formulation for tretinoin and adapalene in terms of stability, drug release and dermal diffusion. Vitamin F cream was the inferior formulation, surpassing Pheroid™ cream only in terms of stability.

Acknowledgements

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Figure legends

Figure 1: Box-plots of the flux values (µg/cm^2.h) for **a)** tretinoin and **b)** adapalene in castor oil cream, Pheroid™ cream and vitamin F cream, respectively

Tables

Table 1: Results obtained from release studies over a period of 8 h

Formulation	Average % released	Average cumulative conc. (µg/ml)	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)
Tretinoin creams				
Castor oil cream	0.763	1.907	0.448 ± 0.032	0.465
Pheroid™ cream	0.162	0.405	0.079 ± 0.0096	0.084
Vitamin F cream	0.219	0.548	0.119 ± 0.108	0.083
Adapalene creams				
Castor oil cream	0.660	6.605	1.51 ± 0.15	1.51
Pheroid™ cream	0.584	5.842	1.29 ± 0.18	1.27
Vitamin F cream	0.374	3.746	0.90 ± 0.20	0.92

Figures

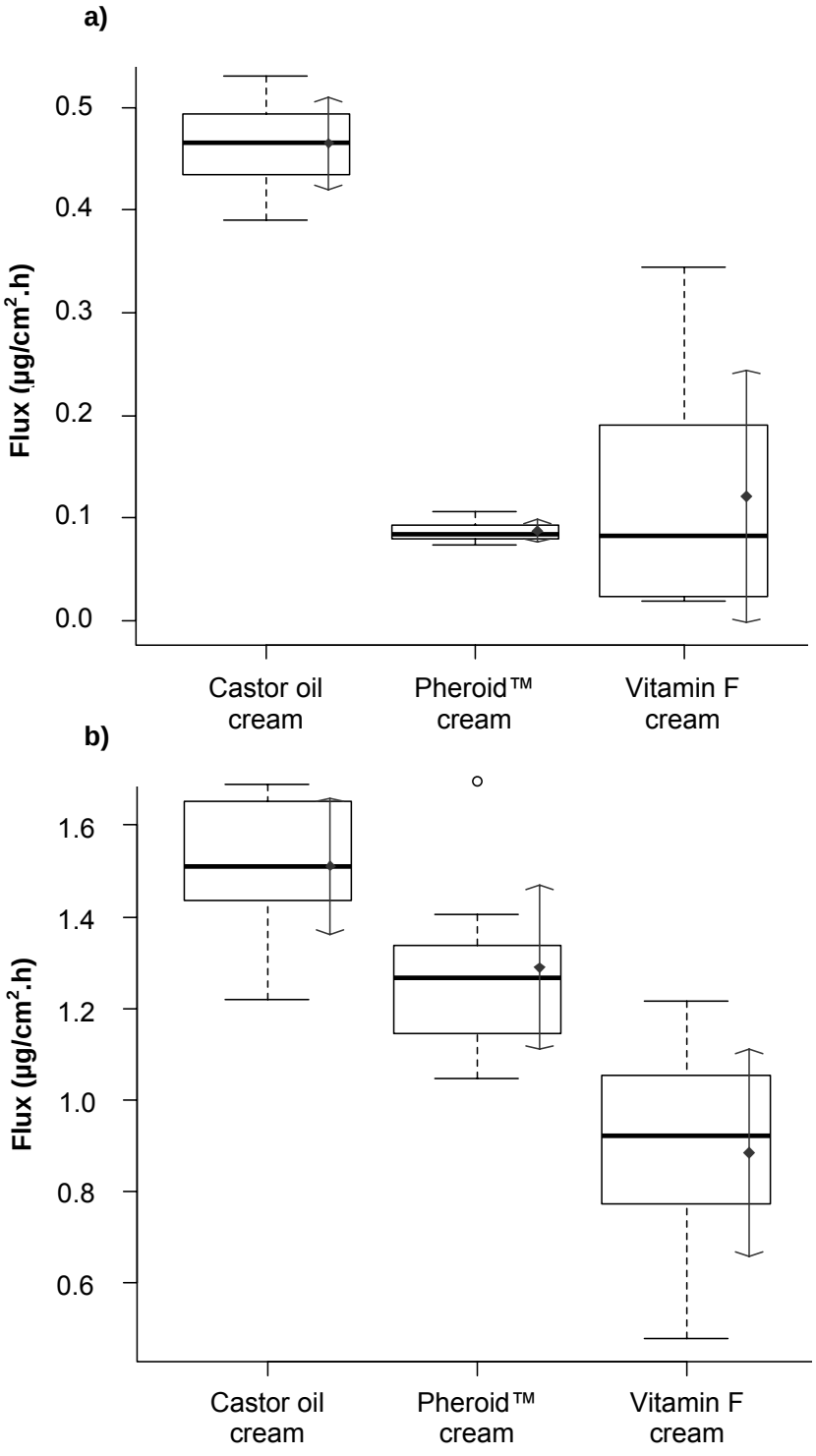

Figure 1: Box-plots of the flux values (µg/cm^2.h) for **a)** tretinoin and **b)** adapalene in castor oil cream, Pheroid™ cream and vitamin F cream, respectively

Chapter 4

Final conclusions

The main aim of this study was to formulate a topical product containing either tretinoin or adapalene that was stable and showed enhanced dermal delivery and retinoid release. This was done by formulating three creams for both tretinoin and adapalene and subjecting the creams to *in vitro* drug release studies, conducting *in vitro* transdermal diffusion studies, and evaluating the stability of the creams over a period of six months. The results of these experiments are summarised in the following paragraphs.

Topical cream formulations were developed and incorporated tretinoin or adapalene in different oil phases. Three creams were formulated for each of tretinoin (0.025 %) and adapalene (0.1 %), namely castor oil cream, vitamin F cream and Pheroid™ cream.

The castor oil creams were the most stable creams during a period of six months, followed closely by the vitamin F creams. Concentration assay showed significant degradation of the analytes of all the cream formulations. The castor oil creams were the most stable formulations. Adapalene was more stable than tretinoin in similar formulations. The α-tocopherol in the Pheroid™ creams was completely degraded at 40 °C and 75 % RH after 6 months, which was possibly due to insufficient protection by the antioxidants or a pro-oxidative effect of α-tocopherol.

All the creams demonstrated a significant increase in viscosity due to additional hydration of the Veegum® and Xanthan® gum during the stability testing period. The creams remained constant during the six months in terms of pH, homogeneity, particle size, visual assessment, and mass variation. Only the Pheroid™ creams depicted significant changes in colour, which were possibly due to oxidation reactions involving α-tocopherol.

The *in vitro* drug release studies showed that tretinoin and adapalene were released from all their respective formulations. No cream released more than 1 % of its retinoid. Castor oil cream was significantly superior in tretinoin release, with 0.763 % of the initial concentration released after eight hours. Most adapalene was released by the castor oil and Pheroid™ creams (0.660 % and 0.584 %, respectively). Tretinoin metabolites were detected in the receptor phases of both castor oil cream (0.018 %) and vitamin F cream (0.014 %), whereas no metabolites were present in the Pheroid™ samples.

No retinoids were present in the epidermis or receptor phase. Tretinoin from only castor oil cream reached the dermis, whereas adapalene from all three creams was detected in the dermis, implying that the retinoids from these four creams reached the target site. Castor oil and Pheroid™ creams delivered the most adapalene to the dermis. The concentrations of tretinoin

and adapalene that reached the dermis were, however, insufficient. Factors that influenced the diffusion results included excessive lipophilicity of the retinoids, insufficient concentration gradients, and a low percentage of unionised molecules. A relation was drawn between retinoid release and dermal delivery and, therefore, the suboptimal release of tretinoin and adapalene from the cream matrices led to decreased concentrations of retinoids available for diffusion.

It may be concluded that the castor oil cream was the most suitable formulation for tretinoin and adapalene in terms of drug release, dermal diffusion and stability. Vitamin F cream was the inferior formulation, surpassing Pheroid™ cream only in terms of stability.

Proposed future research includes investigating the formulation of the creams in order to increase their stability. The use of different antioxidants should be considered and conditions for the optimum hydration of the Veegum®-Xanthan®-blend need to be determined. The cause of the discolouration of the Pheroid™ creams should be investigated, and possible oxidative and pro-oxidative reactions of α-tocopherol should be examined. Methods to increase retinoid release and dermal delivery should be investigated. The absence of retinoids in the epidermis also necessitates further investigation.

Appendix A

Validation of analytical HPLC methods for transdermal diffusion and drug release studies

A.1 Purpose

A sensitive, accurate and reliable method was needed in order to accurately determine the amount of tretinoin or adapalene released by the creams formulations, as well as the amount of retinoids that diffused into and through skin during *in vitro* studies. An HPLC method for the analysis of each of the retinoids was developed and validated, with differences found only in the detection wavelengths, retention times and, therefore, in the run times.

A.2 Chromatographic conditions

A.2.1 Tretinoin

Analytical instrument:	Agilent® 1200 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1200 quaternary pump, degasser, thermostat, autosampler injection mechanism, UV detector and ChemStation software (Rev. A.10.02) for the acquisition and analysis of data.
Column:	Phenomenex® Luna 5 µm C18 (2), 250 x 4.60 mm.
Mobile phase:	Filtered and degassed mixture of gradient grade acetonitrile and 1 % CH_3COOH in the ratio 9:1.
Run time:	10 min.
Flow rate:	1.300 mL/min.
Injection volume:	50 µL.
Detection:	349 nm using UV detection (Ioele *et al.*, 2005:253).
Retention time:	Tretinoin eluted after 7.2 minutes.
Solvent:	Tetrahydrofuran (THF) (Chromasolv®, Sigma Aldrich).
Precautions:	All actions involving tretinoin were conducted under reduced lighting using amber glassware. The thermostat was set at 5°C.

A.2.2 Adapalene

Analytical instrument:	Agilent® 1200 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1200 quaternary pump, degasser, thermostat, autosampler injection mechanism, UV detector and ChemStation software (Rev. A.10.02) for the acquisition and analysis of data.
Column:	Phenomenex® Luna 5 µm C18 (2), 250 x 4.60 mm.
Mobile phase:	Degassed and filtered mixture of gradient grade acetonitrile and 1 % CH_3COOH in the ratio 9:1.
Run time:	16 minutes.
Flow rate:	1.300 mL/min.
Injection volume:	50 µL.
Detection:	241 nm using UV detection.
Retention time:	Adapalene eluted after 11.4 minutes.
Solvent:	THF (Chromasolv®, Sigma Aldrich).

A.3 Standard preparation

A.3.1 Tretinoin

Weigh approximately 2 mg tretinoin accurately and transfer to a 10 ml volumetric flask. Make up to volume with the solvent. Dilute 5 ml of the solution to 50 ml with solvent. This was the 100% standard. Transfer to an HPLC vial and analyse.

A.3.2 Adapalene

Weigh approximately 2 mg adapalene accurately and transfer to a 20 ml volumetric flask. Make up to volume with the solvent. Dilute 5 ml of the solution to 50 ml with solvent. This was the 100% standard. Transfer to an HPLC vial and analyse.

A.4 Validation parameters

A.4.1 Specificity

Specificity is the ability to clearly differentiate between an analyte and other compounds expected to be present in the sample such as impurities, degradation products, other active ingredients or matrix (Hong & Shah, 2000:363; ICH, 2005:4). In the case of tretinoin where degradation products (with isotretinoin as the main degradant) are closely related and can easily interfere with analysis, specificity testing is indispensable.

Forced degradation was used to determine the specificity of the HPLC method. A standard was prepared as described in section 3 and diluted (1:1) with distilled water, 0.1 M HCl, 0.1 M NaOH, and 10 % H_2O_2, respectively. Retinoids are more prone to degradation; therefore the solutions were tested after only three hours. Specificity for tretinoin was also investigated by exposing the standard solution to light for one hour and by spiking a sample with isotretinoin. In order to comply with the acceptance criteria, no peaks must interfere with that of the retinoid.

A.4.2 Linearity and range

Within a given range, the analytical method must be able to obtain test results in direct proportion to the concentration of the analyte in the sample (ICH, 2005:5).

Linearity was investigated by analysing the following different samples of known concentrations: 0.02, 0.2, 2.0, 20 and 200 µg/ml for tretinoin, and 0.1, 1.0, 10 and 100 µg/ml for adapalene. Of each of the mentioned standards, 10, 20, 30, 40 and 50 µl were injected in duplicate.

By using the peak area ratios and concentrations, linear regression analysis was used to determine the slope, y-intercept and regression coefficient. In order to meet the acceptance criteria, the regression coefficient (R^2) had to be equal to or larger than 0.99.

The range of an analytical procedure is the interval from the upper to the lower concentration of an analyte in a sample which demonstrated to be precise, accurate and linear, and is normally derived from linearity studies (ICH, 2005:5, 8).

A.4.3 Accuracy

The ICH (2005:4) defined accuracy as an indication of the closeness of experimental results to the true value.

Three standard solutions of different concentrations were used to determine the accuracy of the HPLC method. The concentrations used for the tretinoin standards were 0.2, 2.0, 20, and 200 µg/ml, and for the adapalene standards: 1.7, 17 and 170 µg/ml. In order to be considered accurate, the recovery had to be between 98 and 102 %.

A.4.4 Precision

Precision is defined as the closeness of a number of measurements from multiple samples of the same homogenous sample to each other (ICH, 2005:4). Hong and Shah (2000:361) described precision more simplistically as the distribution of test results around their average.

Precision can be divided into repeatability (also known as intraday precision), intermediate precision (also called interday precision) and reproducibility (ICH, 2005:5).

A.4.4.1 Repeatability

Repeatability is the precision measured over a short period of time under the same conditions (ICH, 2005:5).

Results of the accuracy testing were used to determine the repeatability. The relative standard deviation (RSD) should be less than 3 % for tretinoin (US Pharmacopoeial Convention. 2010) and less than 2 % for adapalene.

A.4.4.2 Intermediate precision

Intermediate precision is evaluated by using various operating conditions (e.g. different analysts or equipment, or analysis on different days) in the same laboratory (ICH, 2005:5).

The three standard solutions of average concentration used for repeatability testing (i.e. the three 20 µg/ml tretinoin standards and the three 17 µg/ml adapalene standards) were analysed on two other days. The intermediate precision had to be less than or equal to 5 %.

A.4.5 Ruggedness

Hong and Shah (2000:368) defined ruggedness as the measure in which test results can be reproduced under normal conditions, whether using different laboratories or different analysts. It can be seen as a test for environmental or experimental factors or variables influencing the analytical method. Examples of possible variables include pH, temperature, mobile phase composition, column length and flow rate. System repeatability and stability of sample solutions are subdivisions of ruggedness.

A.4.5.1 Stability of sample solutions

The stability was evaluated by preparing a standard solution (see section A.3) and analysing it immediately and at hourly intervals for up to 15 hours. Acceptance criteria suggested that degradation might be no more than 2 %.

A.4.5.2 System repeatability

System repeatability was tested by preparing a standard, as described in section A.3, and injecting it six times consecutively. In order to comply, the RSD of the peak area and the retention time should be equal to or less than 2 %.

A.5 Results and discussion

A.5.1 Tretinoin

The HPLC method for the analysis of tretinoin (section A.2.1) was validated in order to determine its specificity, linearity, range, accuracy, precision and ruggedness. The results obtained are provided in this section.

A.5.1.1 Specificity

The following eight chromatograms (figures A.1 to A.8) were obtained during specificity testing. All the chromatograms reflect absorbance at a wavelength of 349 nm over a period of ten minutes. In figure A.1, a chromatogram of a standard tretinoin solution is shown.

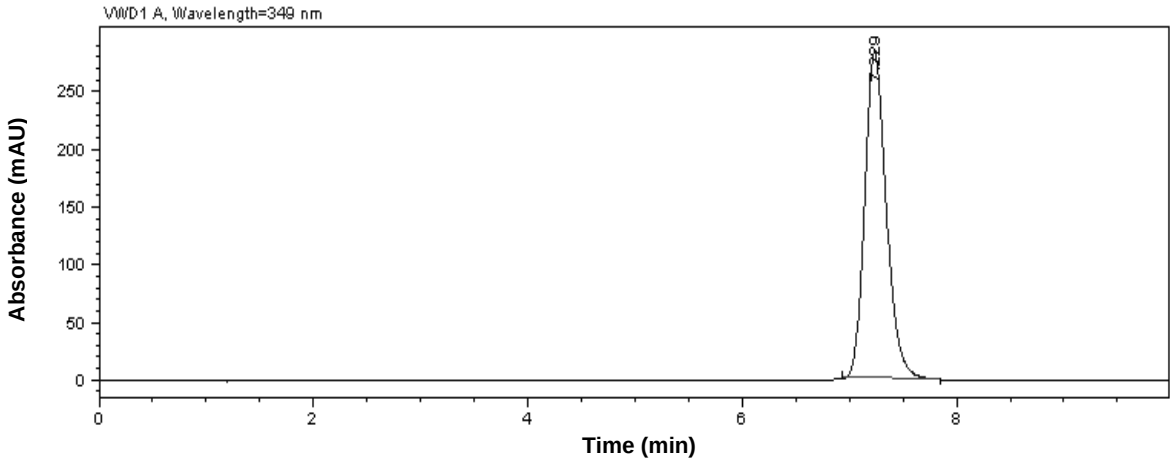

Figure A.1: Chromatogram of a standard tretinoin solution

As could be seen in figure A.2, degradation occurred when the standard solution was stressed in water.

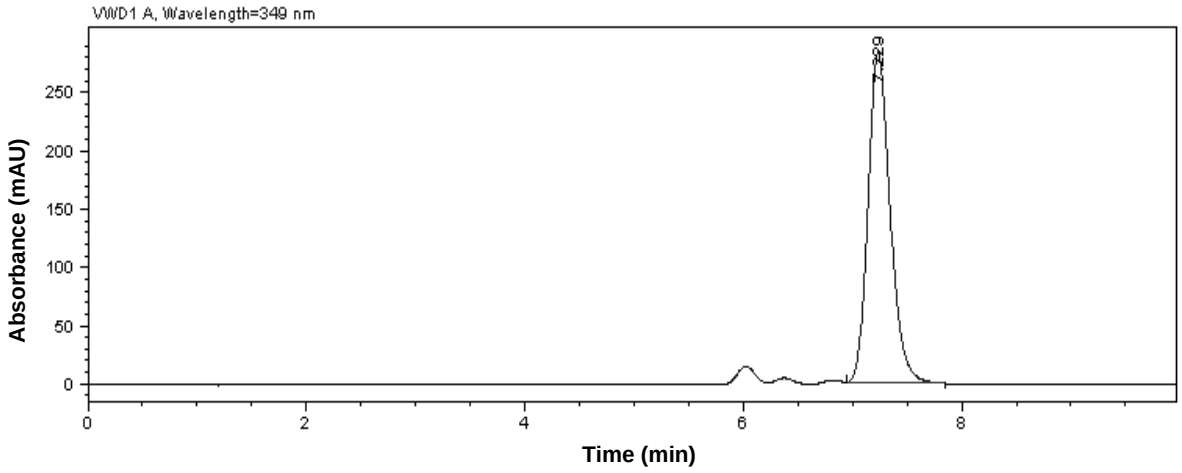

Figure A.2: Chromatogram of a standard solution stressed in water for three hours

A standard solution stressed in hydrochloric acid was more prone to degradation, as shown in figure A.3.

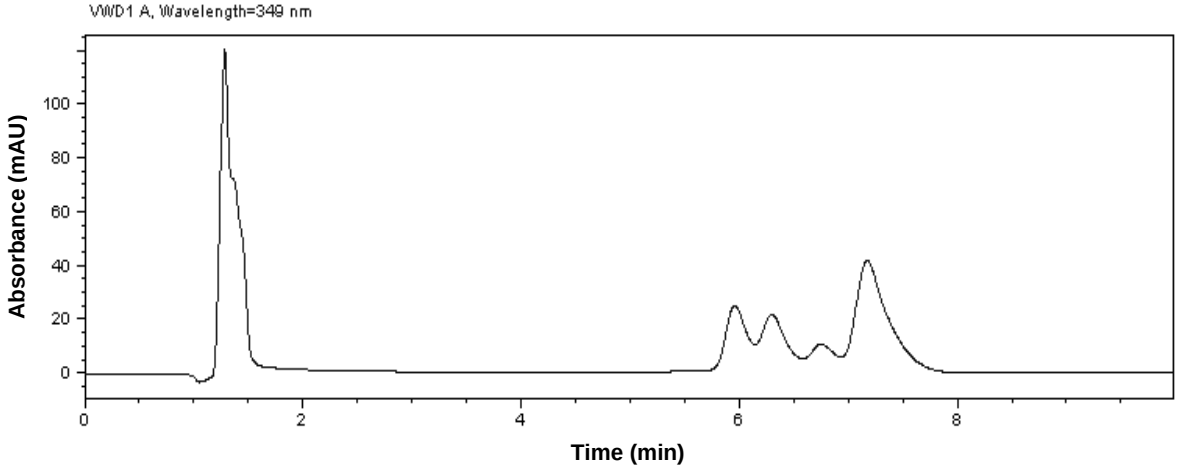

Figure A.3: Chromatogram of a standard solution stressed in 0.1 M HCl for three hours

After being stressed in 0.1 M sodium hydroxide for three hours, the tretinoin was completely degraded, as shown in figure A.4.

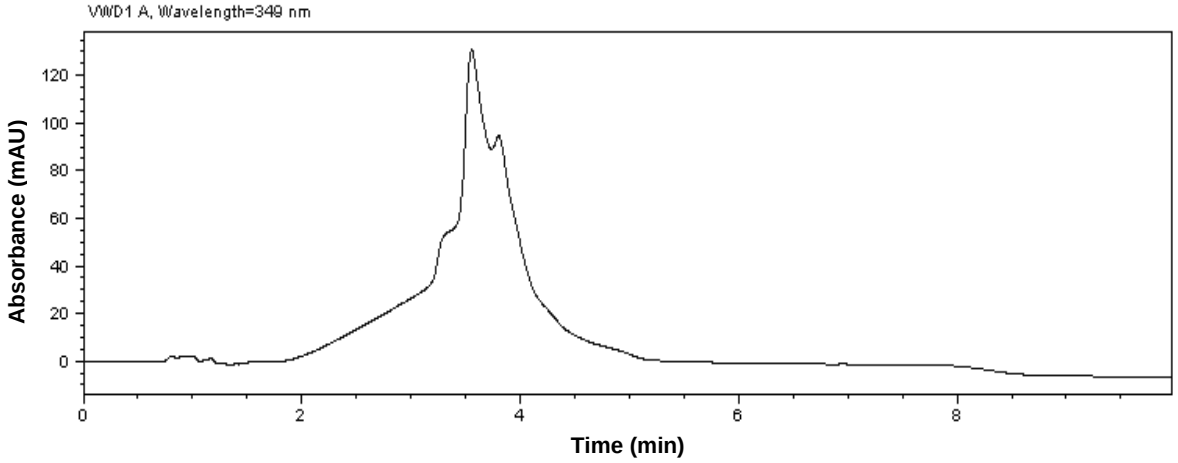

Figure A.4: Chromatogram of a standard solution stressed in 0.1 M NaOH for three hours

Another test was conducted where tretinoin was stressed with 0.1 M NaOH for only 10 minutes. The result is shown in figure A.5. After a short period of 10 minutes, tretinoin was completely degraded.

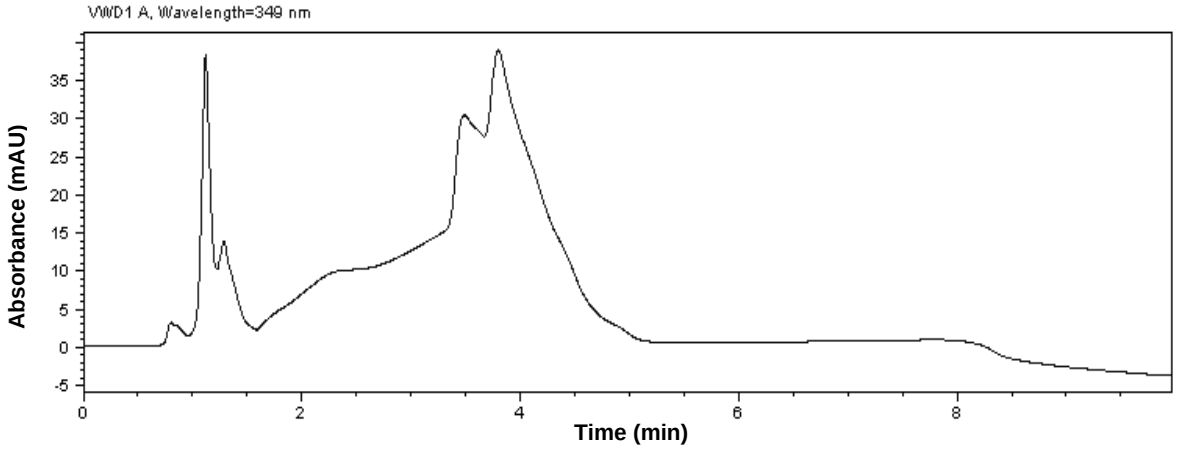

Figure A.5: Chromatogram of a standard solution stressed in 0.1 M NaOH for ten minutes

As could be observed in figure A.6, tretinoin was degraded significantly in the presence of hydrogen peroxide.

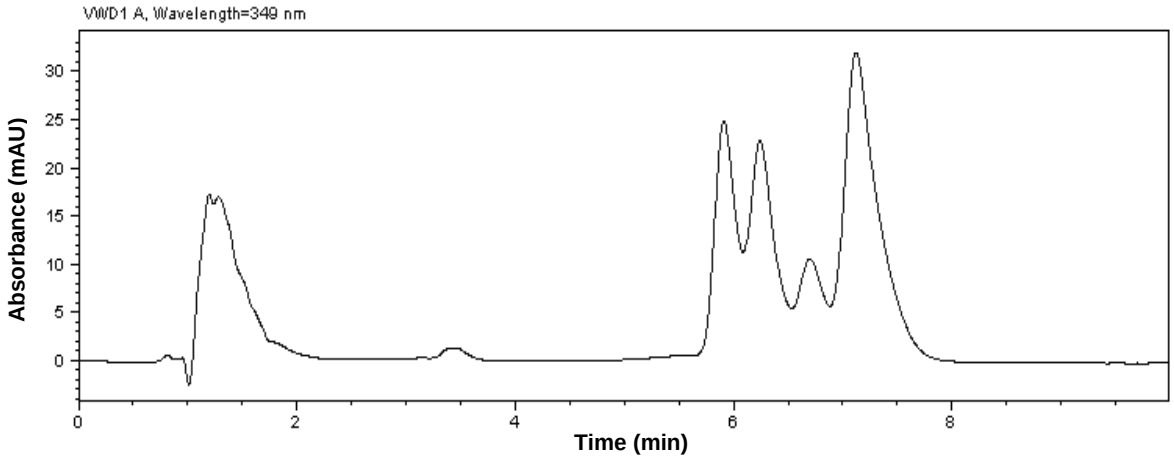

Figure A.6: Chromatogram of a standard solution stressed in 10 % H_2O_2 for three hours

A solution of tretinoin in tetrahydrofuran was exposed to light for a period of one hour. Three degradation products can be observed in figure A.7.

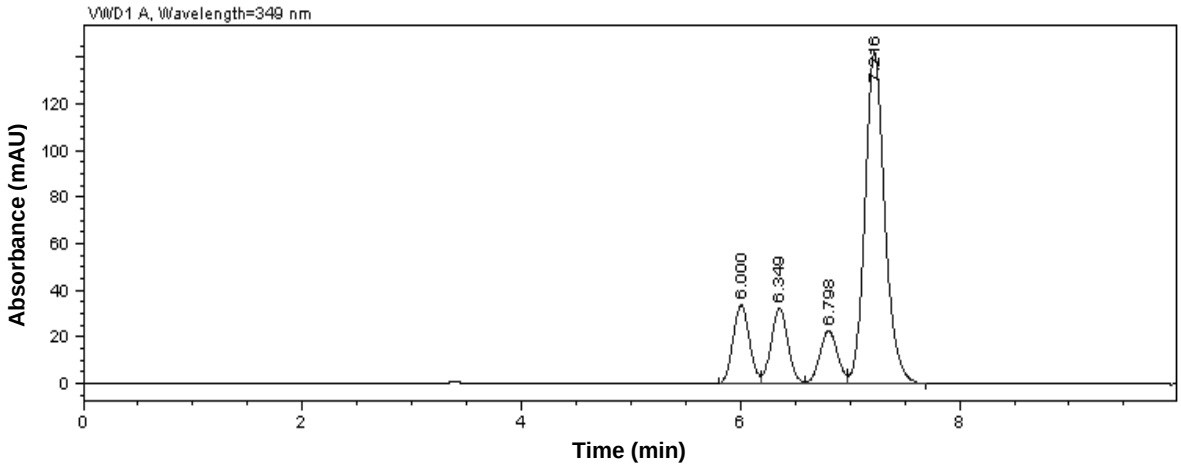

Figure A.7: Chromatogram of a standard solution exposed to light for one hour

In order to identify the degradation products, a tretinoin solution was spiked with isotretinoin (figure A.8). The peak eluting first was identified as isotretinoin. One of the two peaks between isotretinoin and tretinoin was alitretinoin (Tashtoush *et al.*, 2007:863; Gatti *et al.*, 2000:148).

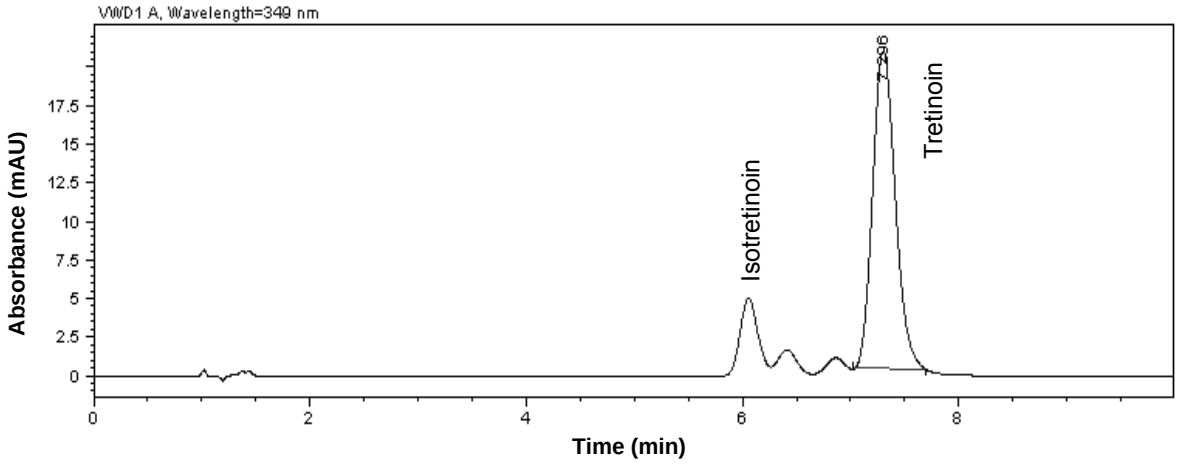

Figure A.8: Chromatogram of a standard solution spiked with isotretinoin.

None of the degradation products interfered with the tretinoin peak, thereby proving the method's specificity. Tretinoin is extremely susceptible to degradation in the presence of sodium hydroxide, as no tretinoin remained after just 10 minutes of exposure. The method can also be used for the detection of isotretinoin only, and for the simultaneous detection of tretinoin, isotretinoin and other degradation products, such as alitretinoin.

A.5.1.2 Linearity and range

Table A.1 presents the peak area ratios obtained after analysing samples of known concentrations over a specified concentration range. Regression statistics were used to calculate the regression coefficient (R^2). The linear regression graph is shown in figure A.9.

Table A.1: Peak area ratios obtained after analysing samples of known concentrations

µg/ml	Area 1	Area 2	Mean
0.392	104.558	104.291	104.425
0.784	205.794	204.504	205.149
1.176	302.714	300.406	301.56
1.568	394.2	393.989	394.095
1.96	481.0	481.7	481.4
3.92	1202.7	1196.6	1199.4
7.84	2385.1	2382.0	2383.6
11.76	3528.0	3534.3	3531.2
15.68	4724.4	4720.3	4722.4
19.6	5861.2	5861.6	5861.4
39.2	12775.0	12773.5	12774.3
78.4	25454.1	25451.4	25452.8
117.6	37506.6	37470.2	37488.4
156.8	49353.2	49342.5	49347.9
196	61016.0	60938.8	60977.4

Regression statistics	
R^2	0.9997
Intercept	-5.5537
Slope	314.56

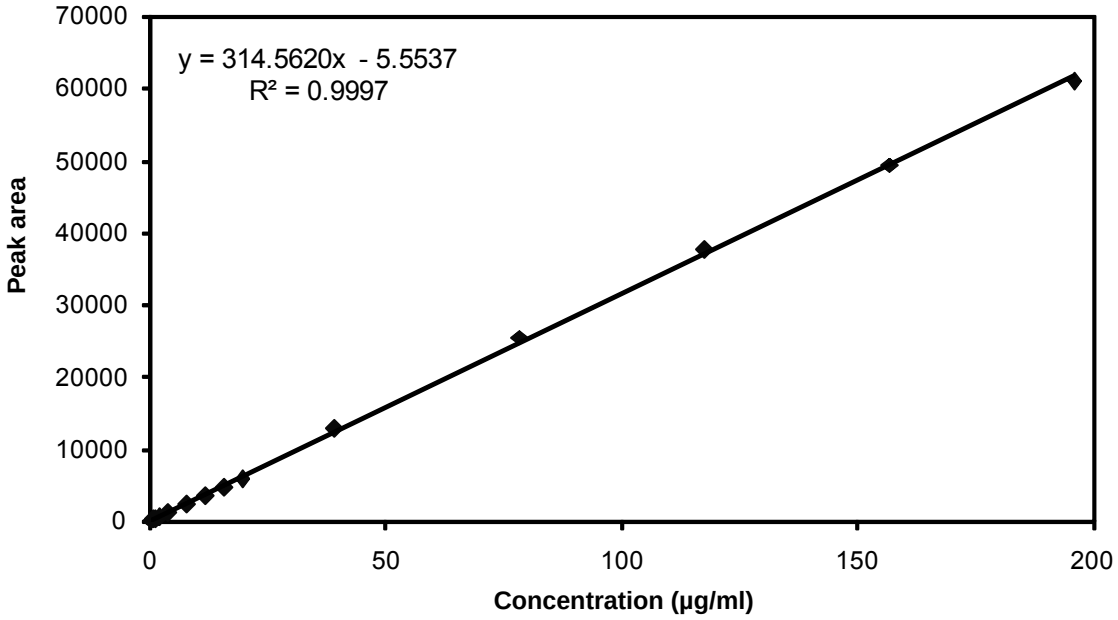

Figure A.9: Linear regression graph for tretinoin

With a regression coefficient of 0.9997, the method showed linearity in the range of 0.39 to 196 µg/ml. This method proved to be suitable for single point calibration.

A.5.1.3 Accuracy and repeatability

The accuracy of the HPLC method was determined by analysing standards with three different concentrations. The mean recovery (table A.2) was an indication of the accuracy, and the RSD indicated the method's repeatability.

Table A.2: Peak area ratios and percentage recovery of tretinoin

Conc. Spiked (µg/ml)	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
2.02	375.2	373.4	374.3	2.0	100.6
2.02	398.5	392.5	395.5	2.1	106.3
2.02	359.4	359.3	359.4	2.0	96.6
20.2	4997.1	4987.2	4992.2	19.4	96.1
20.2	5255.8	5251.4	5253.6	20.4	101.1
20.2	5388.9	5382.2	5385.6	20.9	103.7
121.2	36755.9	36737.2	36746.6	122.1	100.8
121.2	37258.1	37233.1	37245.6	123.8	102.1
121.2	37422.1	37399.7	37410.9	124.4	102.6
				Mean	101.1
				SD	3.03
				% RSD	3.00

In table A.2 it can be seen that a recovery of 101.1 % was obtained, proving the method to be accurate. With an RSD of 3.00%, the repeatability was just within the acceptable limit.

A.5.1.4 Intermediate precision

The 20.2 µg/ml standard used for testing the method's accuracy and precision (section A.5.1.3), were analysed on two more days. The results obtained are shown in table A.3. The RSD indicated the intermediate precision, which was well within acceptable levels.

Table A.3: Peak areas obtained on three different days

	Area 1	Area 2	Mean	SD	% RSD
Day 1	6125.5	6117.1	6121.3	4.20	0.069
Day 2	5982.7	5976.8	5979.8	2.95	0.049
Day 3	5856.3	5856.6	5856.5	0.15	0.003
Between days			5985.83	108.21	1.81

A.5.1.5 Stability of sample solutions

The stability of sample solutions was evaluated over a period of 15 hours. No degradation of tretinoin was observed, as shown in table A.4,

Table A.4: Percentage of tretinoin remaining in solution during 15 hours, indicating its stability

Time (hours)	Peak area	% Remaining
0	15449.9	100.0
1	15464.6	100.1
2	15441.9	99.9
3	15455.4	100.0
4	15438.2	99.9
5	15435.1	99.9
6	15445.0	100.0
7	15459.7	100.1
8	15459.0	100.1
9	15472.3	100.1
10	15457.1	100.0
11	15485.8	100.2
12	15488.2	100.2
13	15513.6	100.4
14	15521.3	100.5
15	15507.3	100.4
Mean	15468.4	100.1
SD	26.40	0.17
% RSD	0.17	0.17

A.5.1.6 System repeatability

A tretinoin sample was analysed six times consecutively. The peak areas and retention times indicated the repeatability of the system. The results are shown in table A.5.

Table A.5: Peak areas and retention times indicating system repeatability

	Peak area	Retention time (minutes)
	6240.0	6.965
	6231.4	6.970
	6230.5	6.965
	6222.4	6.965
	6213.0	6.960
	6207.9	6.949
Mean	6224.2	6.962
SD	11.07	0.007
% RSD	0.18	0.095

The repeatability of the peak area and the retention time were well within limits. Therefore, the system proved to have adequate repeatability.

A.5.1.7 Conclusion

The method was reliable, accurate and specific. It could be used for the analysis of tretinoin containing samples obtained from *in vitro* drug release and diffusion studies. This method complied with the criteria for specificity, linearity, range, accuracy, precision and ruggedness.

A.5.2 Adapalene

The HPLC method developed for analysing adapalene (section A.2.2) was validated in order to determine its specificity, linearity, range, accuracy, precision and ruggedness. This section presents the obtained results.

A.5.2.1 Specificity

The following five chromatograms (figures A.10 to A.14) are indications of the specificity of the HPLC method. All the chromatograms reflect absorbance at a wavelength of 241 nm over a period of 16 minutes. In figure A.10, a chromatogram of a standard adapalene solution is shown.

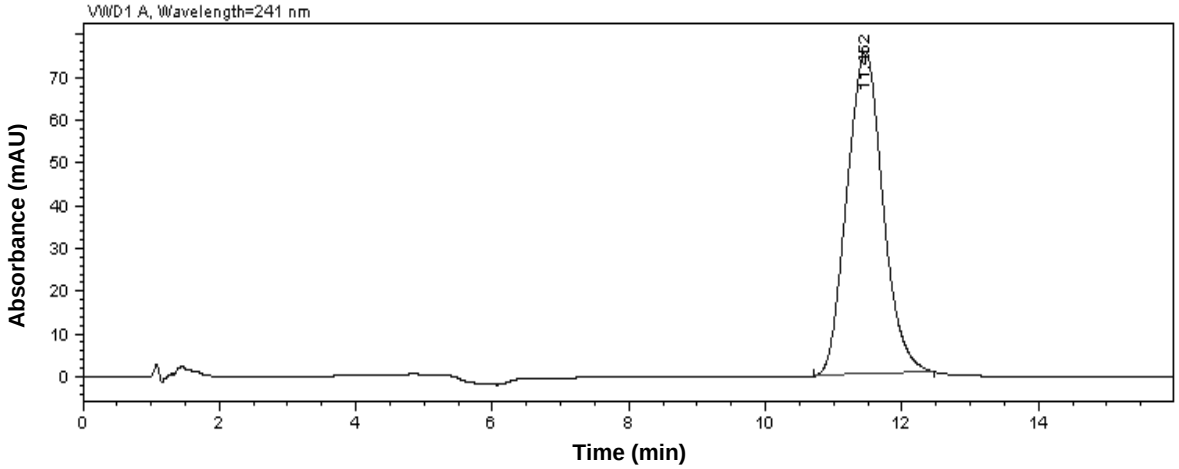

Figure A.10: Chromatogram of a standard adapalene solution

After stressing a standard solution in water, no degradation of adapalene was observed (figure A.11).

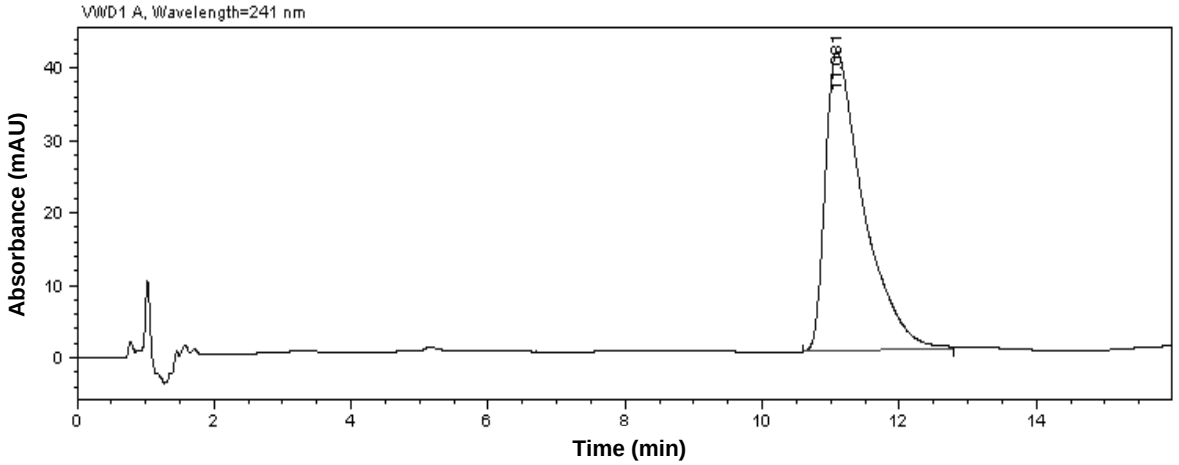

Figure A.11: Chromatogram of a standard adapalene solution stressed in water for three hours

Adapalene did not degrade in the presence of hydrochloric acid, as shown in figure A.12.

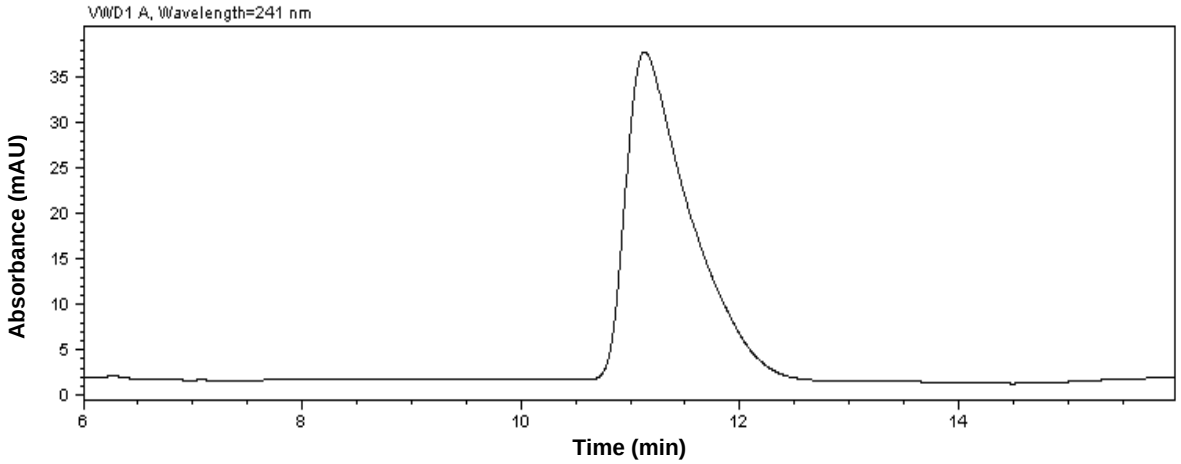

Figure A.12: Chromatogram of a standard solution stressed in 0.1 M HCl for three hours

Adapalene was completely degraded after 10 minutes' exposure to sodium hydroxide (figure A.13).

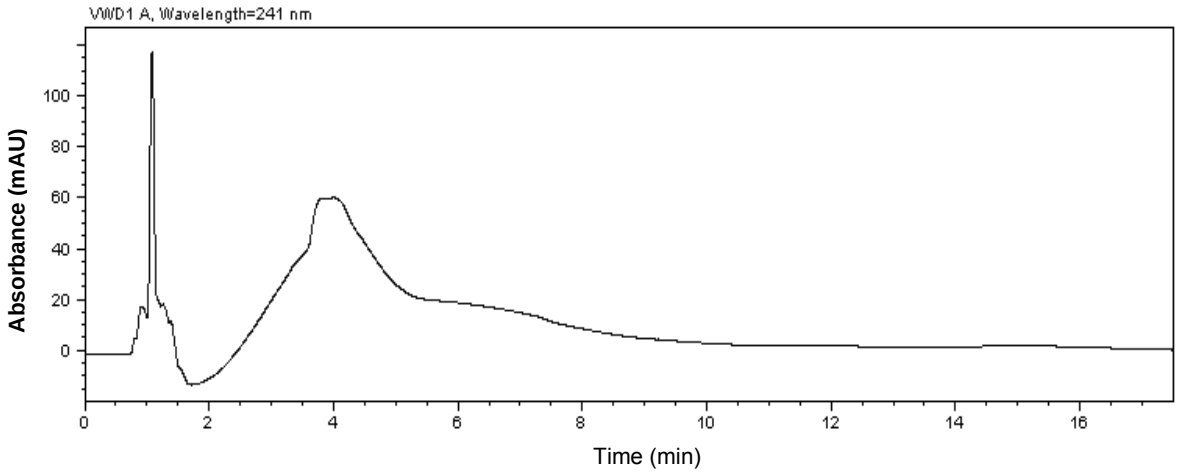

Figure A.13: Chromatogram of a standard solution stressed in 0.1 M NaOH for ten minutes

Figure A.14 indicates that no degradation of adapalene was observed after stressing a standard solution with hydrogen peroxide.

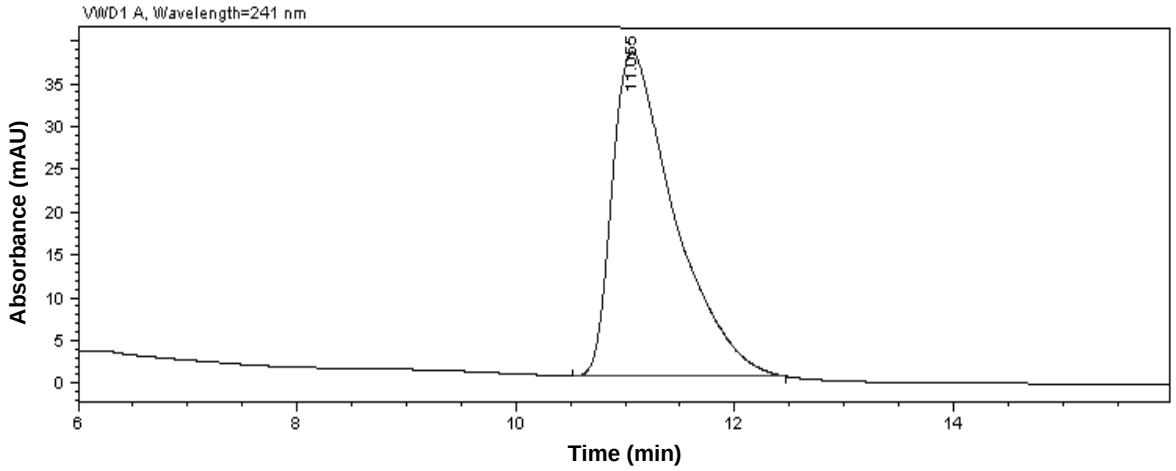

Figure A.14: Chromatogram of a standard solution stressed in 10 % H_2O_2 for three hours

No degradation occurred when adapalene was submitted to stress testing in the presence of HCl, H_2O or H_2O_2. In the presence of NaOH, adapalene, like tretinoin, underwent complete degradation in just 10 minutes. No peak interference was observed for adapalene. The method can thus be regarded as specific.

A.5.2.2 Linearity

Table A.6 presents the peak area ratios obtained after analysing samples of known concentrations over a specified concentration range. Regression statistics were used to calculate the regression coefficient (R^2). The linear regression graph is shown in figure A.15.

Table A.6: Peak area ratios obtained after analysing samples of known concentrations

µg/ml	Area 1	Area 2	Mean
0.021	3.18	2.77	2.97
0.042	5.72	5.80	5.76
0.063	8.93	8.77	8.85
0.084	10.73	10.68	10.70
0.105	13.70	14.06	13.88
0.21	33.98	34.12	34.05
0.42	69.00	68.76	68.88
0.63	102.99	102.65	102.82
0.84	136.89	136.97	136.93
1.05	171.11	171.15	171.13
2.1	352.78	352.74	352.76
4.2	701.89	704.86	703.37
6.3	1058.66	1056.51	1057.59
8.4	1400.51	1399.44	1399.98
10.5	1764.72	1764.99	1764.86
21	3634.72	3634.65	3634.69
42	7265.13	7223.72	7244.43
63	10732.00	10733.90	10732.95
84	14306.40	14302.80	14304.60
105	17875.00	17869.00	17872.00
		R^2	0.99997
		Intercept	-1.8323
		Slope	170.4737

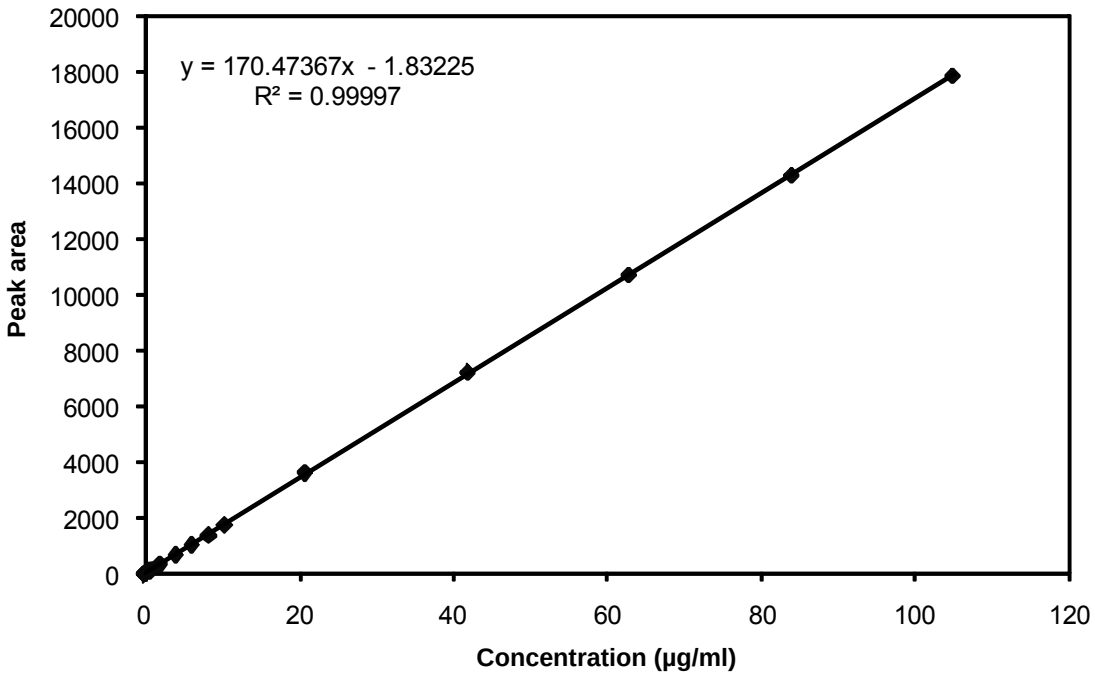

Figure A.15: Linear regression graph for adapalene

The method was linear in the range of 0.021 to 105 µg/ml. It could be used for single point calibration.

A.5.2.3 Accuracy and repeatability

The accuracy of the HPLC method was determined by analysing standards with four different concentrations. The mean recovery was an indication of the accuracy, while the RSD indicated the method's repeatability (table A.7).

Table A.7: Peak area ratios and percentage recovery of adapalene

Conc. Spiked (µg/ml)	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
0.34	52.9	52.3	52.6	0.319	93.87
0.34	51.9	52.9	52.4	0.318	93.55
0.34	54.5	52.1	53.3	0.323	95.11
1.7	243.9	245.2	244.6	1.446	85.03
1.7	244.5	243.2	243.9	1.441	84.77
1.7	244.8	243.1	243.9	1.442	84.80
17	2824.8	2833.7	2829.3	16.61	97.69
17	2834.2	2820.8	2827.5	16.60	97.63
17	2815.3	2826.9	2821.1	16.56	97.41
102	17142.5	17147.6	17145.1	100.59	98.61
102	17156.7	17135.7	17146.2	100.59	98.62
102	17146.2	17123.6	17134.9	100.53	98.55
				Mean	93.81
				SD	5.42
				% RSD	5.78

The method's accuracy and repeatability were not within the specified limits, even though the analysis was performed several times.

A.5.2.4 Intermediate precision

A standard of known concentration was analysed in duplicate on three different days. The results are shown in table A.8. The RSD indicated the intermediate precision, which was well within acceptable levels.

Table A.8: Peak area ratios obtained on three different days

	Area 1	Area 2	Mean	SD	% RSD
Day 1	683.0	682.5	682.8	0.25	0.037
Day 2	668.0	670.8	669.4	1.40	0.209
Day 3	613.8	620.1	616.9	3.18	0.515
Between days			656.37	28.44	4.33

A.5.2.5 Stability of sample solutions

The stability of a sample solution was evaluated over a period of 15 hours. As shown in table A.9, no degradation of adapalene was observed.

Table A.9: Percentage of adapalene remaining in solution during 15 hours, indicating its stability

Time (hours)	Peak area	% Remaining
0	3929.3	100.0
1	3974.1	101.1
2	4005.0	101.9
3	4003.5	101.9
4	4018.1	102.3
5	4037.2	102.7
6	4063.4	103.4
7	4060.5	103.3
8	4066.8	103.5
9	4048.9	103.0
10	4050.4	103.1
11	4053.1	103.2
12	4058.3	103.3
13	4054.9	103.2
14	4057.8	103.3
15	4065.1	103.5
Mean	4034.2	102.7
SD	37.63	0.96
% RSD	0.93	0.93

A.5.2.6 System repeatability

An adapalene sample was analysed six times consecutively. The peak areas and retention times indicated the repeatability of the system. The results are shown in table A.10.

Table A.10: Peak areas and retention times indicating system repeatability

	Peak area	Retention time (minutes)
	690.5	11.121
	690.3	11.158
	688.6	11.216
	689.1	11.238
	688.3	11.247
	686.9	11.246
Mean	689.0	11.204
SD	1.22	0.048
% RSD	0.18	0.430

The repeatability of the peak area and the retention time were well within limits. Therefore, the system proved to have adequate repeatability.

A.5.2.7 Conclusion

Although the method was specific and showed excellent linearity, the accuracy and repeatability could not be improved. Bearing this in mind, the method was still suitable for the analysis of samples obtained from *in vitro* drug release and diffusion studies.

A.6 References

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Appendix B

Validation of analytical HPLC methods for assay testing

B.1 Purpose

HPLC methods were developed and validated to accurately determine the amount of degradable components (i.e. parabens, antioxidants, retinoid and tocopherol) in the different cream formulations subjected to stability studies. The only difference between the methods used for creams containing either tretinoin or adapalene was the change in detection wavelength to facilitate the detection of tretinoin.

B.2 HPLC method for tretinoin containing creams

B.2.1 Chromatographic conditions

Analytical instrument: Agilent® 1200 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1200 quaternary pump, degasser, thermostat, autosampler injection mechanism, UV detector and ChemStation software (Rev. A.10.02) for the acquisition and analysis of data.

Column: Phenomenex® Luna 5 µm C18 (2), 250 × 4.60 mm.

Mobile phase: Gradient elution with two mobile phases was used.

Mobile phases:

A) Make 1 g octane-1-sulphonic acid sodium salt up to 1000 ml with deionised HPLC grade water. Adjust pH to 3.5 with 10 % ortophosphoric acid. Filter and degas the solution.

B) Methanol (gradient grade).

Gradient:

Mobile phase composition (A : B)	Time (min)
50 : 50	1.0
0 : 100	8.0

After each run, the system was re-equilibrated for five minutes.

Run time: 25 minutes.

Injection volume: 20 µl.

Flow rate: 1.000 ml/min.

Detection: UV at two consecutive wavelengths:

Wavelength (nm)	Time (min)
220	0 - 13
349	13 - 16
220	16 - 25

Retention times: Methylparaben: 7.0 min.

Propylparaben: 9.3 min.

BHA: 10.3 min.

BHT: 12.6 min.

Tretinoin: 13.8 min.

α-tocopherol: 22.7 min.

Solvent: THF (Chromasolv®, Sigma Aldrich).

Precautions: All actions involving tretinoin were conducted using amber glassware under reduced lighting.

B.2.2 Preparation of standard solution

a. Accurately weigh approximately 5 mg tretinoin, 40 mg methylparaben, 8 mg propylparaben, 4 mg BHA, 40 mg BHT and 40 mg α-tocopherol. These represented the amounts of each analyte in 20 g cream. Place in a 50 ml volumetric flask and make up to volume with the solvent. This was taken as the 1000 % standard.

b. Transfer 5 ml of the solution in (a) to another 50 ml volumetric flask. Make up to volume with solvent. This standard solution was equal to the amount in 2 g cream and was taken to be the 100 % standard solution.

B.2.3 Preparation of placebo cream

Prepare a vitamin F cream (appendix C, section C.5.1), without tretinoin, parabens, BHA and BHT.

B.2.4 Preparation of sample solution

a. Thoroughly stir the cream to be analysed with a spatula in order to ensure homogeneity.

b. Fill a 5 ml syringe with the cream and attach a piece of plastic tubing (approximately 10 cm) to the syringe.

c. Place a 50 ml volumetric flask on a balance and tare.

d. Place the loose end of the plastic tubing into the flask and accurately weigh approximately 2 g of the cream.

e. Make up to volume with the solvent and ultrasonicate for 8 minutes.

f. Filter the solution by using a 0.45 µm pre-filter and transfer it to an HPLC autosampler vial. This was the 100 % sample solution.

B.2.5 Chromatographic parameters

B.2.5.1 Specificity

Specificity was tested by evaluating the elution of the different analytes. Neither peaks of degradation products nor peaks of the matrix should interfere with the peaks to be analysed. The presence of isotretinoin should also not interfere with the peak of tretinoin.

B.2.5.2 Accuracy

a. Accurately weigh approximately 1.6 g (80 %), 2 g (100 %) and 2.4 g (120 %) of placebo cream (see section B.2.3), respectively, into different 50 ml volumetric flasks. Repeat three times.

b. Add 1000 % standard to each of the volumetric flasks. Add 4 ml to the flasks containing 1.6 g placebo; 5 ml to the flasks containing 2 g placebo; and 6 ml to the flasks containing 2.4 g placebo.

c. Fill to volume with solvent and ultrasonicate for 8 minutes. Filter with a 0.45 µm pre-filter, transfer to HPLC autosampler vials and analyse in duplicate.

B.2.5.3 Repeatability

a. Thoroughly stir the cream to be tested by hand in order to ensure homogeneity.

b. Accurately weigh approximately 1.6 g (80%), 2g (100%) and 2.4 g (120%) of the sample cream, respectively, into different 50 ml volumetric flasks, using the method described in section B.2.4. Fill to volume with THF. Repeat three times. Ultrasonicate the nine volumetric flasks for eight minutes.

c. Filter with a 0.45 µm pre-filter and transfer to HPLC vials. Analyse in duplicate.

d. Prepare a 100 % standard solution as described in section B.2.2 and analyse in duplicate.

B.2.5.4 Intermediate precision

Analysis of the same standard and samples at 100 % concentration, used for repeatability above, was conducted on two other days.

B.2.6 Results and discussion

After developing an HPLC method (described in section B.2.1) to analyse the concentration of degradable components in tretinoin creams, the method was validated in terms of its specificity, accuracy, repeatability and intermediate precision. The results are presented and discussed in this section.

B.2.6.1 Specificity

Figures B.1 to B.5 were obtained during specificity testing. A chromatogram of a standard solution is shown in figure B.1, with the peaks of the degradable compounds labelled. All peaks had adequate resolution.

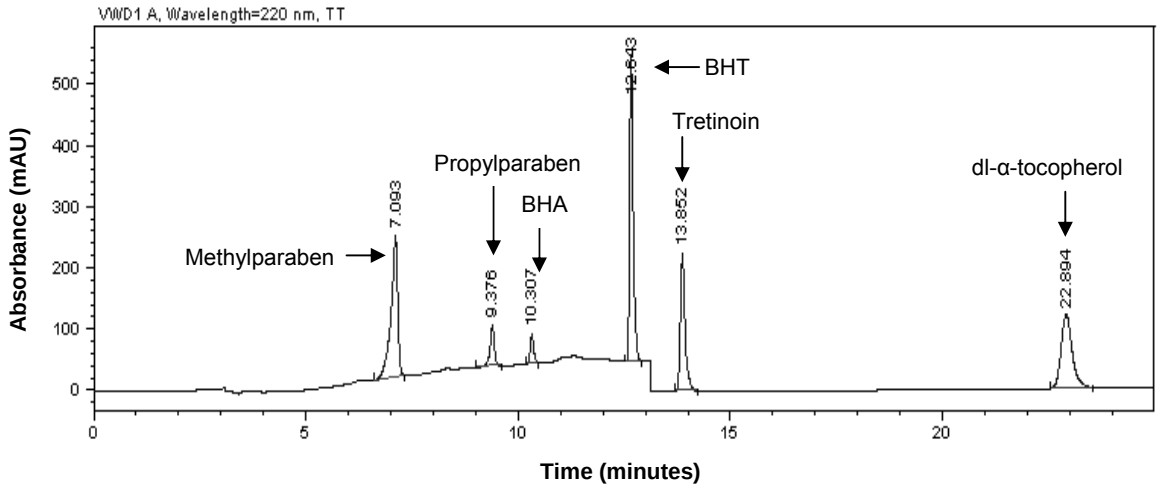

Figure B.1: Chromatogram of a standard solution

Analysis of a sample of Pheroid™ cream showed an extra peak eluting at 12.327 minutes (figure B.2). The peak was identified as vitamin F. Furthermore, the area of the BHT peak was larger than expected.

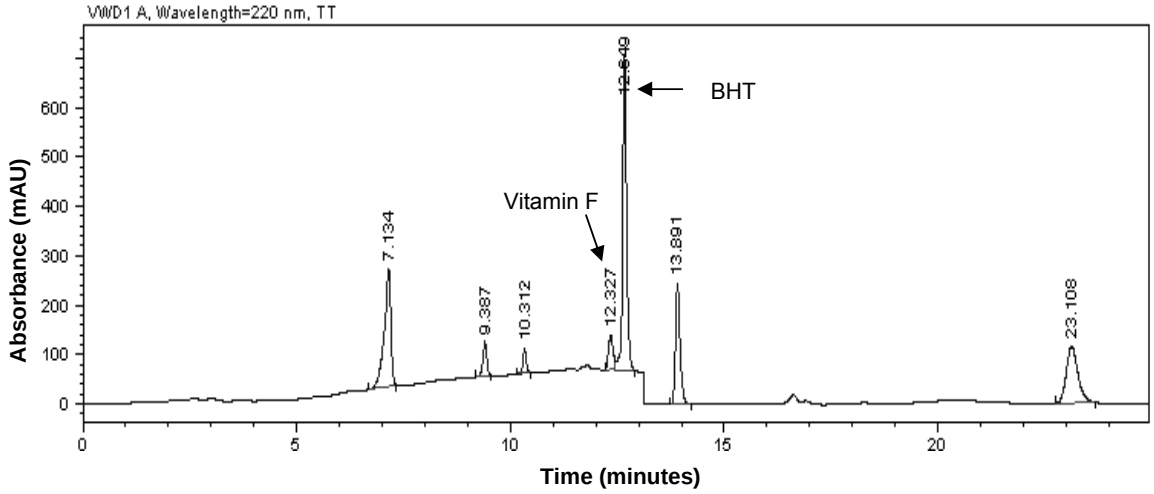

Figure B.2: Chromatogram of a Pheroid™ cream sample solution

As no information regarding the preservation of vitamin F could be obtained, it was necessary to analyse vitamin F and BHT separately. Figure B.3 shows the chromatogram of vitamin F. The UV spectrum of the peak at 12.109 minutes was the same as for the peak of BHT in figure B.2. It was hence deducted that BHT was used to preserve vitamin F.

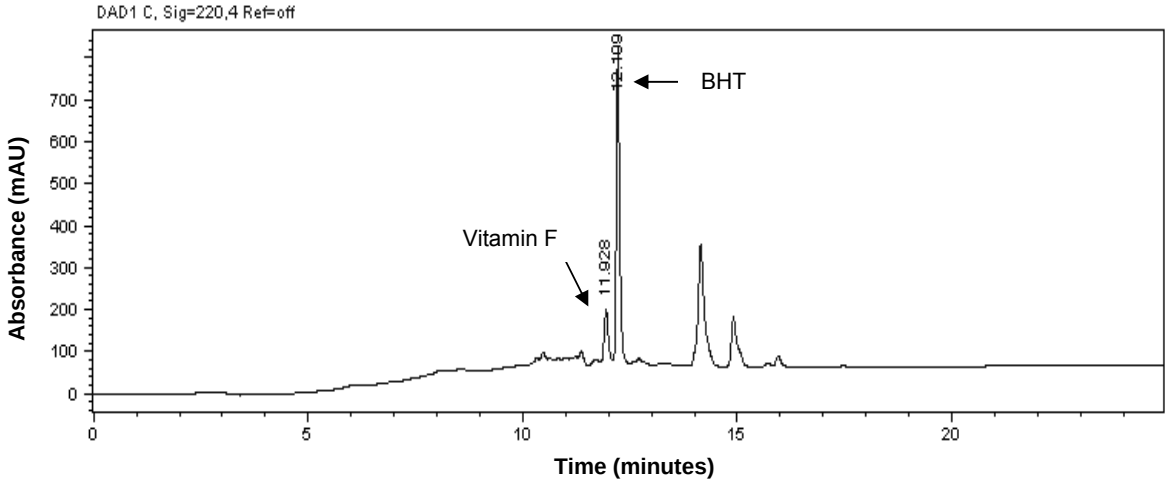

Figure B.3: Chromatogram of vitamin F

Analysis of a standard solution exposed to light showed a small extra peak just before the peak of tretinoin (figure B.4).

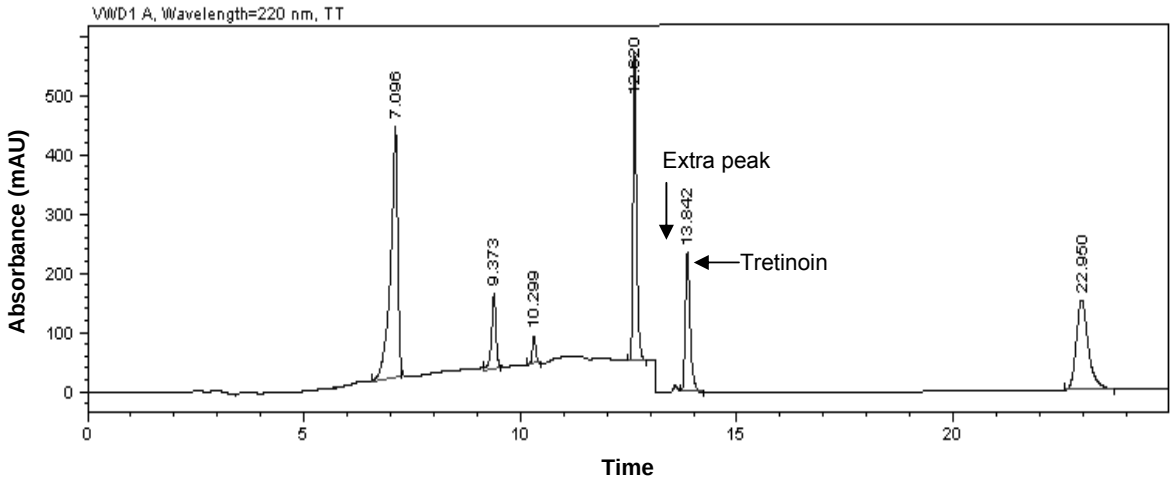

Figure B.4: Chromatogram of a standard solution after exposure to light

A standard solution containing isotretinoin instead of tretinoin was analysed (figure B.5). The resulting chromatogram was overlaid with figure B.4. Isotretinoin's peak in figure B.5 corresponded to the extra peak in figure B.4.

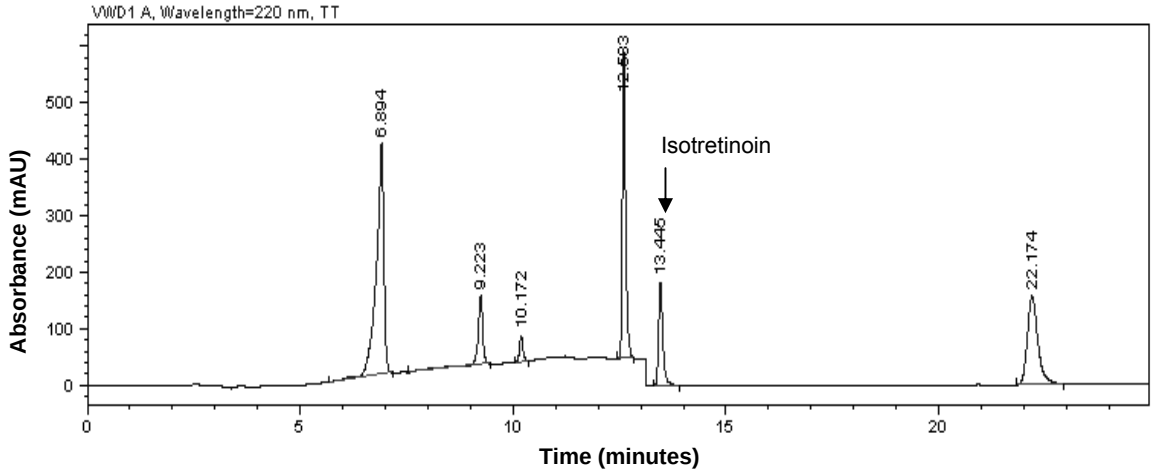

Figure B.5: Chromatogram of a standard solution containing isotretinoin instead of tretinoin

The HPLC method illustrated adequate specificity for the analytes. Isotretinoin did not interfere with the peak of tretinoin. BHT was used as preservative in vitamin F. Therefore, BHT recovery could be expected to be higher in creams containing vitamin F.

B.2.6.2 Accuracy

Peak area ratios were obtained when analysing samples of known concentrations over a specified concentration range. Tables B.1 to B.6 show the recovery values for methylparaben, propylparaben, BHA, BHT, tretinoin and α-tocopherol, respectively.

Table B.1: Recovery values for methylparaben

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
64.18	2031.3	2046.5	2038.9	64.9	101.2
64.18	2033.7	2040.7	2037.2	64.9	101.1
64.18	2053.8	2044.6	2049.2	65.3	101.7
80.22	2527.1	2530.2	2528.7	80.5	100.4
80.22	2584.2	2572.7	2578.5	82.1	102.3
80.22	2558.8	2525.1	2542.0	80.9	100.9
96.26	3044.9	3021.1	3033.0	96.6	100.3
96.26	2922.4	3051.3	2986.9	95.1	98.8
96.26	2998.6	3088.8	3043.7	96.9	100.7
				Mean	100.8
				SD	0.93
				% RSD	0.92

Table B.2: Recovery values for propylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
12.96	356.9	355.8	356.4	12.8	99.0
12.96	355.5	351.5	353.5	12.7	98.2
12.96	344.3	358.1	351.2	12.6	97.6
16.20	458.7	432.1	445.4	16.0	99.0
16.20	453.1	461.5	457.3	16.5	101.6
16.20	432.3	442.4	437.4	15.7	97.2
19.44	540.8	533.1	537.0	19.3	99.4
19.44	510.2	520.3	515.3	18.6	95.4
19.44	526.7	543.1	534.9	19.3	99.1
				Mean	98.5
				SD	1.62
				% RSD	1.64

Table B.3: Recovery values for BHA

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
6.38	219.4	215.1	217.3	6.4	100.8
6.38	220.4	219.9	220.2	6.5	102.1
6.38	222.7	218.8	220.8	6.5	102.4
7.97	277.2	278.0	277.6	8.2	103.0
7.97	273.2	281.0	277.1	8.2	102.9
7.97	275.8	279.7	277.8	8.2	103.1
9.56	320.7	329.1	324.9	9.6	100.5
9.56	327.2	325.5	326.4	9.7	100.9
9.56	342.2	340.5	341.4	10.1	105.6
				Mean	102.4
				SD	1.48
				% RSD	1.44

Table B.4: Recovery values for BHT

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
63.91	3216.2	3218.7	3217.5	89.3	139.7
63.91	3164.5	3190.6	3177.6	88.2	138.0
63.91	3273.7	3311.2	3292.5	91.4	143.0
79.89	4049.5	4064.7	4057.1	112.6	140.9
79.89	3695.0	3693.4	3694.2	102.5	128.3
79.89	3965.1	4042.3	4003.7	111.1	139.1
95.87	4744.9	4759.5	4752.2	131.9	137.6
95.87	4899.1	4905.1	4902.1	136.0	141.9
95.87	5025.9	4990.2	5008.1	139.0	145.0
				Mean	139.2
				SD	4.48
				% RSD	3.21

Table B.5: Recovery values for tretinoin

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
7.93	1479.3	1473.0	1476.2	8.4	106.4
7.93	1486.2	1505.4	1495.8	8.5	107.8
7.93	1461.6	1483.6	1472.6	8.4	106.1
9.91	1847.5	1857.1	1852.3	10.6	106.8
9.91	1891.8	1880.4	1886.1	10.8	108.7
9.91	1847.1	1855.0	1851.1	10.6	106.7
11.89	2224.0	2219.9	2222.0	12.7	106.7
11.89	2185.2	2200.3	2192.8	12.5	105.3
11.89	2277.0	2218.6	2247.8	12.8	108.0
				Mean	106.93
				SD	0.98
				% RSD	0.92

Table B.6: Recovery values for α-tocopherol

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
63.22	1661.1	1596.6	1628.9	62.4	98.6
63.22	1587.4	1594.0	1590.7	60.9	96.3
63.22	1640.5	1585.2	1612.9	61.7	97.7
79.02	2038.4	2050.4	2044.4	78.3	99.0
79.02	2037.0	2084.2	2060.6	78.9	99.8
79.02	1959.0	2041.8	2000.4	76.6	96.9
94.82	2583.3	2383.0	2483.2	95.1	100.2
94.82	2290.6	2470.0	2380.3	91.1	96.1
94.82	2517.5	2524.0	2520.8	96.5	101.8
				Mean	98.50
				SD	1.81
				% RSD	1.84

The HPLC method for assay testing was found to be accurate for methylparaben, propylparaben, BHA and α-tocopherol. Recovery of tretinoin was nearly 7 % too high, whereas the recovery of BHT was approximately 40 % too high. The exceptionally high recovery of BHT was ascribed to BHT used as preservative in vitamin F, as mentioned in section B.2.6.1. The relative standard deviations (RSD) for all the analytes were well below 2 %, except for BHT, which was 3.2 %, but still acceptable.

B.2.6.3 Repeatability

Nine sample solutions of known concentrations were analysed. Tables B.7 to B.12 show the peak area ratios and relative standard deviations (RSD) for methylparaben, propylparaben, BHA, BHT, tretinoin and α-tocopherol, respectively. The RSD served as an indication of the repeatability of the HPLC method.

Table B.7: Repeatability parameters for methylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
66.04	2598.2	2655.5	2626.85	71.41	108.1
111.17	4614.8	4567.3	4591.05	124.81	112.3
67.07	2677.7	2662.7	2670.2	72.59	108.2
78.95	2671.6	3246.1	2958.85	80.44	101.9
84.29	3379.1	3358.2	3368.65	91.58	108.6
81.79	3374.9	3403.5	3389.2	92.14	112.7
100.33	4166.3	4217.2	4191.75	113.96	113.6
102.78	4262.4	4268.1	4265.25	115.96	112.8
117.77	4820.5	4849.5	4835	131.44	111.6
				Mean	109.98
				SD	3.49
				% RSD	3.18

Table B.8: Repeatability parameters for propylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
15.02	530.9	538.4	534.7	15.85	105.6
25.28	889.1	893.5	891.3	26.43	104.5
15.25	539.1	548.2	543.7	16.12	105.7
17.95	507.4	646.4	576.9	17.11	95.3
19.17	686.2	684.2	685.2	20.32	106.0
18.60	643.4	703.3	673.4	19.97	107.4
22.82	843.4	817.7	830.6	24.63	107.9
23.37	841.7	848.8	845.3	25.06	107.2
26.78	917.9	936.0	927.0	27.49	102.6
				Mean	104.7
				SD	3.66
				% RSD	3.49

Table B.9: Repeatability parameters for BHA

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
6.55	207.8	203.1	205.5	7.44	113.5
6.65	189.0	200.8	194.9	7.06	106.1
7.83	203.6	228.3	216.0	7.82	99.8
8.36	267.2	242.0	254.6	9.22	110.2
8.11	247.1	229.3	238.2	8.63	106.3
9.95	283.8	285.9	284.9	10.31	103.6
10.20	314.1	304.6	309.4	11.20	109.9
11.68	345.4	353.5	349.5	12.65	108.3
11.03	360.9	337.1	349.0	12.64	114.6
				Mean	108.0
				SD	4.41
				% RSD	4.08

Table B.10: Repeatability parameters for BHT

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
63.44	2739.5	2718.2	2728.9	82.98	130.8
106.81	4574.0	4569.7	4571.9	139.02	130.2
64.44	2742.5	2735.1	2738.8	83.28	129.2
75.85	2584.7	3376.0	2980.4	90.62	119.5
80.98	3517.6	3473.2	3495.4	106.29	131.2
78.57	3382.1	3383.8	3383.0	102.87	130.9
96.39	4120.6	4127.7	4124.2	125.40	130.1
98.74	4244.3	4260.0	4252.2	129.30	130.9
113.14	4701.6	4761.3	4731.5	143.87	127.2
				Mean	128.9
				SD	3.53
				% RSD	2.74

Table B.11: Repeatability parameters for tretinoin

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
8.66	1465.9	1471.7	1468.8	9.47	109.4
14.58	2457.7	2468.5	2463.1	15.88	108.9
8.80	1456.6	1453.7	1455.2	9.38	106.7
10.35	1372.4	1741.3	1556.9	10.04	97.0
11.05	1872.6	1866.9	1869.8	12.06	109.1
10.73	1823.0	1825.5	1824.3	11.76	109.7
13.16	2221.2	2210.0	2215.6	14.29	108.6
13.48	2275.6	2271.5	2273.6	14.66	108.8
15.44	2554.8	2576.6	2565.7	16.55	107.1
				Mean	107.2
				SD	3.75
				% RSD	3.50

Table B.12: Repeatability parameters for α-tocopherol

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
70.87	1799.0	1755.8	1777.4	68.19	96.2
119.31	3009.7	3027.5	3018.6	115.81	97.1
71.98	1773.4	1767.5	1770.5	67.92	94.4
84.73	1687.0	2082.2	1884.6	72.30	85.3
90.46	2238.4	2224.3	2231.4	85.61	94.6
87.77	2158.2	2205.5	2181.9	83.71	95.4
107.68	2665.3	2689.6	2677.5	102.72	95.4
110.30	2833.0	2763.0	2798.0	107.34	97.3
126.39	3022.2	3125.4	3073.8	117.93	93.3
				Mean	94.3
				SD	3.40
				% RSD	3.61

All the analytes depicted RSD values of approximately 3 %, with the highest being 4.08 % for BHA. As the acceptance criteria for antioxidants are less strict than for pharmacologically active ingredients, e.g. retinoids, the values obtained were acceptable. It should be borne in mind that recovery and repeatability values were influenced by the cream matrix, as such a complex

matrix was very difficult to work with when preparing samples for HPLC. Tetrahydrofuran also influenced the accuracy and precision of the method due to its high volatility.

B.2.6.4 Intermediate precision

A cream sample was analysed on three different days. The relative standard deviation of the mean recovery values was a measure of the HPLC method's intermediate precision. The following six tables present the intermediate precision values for methylparaben (table B.13), propylparaben (table B.14), BHT (table B.15), BHT (table B.16), tretinoin (table B.17) and α-tocopherol (table B.18).

Table B.13: Intermediate precision values for methylparaben

	% Recovery			Mean	SD	% RSD
Day 1	101.9	108.6	112.7	107.7	4.45	4.13
Day 2	110.9	112.0	110.6	111.1	0.61	0.55
Day 3	115.4	116.3	116.6	116.1	0.53	0.46
Between days				111.7	3.43	3.07

Table B.14: Intermediate precision values for propylparaben

	% Recovery			Mean	SD	% RSD
Day 1	95.3	106.0	107.4	102.9	5.40	5.25
Day 2	98.5	104.9	95.8	99.7	3.83	3.84
Day 3	101.9	108.1	107.8	105.9	2.88	2.72
Between days				102.8	2.54	2.47

Table B.15: Intermediate precision values for BHA

	% Recovery			Mean	SD	% RSD
Day 1	99.8	110.2	106.3	105.5	4.29	4.07
Day 2	99.5	110.3	114.0	107.9	6.14	5.69
Day 3	114.6	119.7	118.9	117.7	2.21	1.88
Between days				110.4	5.31	4.81

Table B.16: Intermediate precision values for BHT

	% Recovery			Mean	SD	% RSD
Day 1	119.5	131.2	130.9	127.2	5.47	4.30
Day 2	131.1	129.9	131.3	130.8	0.62	0.47
Day 3	133.9	135.6	137.5	135.7	1.47	1.09
Between days				131.2	3.47	2.65

Table B.17: Intermediate precision values for tretinoin

	% Recovery			Mean	SD	% RSD
Day 1	97.0	109.1	109.7	105.2	5.86	5.56
Day 2	109.3	109.9	109.6	109.6	0.25	0.23
Day 3	115.7	116.4	119.4	117.2	1.62	1.38
Between days				110.7	4.92	4.45

Table B.18: Intermediate precision values for α-tocopherol

	% Recovery			Mean	SD	% RSD
Day 1	85.3	94.6	95.4	91.8	4.57	4.98
Day 2	91.2	95.2	94.9	93.8	1.83	1.96
Day 3	97.9	99.3	101.6	99.6	1.51	1.52
Between days				95.0	3.31	3.48

The intermediate variances of all the analytes were well within the specified 5 % limit and did not differ significantly from the repeatability values.

B.2.7 Conclusion

This HPLC method was suitable for conducting assay tests on creams containing tretinoin. No degradation products or matrix interfered with the analysis of the preservatives, antioxidants, retinoid or tocopherol. The method was accurate with adequate repeatability and should perform well. BHT recovery would be much higher than 100 % for creams containing vitamin F.

B.3 HPLC method for adapalene containing creams

B.3.1 Chromatographic conditions

Analytical instrument: Agilent® 1200 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1200 quaternary pump, degasser, thermostat, autosampler injection mechanism, UV detector and ChemStation software (Rev. A.10.02) for the acquisition and analysis of data.

Column: Phenomenex® Luna 5 µm C18 (2), 250 × 4.60 mm.

Mobile phase: Gradient elution with two mobile phases was used.

Mobile phases:

A) Make 1 g octane-1-sulphonic acid sodium salt up to 1000 ml with deionised HPLC grade water. Adjust pH to 3.5 with 10% phosphoric acid. Filter and degas the solution.

B) Methanol (gradient grade).

Gradient:

Mobile phase composition (A : B)	Time (min)
50 : 50	1.0
0 : 100	8.0

After each run, the system was re-equilibrated for five minutes.

Run time: 25 minutes.

Injection volume: 20 µl.

Flow rate: 1.000 ml/min.

Detection: 220 nm.

Retention times: Methylparaben: 7.0 min.

Propylparaben: 9.3 min.

BHA: 10.3 min.

BHT: 12.6 min.

Adapalene: 16.0 min.

α-tocopherol: 22.7 min.

Solvent: THF (Chromasolv®, Sigma Aldrich).

B.3.2 Preparation of standard solution

a. Accurately weigh approximately 20 mg adapalene, 40 mg methylparaben, 8 mg propylparaben, 4 mg BHA, 40 mg BHT and 40 mg α-tocopherol. These represented the amounts of each analyte in 20 g of cream. Place in a 50 ml volumetric flask and make up to volume with solvent. This was the 1000 % standard solution.

b. Transfer 5 ml of the solution in (a) to another 50 ml volumetric flask. Make up to volume with the solvent. This standard solution was equal to the amount in 2 g of cream and was taken to be the 100 % standard solution.

B.3.3 Preparation of placebo cream

Prepare a vitamin F cream (appendix C, section C.6.1), without adapalene, parabens, BHA and BHT.

B.3.4 Preparation of sample solution

Prepare as for the tretinoin sample solution (see section B.2.4).

B.3.5 Chromatographic parameters

B.3.5.1 Specificity

Specificity was tested by evaluating the elution of the different analytes. No peaks, neither that of the degradation products nor that of the matrix, should interfere with the peaks that have to be analysed.

B.3.5.2 Accuracy

Accuracy testing was conducted in the same manner as for tretinoin (see section B.2.5.2).

B.3.5.3 Repeatability

The same procedure was followed as for tretinoin (see section B.2.5.3).

B.3.5.4 Intermediate precision

Analysis of the same standard and samples at 100 % concentration, used for repeatability above, was conducted on two other days.

B.3.6 Results and discussion

After developing an HPLC method (described in section B.3.1) to analyse the concentration of degradable components in adapalene creams, the method was validated in terms of its specificity, accuracy, repeatability and intermediate precision. The results are presented and discussed in this section.

B.3.6.1 Specificity

Figures B.8 and B.5 were obtained during specificity testing. A chromatogram of a standard solution is shown in figure B.8. with the peaks of the degradable compounds labelled. All peaks had adequate resolution. Figure B.9 shows the chromatogram of a Pheroid™ cream sample.

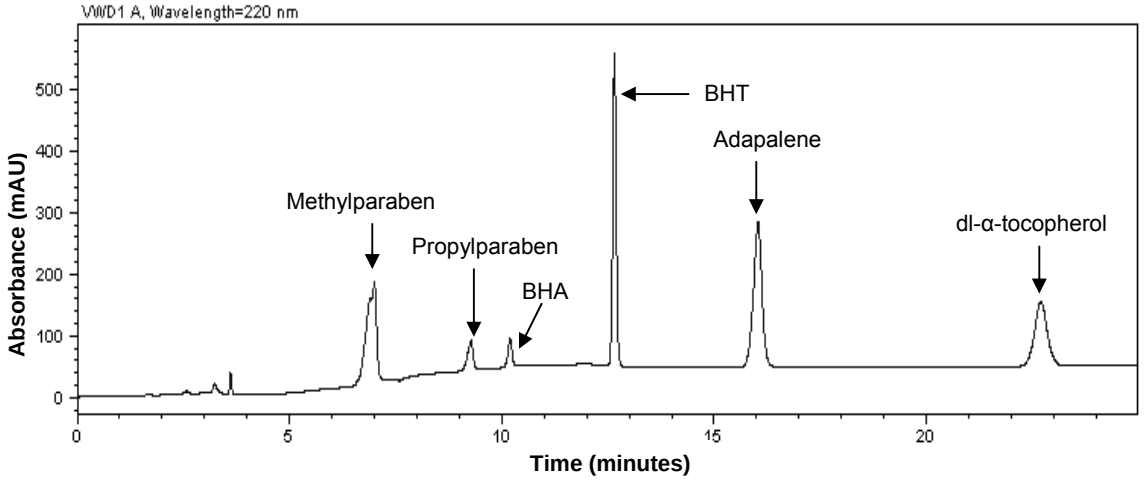

Figure B.8: Chromatogram of a standard solution

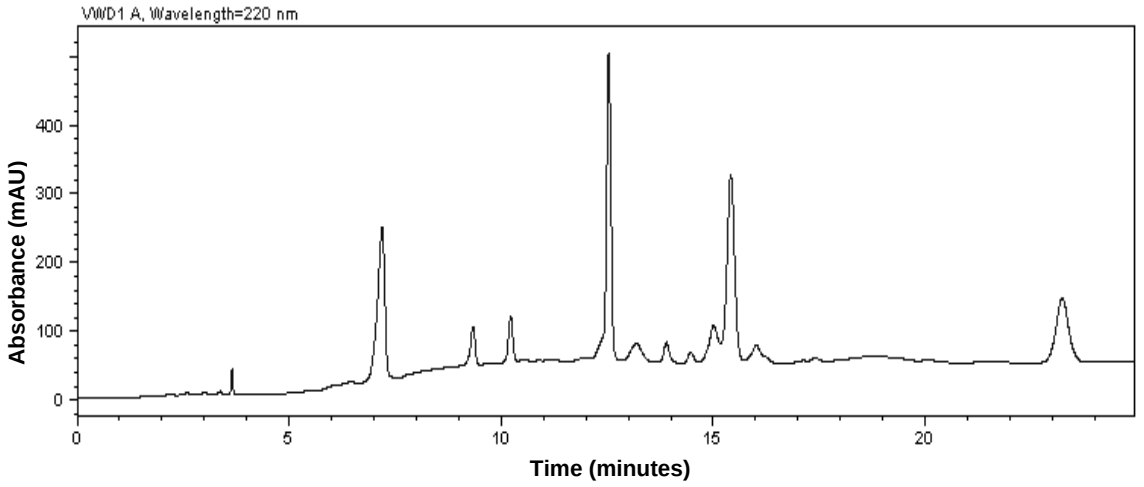

Figure B.9: Chromatogram of a Pheroid™ cream sample solution

No peaks interfered with those of the analytes, thereby demonstrating the method's specificity.

B.3.6.2 Accuracy

Tables B.19 to B.24 present the recovery values for methylparaben, propylparaben, BHA, BHT, adapalene and α-tocopherol, respectively. The mean recovery was used as a measure of the method's accuracy.

Table B.19: Recovery values for methylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
64.05	2052.5	2063.4	2058.0	66.38	103.63
64.05	2063.9	2038.2	2051.1	66.15	103.28
64.05	2070.9	2015.1	2043.0	65.89	102.88
80.06	2522.0	2522.2	2522.1	81.35	101.60
80.06	2597.6	2566.7	2582.2	83.28	104.02
80.06	2576.5	2557.8	2567.2	82.80	103.42
96.08	2992.6	3005.4	2999.0	96.73	100.68
96.08	2983.9	3003.8	2993.9	96.56	100.51
96.08	3010.0	2998.7	3004.4	96.90	100.86
				Mean	102.32
				SD	1.32
				% RSD	1.29

Table B.20: Recovery values for propylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
12.53	335.3	342.1	338.7	12.84	102.49
12.53	331.0	339.0	335.0	12.70	101.37
12.53	342.6	346.4	344.5	13.06	104.24
15.66	413.6	426.7	420.2	15.93	101.71
15.66	429.4	428.5	429.0	16.27	103.84
15.66	430.8	429.0	429.9	16.30	104.07
18.80	499.9	505.3	502.6	19.06	101.39
18.80	507.2	508.5	507.9	19.26	102.45
18.80	501.1	512.8	507.0	19.22	102.27
				Mean	102.65
				SD	1.07
				% RSD	1.04

Table B.21: Recovery values for BHA

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
6.34	215.1	213.2	214.2	6.50	102.60
6.34	210.2	214.1	212.2	6.44	101.64
6.34	212.7	215.1	213.9	6.49	102.48
7.92	265.7	271.6	268.7	8.16	102.97
7.92	266.9	273.3	270.1	8.20	103.53
7.92	272.6	269.6	271.1	8.23	103.91
9.50	312.6	322.4	317.5	9.64	101.41
9.50	322.0	322.2	322.1	9.78	102.88
9.50	319.9	328.0	324.0	9.83	103.47
				Mean	102.77
				SD	0.79
				% RSD	0.77

Table B.22: Recovery values for BHT

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
64.26	3143.8	3119.6	3131.7	87.31	135.87
64.26	3007.5	2981.6	2994.6	83.48	129.92
64.26	3181.7	3156.2	3169.0	88.35	137.49
80.32	3849.8	3872.2	3861.0	107.64	134.01
80.32	3731.5	3711.1	3721.3	103.75	129.16
80.32	3740.1	3741.2	3740.7	104.28	129.84
96.38	4504.4	4536.8	4520.6	126.03	130.76
96.38	4388.4	4409.2	4398.8	122.63	127.23
96.38	4206.4	4234.6	4220.5	117.66	122.08
				Mean	130.71
				SD	4.40
				% RSD	3.36

Table B.23: Recovery values for adapalene

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
32.0	2544.6	2495.0	2519.8	30.73	96.04
32.0	2553.8	2528.0	2540.9	30.99	96.84
32.0	2539.7	2551.0	2545.4	31.04	97.01
40.0	3214.9	3231.4	3223.2	39.31	98.28
40.0	3311.6	3257.1	3284.4	40.06	100.14
40.0	3212.7	3242.4	3227.6	39.36	98.41
48.0	3885.5	3924.8	3905.2	47.63	99.23
48.0	3936.2	3939.3	3937.8	48.03	100.05
48.0	3957.4	3967.3	3962.4	48.33	100.68
				Mean	98.52
				SD	1.54
				% RSD	1.57

Table B.24: Recovery values for α-tocopherol

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
70.4	1834.6	1897.8	1866.2	69.40	98.59
70.4	1831.7	1852.6	1842.2	68.51	97.32
70.4	1850.8	1827.1	1839.0	68.39	97.15
88.0	2230.9	2325.3	2278.1	84.72	96.28
88.0	2420.1	2311.1	2365.6	87.98	99.97
88.0	2213.7	2303.6	2258.7	84.00	95.45
105.6	2887.7	2913.7	2900.7	107.88	102.16
105.6	2868.3	2850.5	2859.4	106.34	100.70
105.6	2872.6	2873.7	2873.2	106.85	101.19
				Mean	98.76
				SD	2.22
				% RSD	2.25

The accuracy of the method was satisfactory. The high recovery value of BHT was attributed to the vitamin F that contained BHT as preservative (see section B.2.6.1 for a more detailed discussion). The RSD of all the analytes were acceptable.

B.3.6.3 Repeatability

Tables B.25 to B.30 show the peak area ratios and relative standard deviations (RSD) for methylparaben, propylparaben, BHA, BHT, adapalene and α-tocopherol, respectively. The RSD indicated the repeatability of the HPLC method.

Table B.25: Repeatability parameters for methylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
90.06	2898.5	2938.0	2918.3	91.60	101.70
87.50	2844.8	2831.1	2838.0	89.08	101.80
83.98	2752.8	2763.1	2758.0	86.57	103.08
107.69	3478.0	3484.5	3481.3	109.27	101.46
112.60	3660.1	3657.5	3658.8	114.84	101.99
115.19	3740.3	3720.3	3730.3	117.09	101.64
133.92	4312.2	4335.5	4323.9	135.72	101.34
164.23	5263.9	5232.4	5248.2	164.73	100.30
167.13	5332.2	5328.2	5330.2	167.30	100.11
				Mean	101.49
				SD	0.84
				% RSD	0.82

Table B.26: Repeatability parameters for propylparaben

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
16.14	424.9	440.3	432.6	15.59	96.61
15.68	419.3	419.4	419.4	15.11	96.39
15.04	402.1	403.7	402.9	14.52	96.50
19.29	529.9	505.7	517.8	18.66	96.71
20.17	544.3	539.0	541.7	19.52	96.75
20.64	565.1	545.3	555.2	20.01	96.94
23.99	636.5	651.3	643.9	23.20	96.71
29.42	778.0	758.6	768.3	27.68	94.09
29.94	798.3	788.7	793.5	28.59	95.50
				Mean	96.24
				SD	0.86
				% RSD	0.89

Table B.27: Repeatability parameters for BHA

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
9.49	405.8	414.9	410.4	12.34	129.99
9.22	393.1	405.0	399.1	12.00	130.11
8.85	385.2	386.2	385.7	11.60	131.03
11.35	507.8	499.5	503.7	15.15	133.43
11.87	516.3	527.4	521.9	15.69	132.22
12.14	536.7	525.1	530.9	15.97	131.49
14.12	640.9	624.5	632.7	19.03	134.79
17.31	750.8	755.1	753.0	22.64	130.80
17.62	802.1	791.4	796.8	23.96	136.01
				Mean	132.21
				SD	2.00
				% RSD	1.51

Table B.28: Repeatability parameters for BHT

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
72.43	3200.7	3208.4	3204.6	89.04	122.93
70.37	3135.5	3146.2	3140.9	87.27	124.02
67.54	3019.6	3026.6	3023.1	84.00	124.38
86.61	3871.6	3879.1	3875.4	107.68	124.33
90.56	3982.9	4045.9	4014.4	111.55	123.17
92.64	4101.7	4148.9	4125.3	114.63	123.73
107.70	4772.9	4796.2	4784.6	132.95	123.44
132.08	5886.4	5901.8	5894.1	163.78	124.00
134.41	6048.1	6005.2	6026.7	167.46	124.59
				Mean	123.84
				SD	0.54
				% RSD	0.43

Table B.29: Repeatability parameters for adapalene

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
38.25	2645.6	2686.4	2666.0	31.71	82.89
37.16	2642.8	2681.7	2662.3	31.66	85.20
35.67	2571.7	2511.4	2541.6	30.23	84.75
45.74	3291.8	3293.7	3292.8	39.16	85.62
47.82	3321.3	3421.0	3371.2	40.09	83.84
48.92	3487.0	3482.4	3484.7	41.44	84.71
56.88	3903.8	4017.4	3960.6	47.10	82.82
69.75	4865.1	4901.6	4883.4	58.08	83.27
70.98	5136.2	5015.0	5075.6	60.36	85.04
				Mean	84.24
				SD	1.00
				% RSD	1.18

Table B.30: Repeatability parameters for α-tocopherol

Conc. spiked µg/ml	Area 1	Area 2	Mean	Recovery	
				µg/ml	%
73.03	1962.0	1984.8	1973.4	70.49	96.53
70.95	1938.4	1953.5	1946.0	69.51	97.98
68.09	1876.5	1838.7	1857.6	66.36	97.45
87.32	2412.5	2439.2	2425.9	86.65	99.24
91.30	2523.9	2498.8	2511.4	89.71	98.26
93.40	2576.5	2571.7	2574.1	91.95	98.45
108.58	2894.6	2990.8	2942.7	105.12	96.81
133.16	3700.9	3708.2	3704.6	132.33	99.38
135.51	3820.9	3778.9	3799.9	135.74	100.17
				Mean	98.25
				SD	1.14
				% RSD	1.16

The relative standard deviation (RSD) values for all the analytes were well below 2 %.

B.3.6.4 Intermediate precision

A cream sample was analysed on three different days. The relative standard deviation of the mean recovery values was a measure of the HPLC method's intermediate precision. The following six tables present the intermediate precision values for methylparaben (table B.31), propylparaben (table B.32), BHT (table B.33), BHT (table B.34), adapalene (table B.35) and α-tocopherol (table B.36).

Table B.31: Intermediate precision values for methylparaben

				Mean	SD	% RSD
Day 1	124.2	120.9	118.2	121.1	2.48	2.04
Day 2	138.2	132.2	130.6	133.7	3.29	2.46
Day 3	115.2	116.9	118.0	116.7	1.11	0.95
Between days				123.8	7.18	5.80

Table B.32: Intermediate precision values for propylparaben

				Mean	SD	% RSD
Day 1	115.8	115.9	114.3	115.3	0.76	0.66
Day 2	126.0	126.0	123.5	125.2	1.17	0.94
Day 3	112.4	113.8	114.3	113.5	0.79	0.70
Between days				118.0	5.12	4.34

Table B.33: Intermediate precision values for BHA

				Mean	SD	% RSD
Day 1	171.9	169.4	168.5	169.9	1.42	0.83
Day 2	178.6	176.7	176.4	177.2	1.00	0.56
Day 3	167.0	165.4	166.6	166.3	0.70	0.42
Between days				171.2	4.54	2.65

Table B.34: Intermediate precision values for BHT

				Mean	SD	% RSD
Day 1	152.4	172.5	171.0	165.3	9.12	5.52
Day 2	174.1	176.6	172.4	174.4	1.73	0.99
Day 3	168.7	171.6	170.2	170.2	1.17	0.69
Between days				170.0	3.71	2.18

Table B.35: Intermediate precision values for adapalene

				Mean	SD	% RSD
Day 1	79.3	75.8	76.3	77.1	1.53	1.98
Day 2	85.0	81.8	81.9	82.9	1.50	1.81
Day 3	80.8	78.0	78.2	79.0	1.28	1.62
Between days				79.7	2.41	3.02

Table B.36: Intermediate precision values for α-tocopherol

				Mean	SD	% RSD
Day 1	58.1	58.7	57.7	58.2	0.38	0.66
Day 2	57.3	59.0	59.6	58.6	1.01	1.72
Day 3	55.2	58.4	57.2	56.9	1.30	2.28
Between days				57.9	0.72	1.24

Intermediate variances of the analytes were within the 5 % guideline, except for methylparaben with an RSD value of 0.8% higher which was still acceptable. The intermediate precision values were clearly higher than the repeatability values.

B.3.7 Conclusion

This HPLC method for assay testing demonstrated adequate accuracy, precision and specificity and could be used for assay testing of adapalene containing creams. The method should perform well, even with a different analyst. Recovery of BHT could be expected to be higher for creams containing vitamin F.

Appendix C

Formulation of retinoid containing cosmeceutical creams

C.1 Introduction

Retinoid containing topical products belong to a category known as “cosmeceuticals” or “active cosmetics” (Paye *et al.*, 2006:1). In fact, topical tretinoin was the first product to meet the criteria for cosmeceuticals (Draelos, 2009:431).

“Cosmeceuticals” is a term coined by A.M. Kligman nearly three decades ago, but it is not a category recognised by the Food, Drug and Cosmetic Act of the United States (Lintner *et al.*, 2009:461). It includes products somewhere between drugs and cosmetics, and is presently used as a term for cosmetic products with a pharmaceutical therapeutic benefit, but not necessarily a biological therapeutic benefit (Choi & Berson, 2006:163).

Cosmetics, on the other hand, may not contain a pharmacologically active compound (Buchmann, 2006:99).

In this study, a suitable delivery vehicle was identified and cosmeceutical formulations developed for the dermal delivery of tretinoin and adapalene.

C.2 Selection of a vehicle type

Successful delivery of a topically applied drug to its target site is dependant on its incorporation into a suitable vehicle. Buchmann (2006:99) described a “vehicle” as the inactive principle of a formulation where it serves as the matrix in which the active principle (in this case tretinoin or adapalene) is embedded. Not only does such a vehicle provide ease of drug application, but it may provide additional “vehicle effects” such as hydration, protection, decoration, care or cleansing (Buchmann, 2006:99-100). It may even have an influence on the integrity of the stratum corneum (see chapter 2 for a discussion on penetration enhancers).

The main dermatological vehicles are:

- Liquid preparations, e.g. liniments, lotions, paints, varnishes, tinctures;
- Gels;
- Powders;
- Ointments, including hydrocarbon bases, fats and fixed-oil bases, silicones, absorption bases, emulsifying bases and water-soluble bases;
- Creams, including oil-in-water (o/w) creams, water-in-oil (w/o) creams, and multiple creams such as w/o/w creams;

- Pastes; and
- Aerosols.

(Barry, 2002:528-530).

Semisolids are generally preferred as dermatologicals because they remain *in situ* and facilitate the delivery of the drug over a longer time period (Walters & Brain, 2002:322). As acne-prone skin is the site of application of retinoid dermatologicals, a greasy cream or ointment may aggravate the already oily skin and could be comedogenic. Therefore, several factors were considered in choosing a vehicle.

Oil-in-water (o/w) cream was the vehicle of choice due to the advantages it offers, namely:

- High acceptance due to a light, non-greasy feeling after application;
- Good skin spreadability and penetration;
- A hydration effect of the external water phase which soothes inflamed tissue; and
- A cooling effect due to the evaporation of the external water phase;

(Buchmann, 2006:109).

C.3 Oil-in-water creams

Creams are semisolid emulsions in which two immiscible phases remain in a metastable mixed state due to the effect of an emulsifier. In o/w creams the internal lipophilic phase is dispersed in the external aqueous phase. This is achieved by decreasing the interfacial tension between the two phases with an emulsifier, which also stabilises the emulsion. (Buchmann, 2006:106).

In addition to the two phases and the emulsifier, an o/w cream may contain a lipophilic thickening agent, a co-emulsifier, preservatives, a hydrophilic thickening agent, emollients, moisturisers, viscosity-increasing agents, perfumes, colourants and active ingredients (Buchmann, 2006:107).

In this study, three o/w creams were formulated for both tretinoin and adapalene. The three cream formulations for each active ingredient included a different component in the oil phase, namely castor oil, vitamin F or Pheroid™ vesicles. The stability, drug release and dermal diffusion of these creams were compared in order to determine whether the Pheroid™ cream was superior to the creams with either castor oil or vitamin F as part of the oil phase.

C.4 Selection of ingredients

A trial-and-error approach was used to experiment with existing and new cream formulations. This section provides a brief discussion of the ingredients used in the final cream formulations. All ingredients used were non-comedogenic.

C.4.1 Oil phase

Brisaert and Plaizier-Vercammen (2007:56) studied the solubility and photostability of tretinoin in different oils, as well as the photostability of tretinoin in creams with the different oils as oil phase. They proved that tretinoin was most soluble (0.604 g/100 mL) and most stable in castor oil, which is highly polar. When formulated into a cream, tretinoin was best protected against photodegradation in a cream containing castor oil as oil phase and it was more stable in an oil phase containing olive oil. Castor oil therefore proved to be the most suitable oil in terms of the solubility and photoprotection of tretinoin.

Linoleic, linolenic, and arachidonic acids are essential fatty acids and are collectively known as vitamin F (Nicolaidou & Katsambas, 2000:90), which in turn is the major oily ingredient in Pheroids™ (see chapter 2 for more information regarding Pheroid™ technology).

C.4.2 Rheology-modifying agents

Several cream formulations were tried and tested, experimenting with a natural smectite clay, Veegum® HV (magnesium aluminium silicate) and an organic polysaccharide, Xanthan® gum (R.T. Vanderbilt Company, 2007a:1; Shah & Singh, 2009).

When Veegum® HV was used, it resulted in a light brownish coloured cream with a dry feeling. Cream with Veegum® was prone to phase separation and, when mixed at 13 500 rpm at the time of emulsification (the speed needed for the formation of Pheroid™ vesicles), it produced an unstable, bubbly cream. When only Xanthan® gum was used as thickener, a beautiful white cream was produced. The appearance was more like an emulgel and it had a sticky feeling.

R.T. Vanderbilt Company (2007b:7) described a synergy between Veegum® and organic thickeners in which formulations containing such mixtures had a higher viscosity or stability than either component alone and could reduce the tackiness of the organic thickener. A mixture of Veegum® and Xanthan® gum was subsequently experimented on, which resulted in a cream with a light beige colour, acceptable texture, perfect thickness and excellent homogeneity. No phase separation occurred.

As the degree of hydration of Veegum® is directly proportional to the amount of energy used for its dispersion (R.T. Vanderbilt Company, 2007b:4), hydration of the Veegum®-Xanthan® gum powder blend was conducted with water heated to 75 °C and homogenized at 13 500 rpm for a minimum of ten minutes.

C.4.3 Thickening agents

Due to the small ratio of internal phase, a combination of cetyl alcohol and stearic acid was used to increase the firmness and stability of the creams (Mitsui, 1997:343). Stearic acid possesses additional emulsifying properties (Allen, 2009) and cetyl alcohol also serves as an emollient (Unvala, 2009).

C.4.4 Emulsifiers

Frequently used in combination, the non-ionic surfactants Tween® 80 (polysorbate 80) and Span® 60 (sorbitan monostearate) acted as emulsifiers (Zhang, 2009a; Zhang, 2009b).

C.4.5 Antimicrobials

Methyl and propylparaben are two of the antimicrobial preservatives most frequently used in cosmetics and can be used synergistically. The concentrations of these two parabens were chosen to be within the concentration range for topical preparations, namely 0.02 to 0.3 % for methylparaben and 0.01 to 0.6 % for propylparaben (Haley, 2009a; Haley, 2009b).

C.4.6 Antioxidants

A combination of BHA and BHT are normally used as antioxidants in Pheroid™ formulations. Therefore, both BHA and BHT were used in the same concentrations in this study.

C.4.7 Solvent

Polyethylene glycol 400 (macrogol 400 or PEG 400) was used as a solvent for tretinoin before adding it to the lipophilic phase. However, when PEG 400 was tested as solvent for adapalene, results showed that it repelled adapalene and caused the formation of clusters.

C.5 Formulation of tretinoin creams

C.5.1 Composition

The formula of tretinoin cream with castor oil or vitamin F as oil phase is given in Table C.1.

Table C.1: Components of tretinoin creams with castor oil or vitamin F

	Component	Cream formulation	
		Tretinoin & castor oil (% w/w)	**Tretinoin & vitamin F (% w/w)**
A	Distilled water	79.715	79.715
	Veegum® HV	1.0	1.0
	Xanthan® gum	0.3	0.3
B	Vit F ethyl ester	-	4.0
	Castor oil	4.0	-
	Cetyl alcohol	4.0	4.0
	Stearic acid	4.0	4.0
	Span® 60	0.5	0.5
	Tween® 80	4.5	4.5
	Methylparaben	0.2	0.2
	Propylparaben	0.04	0.04
	BHA	0.02	0.02
	BHT	0.2	0.2
C	PEG 400	1.5	1.5
	Tretinoin	0.025	0.025
	Total	**100**	**100**

C.5.2 Composition of the Pheroid™ cream

To formulate the Pheroid™ cream, the 4 % vitamin F or castor oil was replaced by the oil phase components of Pheroid™ vesicles. Pheroid™ cream was the only cream that contained α-tocopherol (vitamin E). Instead of using distilled water, water saturated with nitrous oxide was used.

C.5.3 Method

Tretinoin was protected from light at all times. The following steps were followed and, due to the photosensitivity of tretinoin, steps 3 to 9 were conducted in an environment that was as dark as possible.

1) Weigh the water and heat to 75 °C. Mix the dry powders of Veegum® HV and Xanthan® gum. Slowly add the mixture to the water while homogenising at 13 500 rpm for at least ten minutes. This is mixture A.
2) Weigh the ingredients listed as B in Table C.1 and heat together to 70 °C until all the ingredients have melted. This is mixture B.
3) Weigh the ingredients listed as C in Table C.1 and heat together to 70 °C until the tretinoin is completely dissolved in the PEG 400. This is mixture C.
4) Heat mixture A to 70°C.
5) Add mixture C to mixture B to obtain mixture D.
6) Immediately add mixture D to mixture A. Homogenise at 13 500 rpm until 40 °C is reached.
7) Homogenise at 200 rpm until 35 °C is reached.
8) Stir occasionally. Leave the final mixture to cool to room temperature (± 25 °C).
9) Transfer to labelled container(s).

C.5.4 Results

All three formulations resulted in fine, homogenous creams with a pale yellow colour. The Pheroid™ cream was slightly deeper yellow. No creams were oily or tacky and all the creams spread easily onto and into the skin. The castor oil cream had a characteristic castor oil smell and the vitamin F cream smelled faintly of vitamin F, whereas the Pheroid™ cream had a distinctly fishy smell.

C.6 Formulation of adapalene creams

C.6.1 Composition

The formula of adapalene cream with castor oil or vitamin F as oil phase is given in Table C.2.

Table C.2: Components of adapalene creams with castor oil or vitamin F

	Component	Cream formulation	
		Adapalene & castor oil (% w/w)	**Adapalene & vitamin F (% w/w)**
A	Distilled water	81.14	81.14
	Veegum® HV	1.0	1.0
	Xanthan® gum	0.3	0.3
B	Vit F ethyl ester	-	4.0
	Castor oil	4.0	-
	Cetyl alcohol	4.0	4.0
	Stearic acid	4.0	4.0
	Span® 60	0.5	0.5
	Tween® 80	4.5	4.5
	Methylparaben	0.2	0.2
	Propylparaben	0.04	0.04
	BHA	0.02	0.02
	BHT	0.2	0.2
	Adapalene	0.1	0.1
	Total	**100**	**100**

C.6.2 Composition of the Pheroid™ cream

To formulate the Pheroid™ cream, the 4 % vitamin F or castor oil was replaced by the oil phase components of Pheroid™ vesicles. Instead of using distilled water, water saturated with nitrous oxide was used.

C.6.3 Method

1) Weigh the water and heat to 75 °C. Mix the dry powders of Veegum® HV and Xanthan® gum. Slowly add the mixture to the water while homogenizing at 13 500 rpm for at least ten minutes. This is mixture A.
2) Weigh adapalene and disperse in Tween® 80 to prevent clotting. Weight and add the liquid ingredients listed as B in Table C.2, followed by the solid ingredients. Heat to 70 °C until all the ingredients have melted. This is mixture B.
3) Heat mixture A to 70 °C.

4) Add mixture B to mixture A. Homogenise at 13 500 rpm until 40 °C is reached.

5) Homogenise at 200 rpm until 35 °C is reached.

6) Stir occasionally. Leave the final mixture to cool to room temperature (± 25 °C).

7) Transfer to labelled container(s).

C.6.4 Results

The adapalene cream formulations resulted in fine, homogenous creams. All the creams spread easily onto and into the skin without being oily or tacky. The castor oil and vitamin F creams were very light beige, whereas the Pheroid™ cream presented with a very light yellow colour. The vitamin F cream smelled faintly of vitamin F and the castor oil cream had a characteristic castor oil smell, whereas the Pheroid™ cream had a distinctly fishy smell.

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Appendix D

Stability studies

D.1 Introduction

A well-developed dermatological formulation must be physically, chemically, biologically and microbiologically stable over a defined period of time. Stability testing is used to determine the influence of environmental factors such as temperature, humidity and light on the quality of the product over a specified time period (ICH, 2003:1, 3).

Physicochemical tests may include evaluation of the following:

- Thermal stability of the drug and preservatives (e.g. antimicrobials and antioxidants);
- Efficacy of antimicrobials;
- Rheological properties;
- Loss of solvents or sensitivity to moisture;
- Phase changes (bleeding, cracking, inhomogeneity);
- Particle size distribution;
- Organoleptic properties (e.g. colour, odour, texture);
- pH; and
- Photostability.

(Barry, 2002:531; ICH, 2003:7-8).

A dermatological product showing no or acceptable changes during stability testing is regarded cosmetically and therapeutically stable under normal storage conditions. The ICH (2003:9) describes significant change for a drug product as:

- A change in assay of 5 % from its initial value;
- Presence of any degradation product in a quantity exceeding its acceptance criterion;
- Failure to meet the acceptance criterion for pH; or
- Not meeting the acceptance criteria for appearance and functionality (e.g. colour, phase separation, caking or hardness). Physical changes (e.g. melting of creams) may occur under accelerated conditions (i.e. 40 °C and 75 % relative humidity).

In this study the six cream formulations to be tested were subjected to three conditions, differing in temperature and relative humidity (RH), namely 25 °C and 60 % RH; 30 °C and 60 % RH; as well as 40 °C and 75 % RH. Labcon Humidity Chambers were used to provide 30 °C and 60 % RH; as well as 40 °C and 75 % RH conditions. A Labcon Low Temperature Humidity Chamber was used for 25 °C and 60 % RH.

Plastic containers, each with a capacity for approximately 30 g of cream, were used to store the creams under the above specified conditions. Wide mouthed Consol® glass jars with a capacity of 125 ml were used to study rheology. Before use, the plastic and glass containers were washed with 70 % ethanol and dried in an aseptic flow cabinet.

Three different creams were prepared for both tretinoin and adapalene, as described in appendix C, namely castor oil, vitamin F and Pheroid™ creams. The plastic and glass containers were filled and stored at the appropriate temperatures and humidities.

Six stability tests were conducted at specific time intervals, namely:

- Assay;
- pH;
- Rheology;
- Homogeneity and particle size;
- Visual assessment; and
- Mass variation.

The creams were tested before being stored in the climate chambers (at time = 0) and again after one, two, three and six months.

D.2 Methods

D.2.1 Assay

HPLC methods were used to determine the concentration of the active ingredients (retinoid, parabens, antioxidants and vitamin E) in tretinoin or adapalene containing creams. The HPLC methods were described and validated in appendix B.

D.2.1.1 Preparation of standard solution

The preparation of a standard solution for tretinoin and adapalene was described in appendix B (section B.2.2 and B.3.2, respectively).

D.2.1.2 Preparation of samples

Samples were prepared as described in appendix B (section B.2.4). For each type of cream at each storage condition, two 100 % sample solutions were prepared and analysed in duplicate.

D.2.2 pH

The pH was determined using a Mettler Toledo MultiSeven pH meter and Mettler Tolodo Inlab® 410 probe. Before use, the pH meter was calibrated at pH 4.01, 7.00 and 10.01 with Mettler

Toledo pH buffer solutions. The pH of each cream formulation at each storage condition was measured once.

D.2.3 Rheology

Rheology is the study of the flow and deformation properties of matter. Knowledge of the rheological properties of pharmaceutical materials is necessary for preparation, development, evaluation and performance of pharmaceutical dosage forms (Marriott, 2002:41).

Viscosity is a fluid's resistance to flow or movement (Marriott, 2002:41). A viscometer acts as a tool of rheology by measuring this internal friction of the fluid (Brookfield Engineering Laboratories, 2006:14).

A Brookfield DV-II+ viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, U.S.A.), together with a Brookfield helipath and water bath, were used to measure viscosity.

The glass jars with the cream samples used to study rheology were stored at 25 °C and 60 % RH only. The creams were heated in the water bath to a temperature of 25 °C. The horizontal bar of the T-bar spindle (spindle T-E) was placed 1 cm below the surface of the cream. While the helipath descended and ascended, the viscometer took one reading every ten seconds to a total of 31 data points, providing enough time for the helipath to complete one bidirectional cycle. The first reading was discarded and the rest used to calculate the average viscosity.

D.2.4 Homogeneity and particle size

Homogeneity and particle size were evaluated by confocal laser scanning microscopy. Samples were prepared by adding 2 µl Nile Red to approximately 200 mg cream. The samples were incubated in a dark room for 15 minutes where after a small amount was placed on a microscope plate and studied under the confocal laser scanning microscope. Visual assessment was also conducted using a light microscope.

D.2.5 Visual assessment

The colours of the creams were evaluated and photos were taken to compare results. Paint colour cards were used to define the colours.

D.2.6 Mass variation

A Shimadzu AUW 120D balance (Shimadzu, Japan) was used to determine the mass of each cream at each condition during the specified storage period.

D.3 Results and discussion

D.3.1 Assay

Tables D.1 to D.3 present the percentage recovery of the active ingredients of the three tretinoin creams at the different conditions: table D.1 for castor oil cream, table D.2 for vitamin F cream, and table D.3 for Pheroid™ cream.

Table D.1: Percentage recovery of the active ingredients of tretinoin cream with castor oil at each storage condition for a period of 6 months

	% Recovery				
	Initial	**Month 1**	**Month 2**	**Month 3**	**Month 6**
25 °C and 60 % RH					
Methylparaben	105.5	99.2	90.6	86.4	106.7
Propylparaben	101.8	101.5	96.3	93.1	110.5
BHA	98.4	97.0	89.0	84.7	113.9
BHT	122.8	113.2	105.3	92.0	125.4
Tretinoin	103.8	94.3	87.1	75.8	104.6
30 °C and 60 % RH					
Methylparaben	105.5	88.9	90.9	55.1	100.4
Propylparaben	101.8	89.1	93.2	55.6	87.3
BHA	98.4	84.6	86.3	50.8	91.0
BHT	122.8	99.2	104.5	52.7	100.5
Tretinoin	103.8	82.5	85.0	42.5	81.8
40 °C and 75 % RH					
Methylparaben	105.5	101.9	85.0	72.8	77.9
Propylparaben	101.8	104.0	87.6	75.1	81.9
BHA	98.4	99.0	82.1	69.2	79.5
BHT	122.8	114.6	96.3	70.3	83.7
Tretinoin	103.8	95.3	77.4	55.3	60.7

Table D.2: Percentage recovery of the active ingredients of tretinoin cream with vitamin F at each storage condition for a period of 6 months

	% Recovery				
	Initial	**Month 1**	**Month 2**	**Month 3**	**Month 6**
25 °C and 60 % RH					
Methylparaben	101.5	94.5	63.7	104.3	71.7
Propylparaben	96.0	92.6	59.6	107.3	80.9
BHA	94.2	89.4	55.2	96.0	70.0
BHT	120.4	111.9	69.0	109.4	80.3
Tretinoin	97.0	87.4	52.6	85.9	60.9
30 °C and 60 % RH					
Methylparaben	101.5	101.8	63.5	125.9	80.5
Propylparaben	96.0	102.1	60.1	132.0	89.7
BHA	94.2	101.8	61.3	120.2	78.6
BHT	120.4	122.3	67.6	134.7	87.5
Tretinoin	97.0	95.5	51.1	106.0	63.9
40 °C and 75 % RH					
Methylparaben	101.5	93.2	69.0	98.7	75.7
Propylparaben	96.0	92.1	65.4	99.3	82.0
BHA	94.2	90.6	60.0	88.6	70.6
BHT	120.4	104.9	72.0	91.9	72.3
Tretinoin	97.0	81.1	53.4	67.9	45.8

Table D.3: Percentage recovery of the active ingredients of tretinoin cream with Pheroid™ at each storage condition for a period of 6 months

	% Recovery				
	Initial	**Month 1**	**Month 2**	**Month 3**	**Month 6**
25 °C and 60 % RH					
Methylparaben	104.0	92.6	73.7	67.3	57.4
Propylparaben	103.0	95.5	75.8	72.9	60.4
BHA	100.5	97.6	73.2	62.1	73.7
BHT	122.8	115.0	93.1	86.6	77.3
Tretinoin	101.2	70.9	49.4	34.0	19.5
α-tocopherol	80.0	77.0	55.5	48.9	25.2
30 °C and 60 % RH					
Methylparaben	104.0	89.3	61.0	70.0	57.6
Propylparaben	103.0	89.5	62.3	74.8	60.3
BHA	100.5	97.5	76.0	70.6	77.3
BHT	122.8	94.7	61.3	71.9	55.7
Tretinoin	101.2	54.1	27.8	29.6	13.6
α-tocopherol	80.0	63.3	38.7	42.5	18.7
40 °C and 75 % RH					
Methylparaben	104.0	79.3	69.1	82.6	89.9
Propylparaben	103.0	80.5	75.2	92.5	91.6
BHA	100.5	139.7	101.7	84.5	81.6
BHT	122.8	32.3	34.3	45.0	38.0
Tretinoin	101.2	38.0	29.0	31.1	13.3
α-tocopherol	80.0	28.4	20.7	22.7	3.8

The percentage recovery of the active ingredients of the three adapalene creams at the different conditions is presented in tables D.4 to D.6.

Table D.4: Percentage recovery of the active ingredients of adapalene cream with castor oil at each storage condition for a period of 6 months

	% Recovery				
	Initial	**Month 1**	**Month 2**	**Month 3**	**Month 6**
25 °C and 60 % RH					
Methylparaben	109.3	94.2	92.4	83.6	107.7
Propylparaben	109.3	95.1	99.2	85.9	105.4
BHA	106.7	88.8	83.8	82.1	101.4
BHT	127.3	112.0	105.7	99.6	129.0
Adapalene	111.2	101.2	87.2	86.9	114.7
30 °C and 60 % RH					
Methylparaben	109.3	101.3	78.7	85.3	82.7
Propylparaben	109.3	101.8	84.1	86.9	83.2
BHA	106.7	94.7	71.5	83.5	73.8
BHT	127.3	121.4	90.6	97.8	91.5
Adapalene	111.2	109.5	74.6	83.0	80.9
40 °C and 75 % RH					
Methylparaben	109.3	100.5	75.9	77.9	104.3
Propylparaben	109.3	101.5	79.7	82.2	106.0
BHA	106.7	91.9	66.3	75.6	95.7
BHT	127.3	116.8	84.5	86.2	117.3
Adapalene	111.2	108.2	71.1	79.3	112.0

Table D.5: Percentage recovery of the active ingredients of adapalene cream with vitamin F at each storage condition for a period of 6 months

	% Recovery				
	Initial	**Month 1**	**Month 2**	**Month 3**	**Month 6**
25 °C and 60 % RH					
Methylparaben	109.1	90.8	97.0	69.2	80.4
Propylparaben	104.8	83.2	98.3	67.6	82.1
BHA	114.4	85.2	92.1	67.3	79.0
BHT	129.2	103.9	115.1	73.5	94.3
Adapalene	98.3	90.3	91.3	68.5	70.5
30 °C and 60 % RH					
Methylparaben	109.1	84.5	63.5	75.1	92.8
Propylparaben	104.8	77.2	63.6	73.7	87.8
BHA	114.4	79.3	56.6	71.1	88.7
BHT	129.2	96.7	68.6	77.2	106.2
Adapalene	98.3	83.4	55.1	70.0	79.8
40 °C and 75 % RH					
Methylparaben	109.1	100.7	78.8	82.1	89.3
Propylparaben	104.8	96.8	79.0	80.0	86.2
BHA	114.4	88.1	71.8	78.1	81.4
BHT	129.2	115.9	82.0	82.3	86.8
Adapalene	98.3	105.4	71.9	79.5	75.8

Table D.6: Percentage recovery of the active ingredients of adapalene cream with Pheroid™ at each storage condition for a period of 6 months

	% Recovery				
	Initial	**Month 1**	**Month 2**	**Month 3**	**Month 6**
25 °C and 60 % RH					
Methylparaben	114.8	102.9	100.1	74.0	85.8
Propylparaben	100.0	87.4	94.8	85.1	80.8
BHA	113.6	93.1	89.5	95.1	108.4
BHT	85.6	90.2	96.0	106.9	118.1
Adapalene	92.9	93.3	83.1	98.0	89.4
α-tocopherol	103.0	90.1	78.0	70.4	48.7
30 °C and 60 % RH					
Methylparaben	114.8	104.8	72.4	101.6	93.1
Propylparaben	100.0	89.8	68.8	90.3	74.1
BHA	113.6	97.4	62.9	100.3	95.2
BHT	85.6	85.0	57.1	95.5	96.1
Adapalene	92.9	97.5	58.2	96.0	75.1
α-tocopherol	103.0	79.3	43.2	51.3	18.0
40 °C and 75 % RH					
Methylparaben	114.8	105.3	93.8	101.5	99.9
Propylparaben	100.0	89.4	88.3	89.6	78.1
BHA	113.6	104.4	82.4	78.3	46.6
BHT	85.6	37.2	38.0	45.9	45.8
Adapalene	92.9	94.8	77.1	93.8	81.4
α-tocopherol	103.0	29.9	17.8	8.3	0.0

The concentrations of the active ingredients in each cream formulation showed significant change. In fact, the recovery values differed exceptionally and inconsistently between the different months. The percentage relative standard deviations (% RSD or precision) also differed significantly, varying from 0.02 % and larger to an occasional 22 %. However, when validated in appendix B, the precision proved to be adequate.

As mentioned in appendix B, the cream matrices complicated HPLC analysis. It is also important to keep in mind that the concentrations of the active ingredients in the creams were significantly small. This enlarged the possible margin of error.

Assay testing usually applies to the pharmacologically active ingredient(s) in a product (in this case tretinoin or adapalene). The 5 % change interpreted by the ICH as significant was therefore applicable to the retinoids only. At this stage it is important to note that the ICH provided only recommendations (and no laws) for stability testing (ICH, 2003:1). Where the ICH

interpreted a change in concentration of more than 5 % as significant in stability testing, the FDA (2001:5) suggested a limit of 15 % (and at low concentrations a limit of 20 %) for bioanalytical studies. Therefore, no fixed rules exist for the interpretation and use of stability data.

Although this study included the determination of the concentrations of the adjuvants, the criteria for these components are different. The purpose of the antioxidants is to protect the formulation, and especially oxidation-prone tretinoin, from oxidation. Antioxidants are thus permitted to decrease in concentration over time. The same applies to the preservatives which serve to prevent microbacterial growth.

Due to the above-mentioned factors, the results obtained during stability studies could not be used quantitatively, but were used comparatively. It was clear that degradation had occurred, but it was impossible to state the exact amount of degradation due to the inherent limitations of the used method.

Adapalene was, as expected, more stable than tretinoin in similar formulations. It was observed that the castor oil creams (tables D.1 and D.4) were the most stable formulations, followed closely by the vitamin F creams (tables D.2 and D.5), with the Pheroid™ creams (tables D.3 and D.6) being far less stable than the other creams. The α-tocopherol in the Pheroid™ creams was completely degraded at 40 °C and 75 % RH after six months, and the tretinoin concentration also decreased significantly. The degradation of the α-tocopherol and tretinoin could be due to insufficient protection by the antioxidants. It could be possible that α-tocopherol exerted a greater antioxidative effect than BHA and BHT, or that it acted as a pro-oxidant. Belhaj *et al.* (2010:190-191) reported the pro-oxidative effect of 0.2% α-tocopherol, which was the same concentration of α-tocopherol used in the Pheroid™ creams. In their study, the high concentration of α-tocopherol accelerated the onset of oxidation of crude salmon oil, as well as of salmon oil in an emulsion.

D.3.2 pH

The following two tables (tables D.7 and D.8) reflect the pH values of the different cream formulations, as well as the increase in pH during six months of storage at the different conditions.

Table D.7: pH values for tretinoin creams, which included castor oil, vitamin F or Pheroid™, over a six month period

Storage conditions	Initial	1 month	2 months	3 months	6 months	Increase
Castor oil cream						
25 °C and 60 % RH	5.298	5.123	6.061	5.622	5.531	0.233
30 °C and 60 % RH	5.298	5.134	5.482	5.571	5.467	0.169
40 °C and 75 % RH	5.298	5.192	5.803	4.871	5.750	0.452
Vitamin F cream						
25 °C and 60 % RH	5.212	5.216	5.217	5.789	5.525	0.313
30 °C and 60 % RH	5.212	5.131	5.289	5.429	5.491	0.279
40 °C and 75 % RH	5.212	4.910	5.494	5.687	5.832	0.620
Pheroid™ cream						
25 °C and 60 % RH	4.881	4.863	5.286	5.396	5.337	0.456
30 °C and 60 % RH	4.881	4.788	5.342	5.277	5.203	0.322
40 °C and 75 % RH	4.881	4.643	5.537	5.371	5.322	0.441

Table D.8: pH values for adapalene creams, which included castor oil, vitamin F or Pheroid™, over a six month period

Storage conditions	Initial	1 month	2 months	3 months	6 months	Increase
Castor oil cream						
25 °C and 60 % RH	5.296	5.047	5.506	5.785	5.551	0.255
30 °C and 60 % RH	5.296	4.987	5.363	5.557	5.428	0.132
40 °C and 75 % RH	5.296	4.898	5.392	5.570	5.738	0.442
Vitamin F cream						
25 °C and 60 % RH	5.245	5.142	5.630	5.324	5.521	0.276
30 °C and 60 % RH	5.245	5.088	5.323	5.272	5.582	0.337
40 °C and 75 % RH	5.245	4.996	5.362	5.598	5.930	0.685
Pheroid™ cream						
25 °C and 60 % RH	5.345	5.095	5.658	5.419	5.439	0.094
30 °C and 60 % RH	5.345	4.982	5.433	5.359	5.425	0.080
40 °C and 75 % RH	5.345	4.865	5.189	5.328	5.370	0.025

No significant change in pH was observed for any cream formulation during the six month period. Any change in pH was relatively small with the largest difference being 0.685 for vitamin F and adapalene cream at 40 °C and 75 % RH. The Pheroid™ and adapalene cream showed the least variation in pH. This was not as expected, because of the chemical degradation observed in the Pheroid™ creams during assay testing (see section D.3.1).

D.3.3 Rheology

The six creams were subjected to a temperature of 25 °C and relative humidity of 60% for six months. Table D.9 presents the average monthly viscosity of each cream, together with the percentage increase in viscosity after six months. Figure D.1 illustrates the changes in viscosity observed during six months.

Table D.9: Average viscosity (cP) of the different cream formulations during storage at 25 °C and 60 % RH for six months. Included in this table is the percentage increase in viscosity after six months

Cream	Initial	Month 1	Month 2	Month 3	Month 6	% Increase
Tretinoin creams						
Castor oil	341437.6	307746.8	352581.0	358100.7	411891.3	20.6
Vitamin F	841903.7	801946.8	942559.3	1004212.8	1027020.4	22.0
Pheroid™	575346.0	561078.2	637780.6	749163.1	742706.1	29.1
Adapalene creams						
Castor oil	265932.8	283585.3	341281.3	355184.6	394759.5	48.4
Vitamin F	836227.8	774938.8	969324.4	879708.1	941101.3	12.5
Pheroid™	582688.2	566493.7	576387.4	685478.7	787852.7	35.2

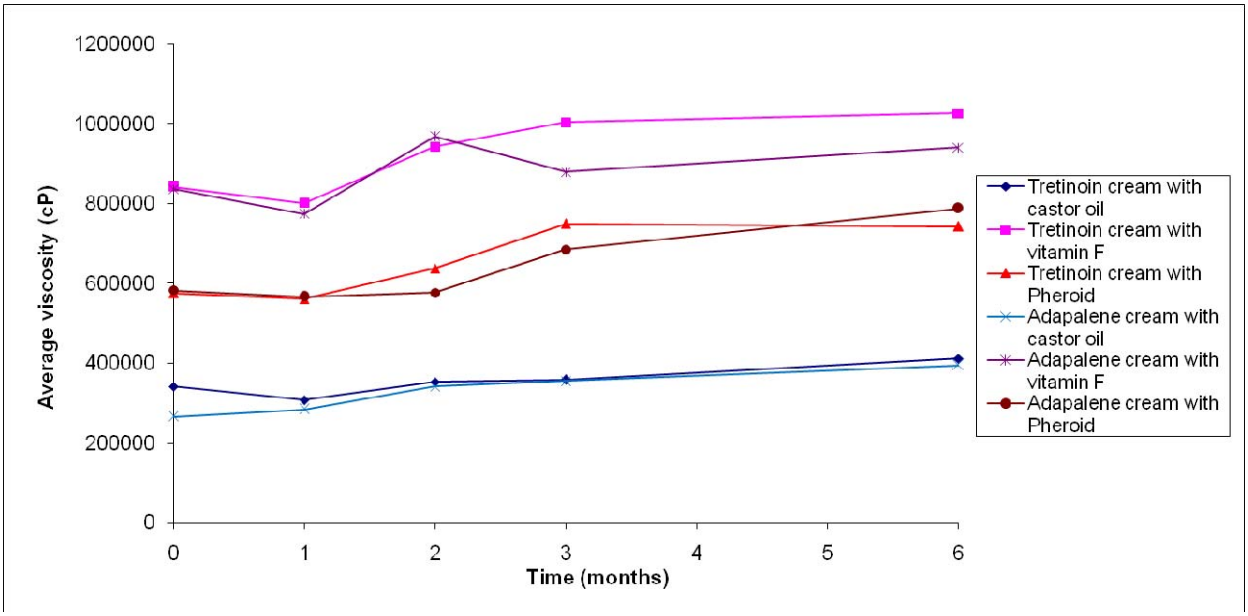
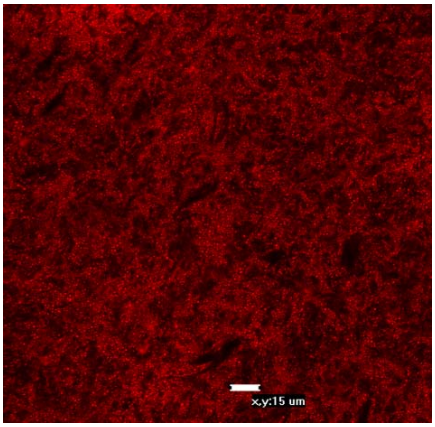


Figure D.1: Average viscosity of the six creams stored at 25 °C and 60% RH during six months.

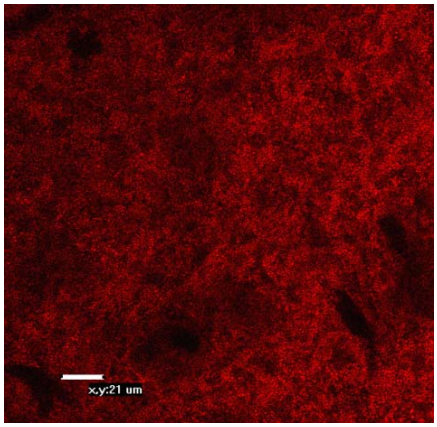
All the creams demonstrated a significant increase in viscosity, with the percentage increase ranging from 12.5 to 48.4 %.

D.3.4 Homogeneity and particle size

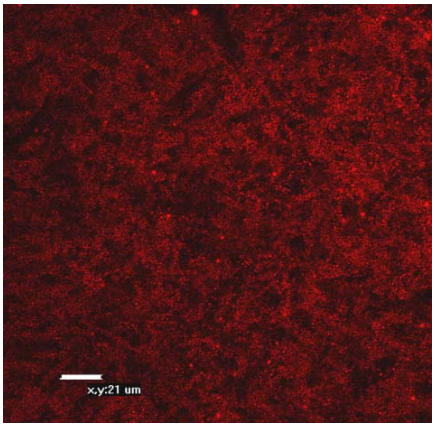
The following images (figures D.2 to D.7) were taken by a confocal laser scanning microscope and were used to compare the homogeneity and particle size of the creams at different conditions over a period of six months.



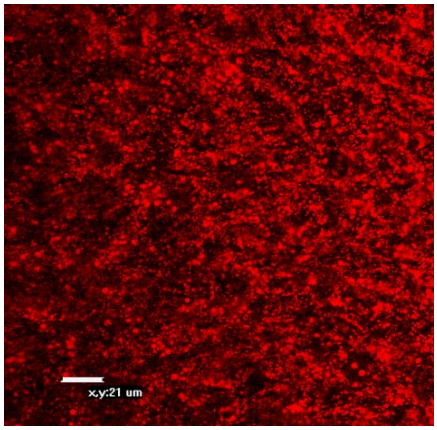
a) Initial



b) 25 °C and 60 % RH

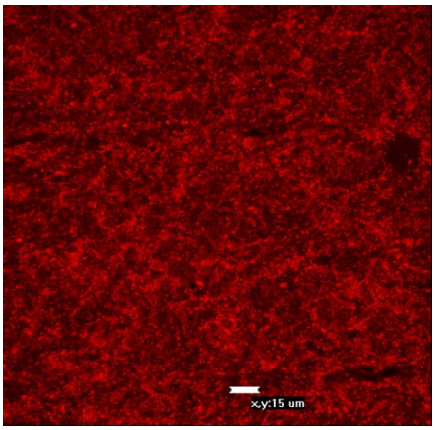


c) 30 °C and 60 % RH

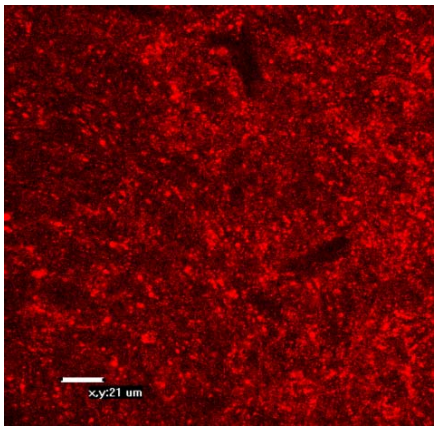


d) 40 °C and 75 % RH

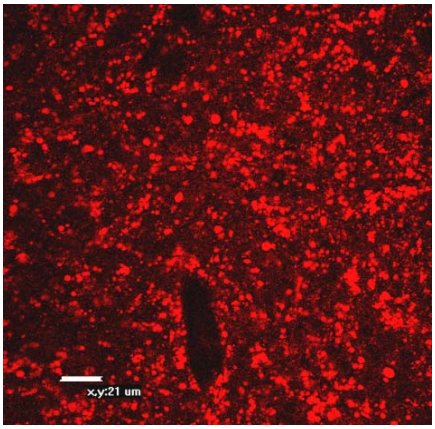
Figure D.2: Homogeneity of tretinoin and castor oil cream (a) initially and (b-d) after six months at the specified conditions



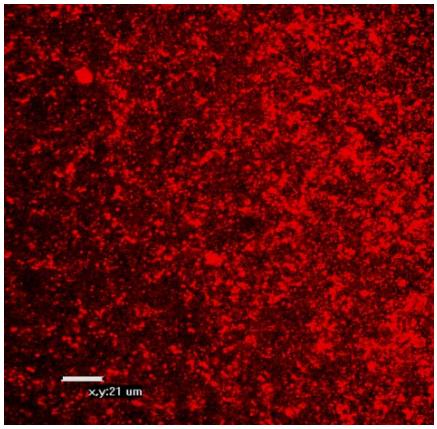
a) Initial



b) 25 °C and 60 % RH

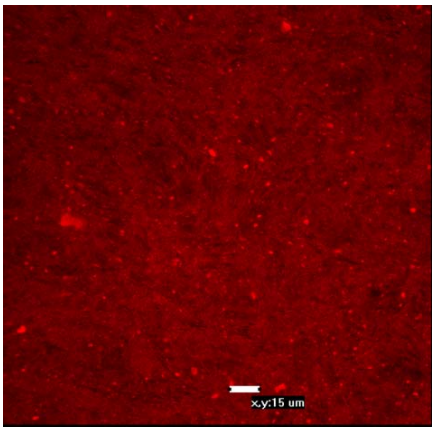


c) 30 °C and 60 % RH

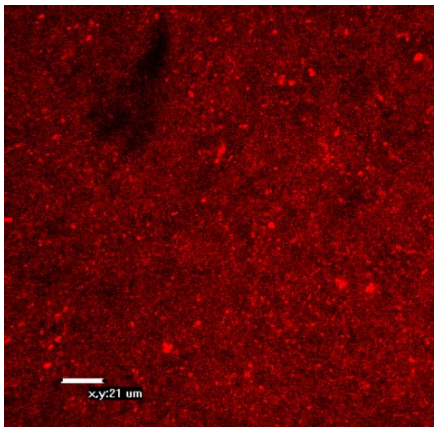


d) 40 °C and 75 % RH

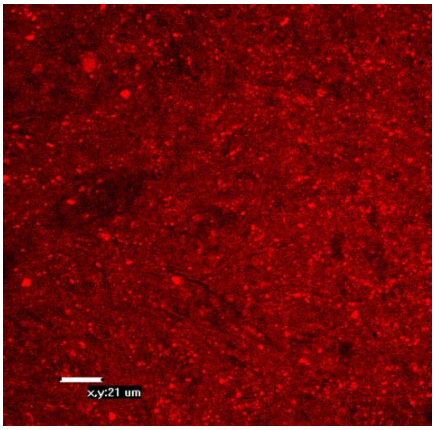
Figure D.3: Homogeneity of tretinoin and vitamin F cream (a) initially and (b-d) after six months at the specified conditions



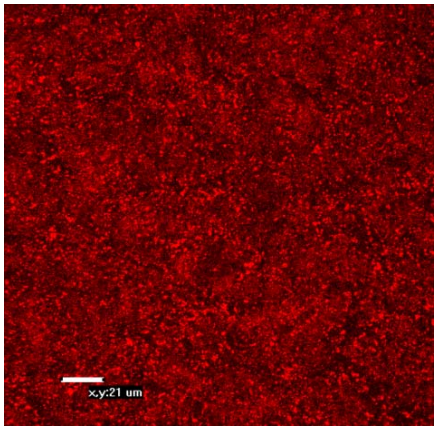
a) Initial



b) 25 °C and 60 % RH

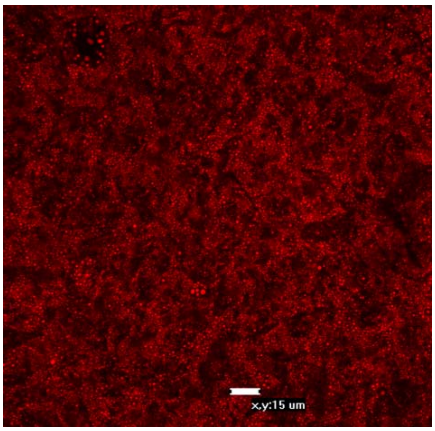


c) 30 °C and 60 % RH

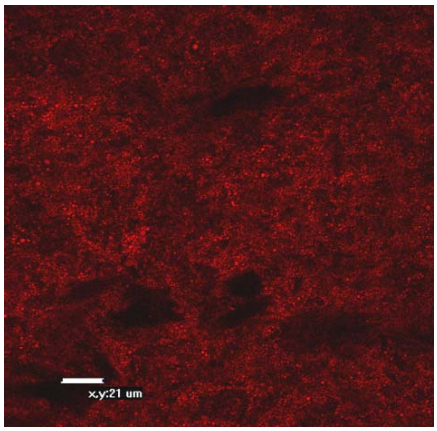


d) 40 °C and 75 % RH

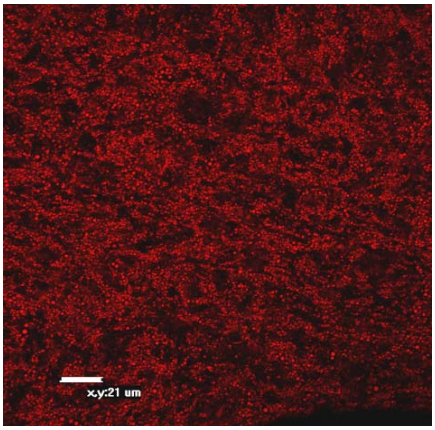
Figure D.4: Homogeneity of tretinoin and Pheroid™ cream (a) initially and (b-d) after six months at the specified conditions



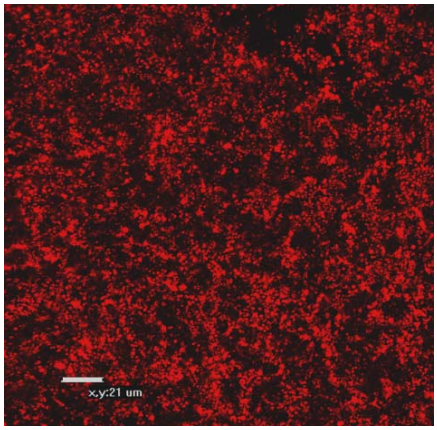
a) Initial



b) 25 °C and 60 % RH

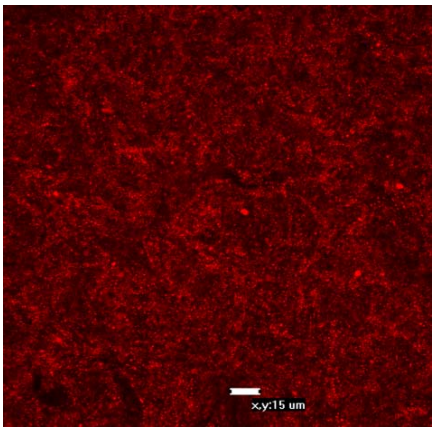


c) 30 °C and 60 % RH

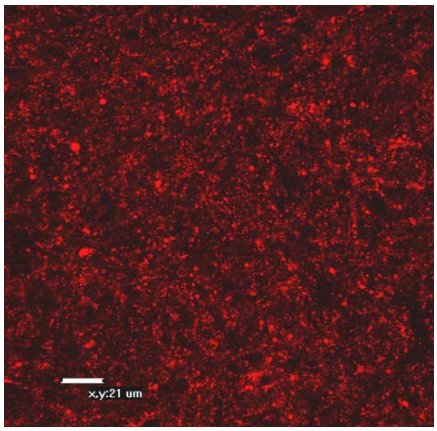


d) 40 °C and 75 % RH

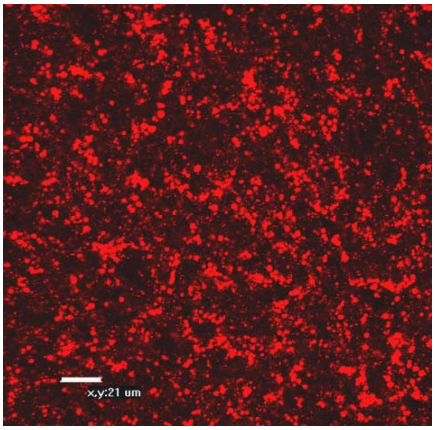
Figure D.5: Homogeneity of adapalene and castor oil cream (a) initially and (b-d) after six months at the specified conditions



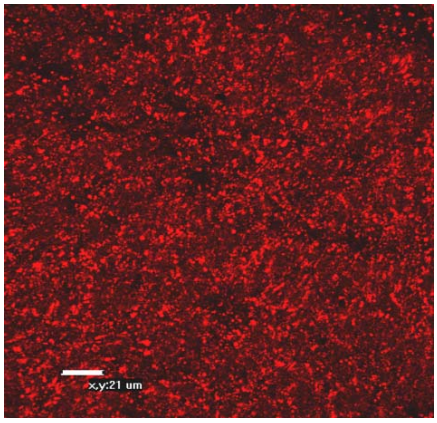
a) Initial



b) 25 °C and 60 % RH

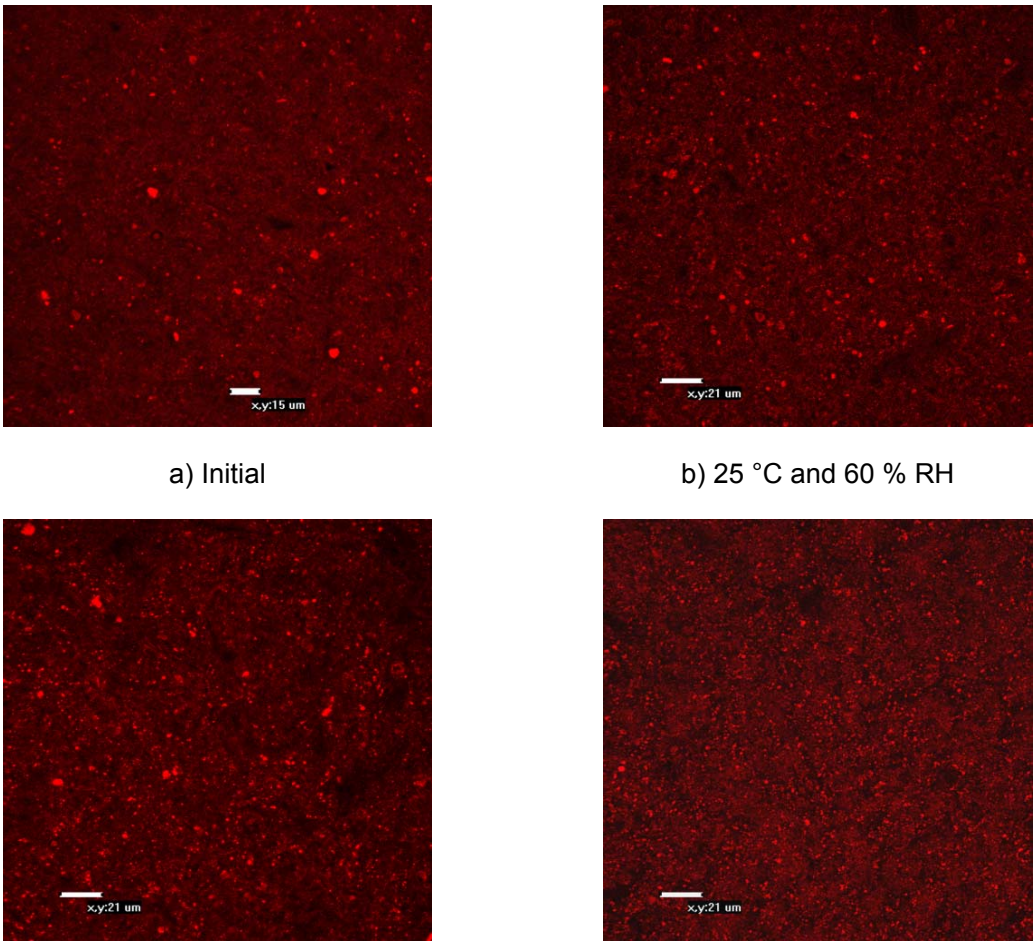


c) 30 °C and 60 % RH



d) 40 °C and 75 % RH

Figure D.6: Homogeneity of adapalene and vitamin F cream (a) initially and (b-d) after six months at the specified conditions



a) Initial

b) 25 °C and 60 % RH

c) 30 °C and 60 % RH

d) 40 °C and 75 % RH

Figure D.7: Homogeneity of adapalene and Pheroid™ cream (a) initially and (b-d) after six months at the specified conditions

The creams remained homogenous at the different storage conditions during six months.

Overall, the particle sizes remained constant. At 40 °C and 75 % RH the castor oil creams depicted larger particles. The vitamin F creams showed larger particles at 30 °C and 60 % RH. These minimal changes were acceptable.

When assessed under the light microscope, all the creams initially showed a significant number of gum stripes (figure D.8). As time passed, these stripes became less and smaller until, at month six, almost no stripes could be observed. In the samples stored at 40 °C and 75 % RH, the stripes disappeared after only two months. The Veegum® and Xanthan® gum were probably not adequately hydrated during manufacturing of the creams. This could be the reason for the increase in viscosity observed in section D.3.3.

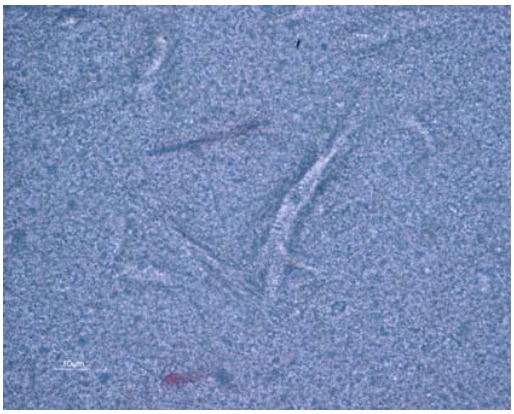


Figure D.8: Gum stripes in adapalene and vitamin F cream as observed with light microscopy. The red crystals are Nile Red

D.3.5 Visual assessment

The following images (figures D.9 to D.14) were used to compare the colours of the creams during the six month stability testing. For each formulation, the photos provided are those taken initially and after six months at each storage condition.

a) Initial

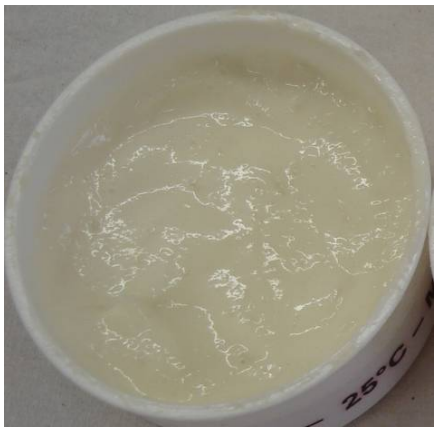
b) 25 °C and 60 % RH

c) 30 °C and 60 % RH

d) 40 °C and 75 % RH

Figure D.9: Colour assessment of tretinoin and castor oil cream (a) initially and (b-d) after six months at the specified conditions

a) Initial



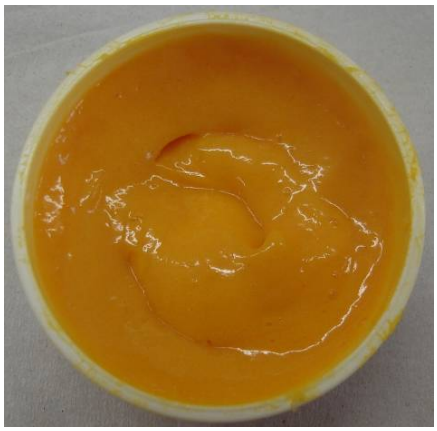
b) 25 °C and 60 % RH

c) 30 °C and 60% RH

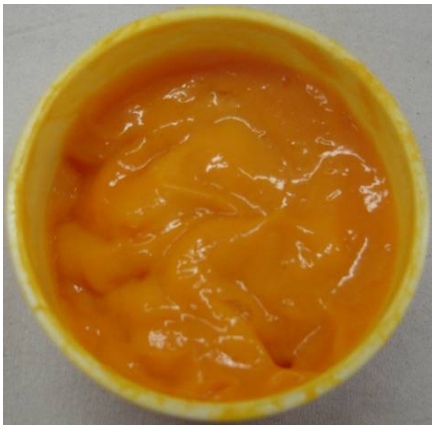
d) 40 °C and 75 % RH

Figure D.10: Colour assessment of tretinoin and vitamin F cream (a) initially and (b-d) after six months at the specified conditions

a) Initial



b) 25 °C and 60 % RH



c) 30 °C and 60 % RH

d) 40 °C and 75 % RH

Figure D.11: Colour assessment of tretinoin and Pheroid™ cream (a) initially and (b-d) after six months at the specified conditions

a) Initial

b) 40 °C and 75 % RH

c) 30 °C and 60 % RH

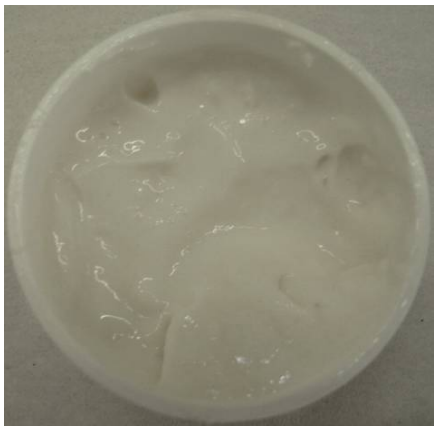
d) 30 °C and 60 % RH

Figure D.12: Colour assessment of adapalene and castor oil cream (a) initially and (b-d) after six months at the specified conditions

a) Initial

b) 25 °C and 60 % RH

c) 30 °C and 60 % RH



d) 40 °C and 75 % RH

Figure D.13: Colour assessment of adapalene and vitamin F cream (a) initially and (b-d) after six months at the specified conditions

a) Initial

b) 25 °C and 60 % RH

c) 30 °C and 60 % RH

d) 40 °C and 75 % RH

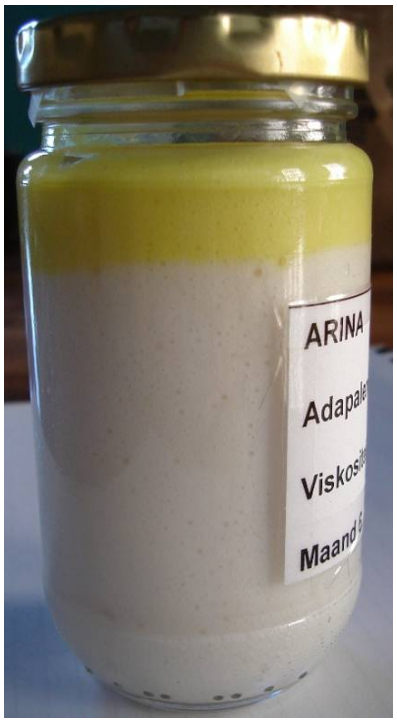
Figure D.14: Colour assessment of adapalene and Pheroid™ cream (a) initially and (b-d) after six months at the specified conditions

The castor oil and vitamin F creams with tretinoin remained a slight yellow colour. However, the Pheroid™ and tretinoin cream changed within six months from the initial light yellow to a bright orange-yellow, increasing in intensity as the temperature and humidity increased, until it became an unattractive mustard colour at 40 °C and 75 % RH. As could be observed in figure D.11 (d), the Pheroid™ and tretinoin cream at 40 °C and 75 % RH was significantly less viscous after six months.

The castor oil and vitamin F creams with adapalene remained an off-white colour during the stability testing period. However, the Pheroid™ and adapalene cream changed to a brilliant yellow at 25 °C and 60 % RH and an even brighter yellow at 30 °C and 60 % RH. At 40 °C and 75 % RH the cream was, like the Pheroid™ and tretinoin cream, mustard coloured. During the six months was observed that the Pheroid™ and tretinoin cream showed a more rapid change in colour than the Pheroid™ and adapalene cream.

Figures D.15 (a-b) demonstrate the discolouration of the Pheroid™ creams. Of interest is the fact that the yellow part remained constant during six months. The reaction was probably caused by oxidation of at least one of the ingredients. As only the Pheroid™ creams contained α-tocopherol, it was concluded that α-tocopherol played an important role in the discolouration of the Pheroid™ creams.

a) Tretinoin and Pheroid™ cream



b) Adapalene and Pheroid™ cream

Figure D.15: Pheroid™ creams after storage at 25 °C and 60 % RH for six months

D.3.6 Mass variation

The mass of the six creams was determined initially, as well as after 1, 2, 3 and 6 months of storage at the specified conditions. Tables D.10 and D.11 present the mass variation for tretinoin and adapalene creams, respectively.

Table D.10: Monthly measurements of the mass (g) of the three tretinoin creams, as well as the percentage of mass loss after six months

Storage conditions	Initial	1 month	2 months	3 months	6 months	% Loss
Castor oil cream						
25 °C and 60% RH	28.11598	28.08176	28.08538	28.04008	27.97481	0.582
30 °C and 60% RH	28.89516	28.86936	28.86835	28.84090	28.78487	0.382
40 °C and 75% RH	29.03994	28.92911	28.86686	28.79577	28.61907	1.449
Vitamin F cream						
25 °C and 60% RH	31.77802	31.74317	31.86258	31.67747	31.60142	0.948
30 °C and 60% RH	32.59652	32.53400	32.51676	32.49747	32.43490	0.496
40 °C and 75% RH	32.67258	32.61069	32.58155	32.53438	32.39346	0.854
Pheroid™ cream						
25 °C and 60% RH	32.01551	32.00020	32.15588	31.98935	31.91971	0.593
30 °C and 60% RH	31.25170	31.21516	31.19751	31.17784	31.13431	0.376
40 °C and 75% RH	32.02902	31.98375	31.97085	31.94372	31.86384	0.516

Table D.10: Monthly measurements of the mass (g) of the three adapalene creams, as well as the percentage of mass loss after six months

Storage conditions	Initial	1 month	2 months	3 months	6 months	% Loss
Castor oil cream						
25 °C and 60% RH	26.67432	26.66128	26.65389	26.64532	26.62052	0.202
30 °C and 60% RH	27.43534	27.41913	27.41111	27.39941	27.36238	0.266
40 °C and 75% RH	26.76163	26.71304	26.69167	26.65893	26.56519	0.734
Vitamin F cream						
25 °C and 60% RH	30.69912	30.66848	30.65410	30.63940	30.60050	0.321
30 °C and 60% RH	31.29719	31.24902	31.23824	31.22311	31.17321	0.396
40 °C; 75% RH	31.44756	31.37822	31.34301	31.27757	31.07217	1.194
Pheroid™ cream						
25 °C and 60% RH	27.02188	26.99805	26.97118	26.94372	26.86411	0.584
30 °C and 60% RH	29.28923	29.25973	29.24866	29.23762	29.20778	0.278
40 °C and 75% RH	29.72989	29.68954	29.67988	29.65742	29.58739	0.479

All the creams showed minimal mass loss. The highest mass loss after six months was depicted by adapalene and vitamin F cream at 40 °C and 75 % RH (1.194 %). Mass variation for each cream was, however, acceptable.

D.4 Conclusion

All the active ingredients showed degradation during six months. Although no exact degradation values could be given, the castor oil creams proved to be the most stable formulations regarding concentration of active ingredients. The Pheroid™ creams were the most unstable with α-tocopherol largely degraded at 25 °C and 60 % RH, as well as 30 °C and 60 % RH after six months. It was completely degraded at 40 °C and 75 % RH. α-Tocopherol could probably provide a more significant antioxidant effect than BHA and BHT, or it could act as a pro-oxidant. Adapalene was more stable than tretinoin in the respective cream formulations.

The pH of all the cream formulations remained relatively stable with only a slight increase during the six months.

Viscosity of the creams changed considerably with vitamin F and adapalene cream being the most stable formulation after six months. This large increase in viscosity could be due to hydration of the gums over time, as observed during assessment with light microscopy. By prolonging the hydration time during manufacturing of the creams, the problem of viscosity changes could possibly be overcome.

The creams remained physically relatively homogenous at all conditions during six months.

Confocal laser scanning microscopy showed no change in homogeneity. Particle size remained constant with only a slight, but acceptable, increase in the particle size of the castor oil and vitamin F creams.

Only the Pheroid™ creams failed to retain their original colours. The colour changes were probably due to oxidation reactions. The role of α-tocopherol in the colour changing reactions should be investigated.

Mass loss was minimal and insignificant for all six cream formulations.

Although the pH, homogeneity, particle size and mass loss tests were passed, the instability shown by assay and colour testing should be investigated. Compatibility and the role of α-tocopherol and the antioxidants in the formulations need to be reviewed.

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Appendix E

In vitro diffusion studies

E.1 Introduction

In vitro skin diffusion studies are used extensively to measure penetration of chemicals into skin and their subsequent permeation across skin (Brain *et al.,* 2002:198; ICPS, 2006:38). These studies may utilise either nonviable or fresh, metabolically active skin (ICPS, 2006:38). By using the correct methodology, *in vitro* studies can be used to predict *in vivo* absorption (ICPS, 2006:79).

An advantage offered by *in vitro* methods is the possibility of exerting precise control over experimental conditions. This control can be used to ensure that the skin and the test material are the only variables (Brain *et al.*, 2002:198-199). A limitation associated with *in vitro* studies is that sink conditions of the peripheral vascular system may not be fully mimicked (OECD, 2004:12). When nonviable skin is used, it may be difficult to maintain metabolic activity in the skin, especially for prolonged periods (Williams, 2003:52).

Some synthetic membranes, e.g. silicone membranes, are not rate-limiting and can therefore be used for *in vitro* studies to measure drug-vehicle interactions affecting drug release from formulations (Barry, 2002:518). These studies are used mainly for quality assurance (Surber & Davis, 2002:408). They do not determine skin absorption (Barry, 2002:518) and may not be predictive of drug bioavailability (Walters & Brain, 2002:324).

In this study, the three 0.025 % tretinoin creams and the three 0.1 % adapalene creams (appendix C) were subjected to *in vitro* studies employing silicone membranes (section E.2) and full thickness human skin (section E.3)

E.2 Drug release studies

Synthetic membranes were used to study the release of tretinoin and adapalene from the cream formulations. The amount of retinoid that diffused through the membrane and into the receptor phase was measured over a period of eight hours.

E.2.1 Receptor phase preparation

PBS with a pH of 7.4 (British Pharmacopoeia, 2009:A143) was prepared by dissolving 6.81 g potassium dihydrogen phosphate (KH_2PO_4) into 250 ml water and 1.5736 g sodium hydroxide (NaOH) into 393.4 ml water. The two solutions were combined, stirred and the pH was set to 7.4 with orthophosphoric acid.

A mixture of PBS (pH 7.4), ethanol and THF in the ratio 9:9:2 was used as receptor phase. Fresh receptor phase was prepared before each study, because crystallisation occurred when it was stored at 4 °C.

E.2.2 Method used for drug release studies

Release studies were performed using vertical Franz diffusion cells (figure E.1) with a receptor capacity of approximately 2 ml and a diffusion area of 1.075 cm^2. Six release studies were performed, i.e. one study per cream formulation. Each release study made use of ten Franz cells.

A silicone membrane was placed on the receptor compartment and the donor compartment was subsequently placed on top of the membrane. The whole unit was sealed with Dow Corning vacuum grease and fastened with a horseshoe clamp. Amber Franz cells were used for studies involving tretinoin creams in order to prevent photo-isomerisation.

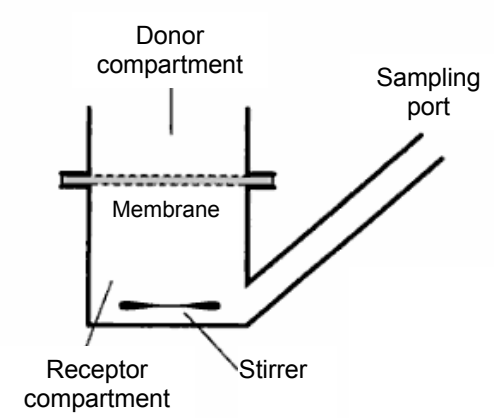

Figure E.1: Illustration of a vertical Franz diffusion cell (Williams, 2003:63)

After calibrating the receptor compartment, it was filled with 2 ml of the receptor phase at 37 °C. Care was taken to prevent entrapment of air bubbles underneath the membrane. The Franz cells were placed in a water bath with a temperature of 37 °C. The receptor phase was stirred continuously using a Variomag® magnetic stirrer plate inside the water bath and a small stirring magnet inside the receptor compartment.

A 1 ml sample of cream was placed in contact with the whole diffusion area of the membrane in the donor compartment. In order to prevent evaporation, the donor compartment was covered with Parafilm®.

The entire receptor volume of each cell was withdrawn after 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 hours. After withdrawal, the receptor compartments were immediately refilled with 2 ml of the receptor phase at 37 °C to maintain sink conditions. Samples obtained from receptor compartments were analysed by means of HPLC for retinoid content (see section E.2.3).

E.2.3 HPLC analysis

HPLC analysis of tretinoin and adapalene was performed as described in appendix A. Some minor adjustments were made in order to reduce the run times. A shorter column (Phenomenex® Luna 5 µm C18 (2), 150 x 4.60 mm) was used and the flow rate was set to 1.500 ml/min. This resulted in a run time of 7 minutes for tretinoin samples and 11 minutes for adapalene samples. Tretinoin and adapalene eluted after 4.0 and 7.8 minutes, respectively. The specificity and reliability of the HPLC methods were not compromised by these changes.

A standard solution was used to draw a standard curve before analysing samples. The standard was reanalysed after every 20 sample runs for control purposes. Injection volumes of 100 µl and 50 µl were used for sample solutions and standard solutions, respectively. Standards were prepared by dissolving 2 mg of the appropriate retinoid in 5 ml THF and adding this solution to 45 ml of a PBS and ethanol mixture with a 1:1 ratio.

E.2.4 Statistical data analysis

For each of the creams, the average (mean) cumulative concentration of tretinoin or adapalene that diffused into the receptor phase over a period of eight hours was determined. This average cumulative concentration was used to calculate the average percentage of retinoid released from the cream matrix.

SAS software (SAS Institute, 2005) was used to calculate the median flux by means of regression coefficients. The median is the preferred method to determine flux (Gerber *et al.,* 2008:190) in the case of asymmetrical (skewed) distributions or when outliers are present, because it is, unlike the mean, not affected by these distributions (Feinstein, 2002:32).

Kruskal-Wallis tests were conducted using Statistica software (Statsoft, 2008). This test is a nonparametric analysis of variance used to compare the medians of three or more independent or unrelated samples. If the result of the test is statistically significant, i.e. the obtained p-value is less than 0.05, at least one of the samples is different from the other samples (Corder & Foreman, 2009:100). One Kruskal-Wallis test was used to compare the median fluxes of the three tretinoin creams and a second test was used for the three adapalene creams.

Multiple comparison tests were used to determine which samples were different from the others. These tests were used when significant differences had been observed with the Kruskal-Wallis test (Shepherd *et al*., 2000).

E.2.5 Results and discussion

E.2.5.1 Tretinoin creams

Figures E.2 to E.4 display the cumulative amount per area of tretinoin released from each cream formulation. The data points plotted at each time interval are indicative of the cumulative amount of tretinoin in the receptor compartment of each of the ten Franz cells.

The distribution of data points in the figures was an indication of the variation in the measurements obtained from different Franz cells. There was a significant difference in the cumulative amounts of vitamin F cream among the ten Franz cells, as could be observed in figure E.4. Castor oil cream (figure E.2) and Pheroid™ cream (figure E.3) showed less variation between different cells.

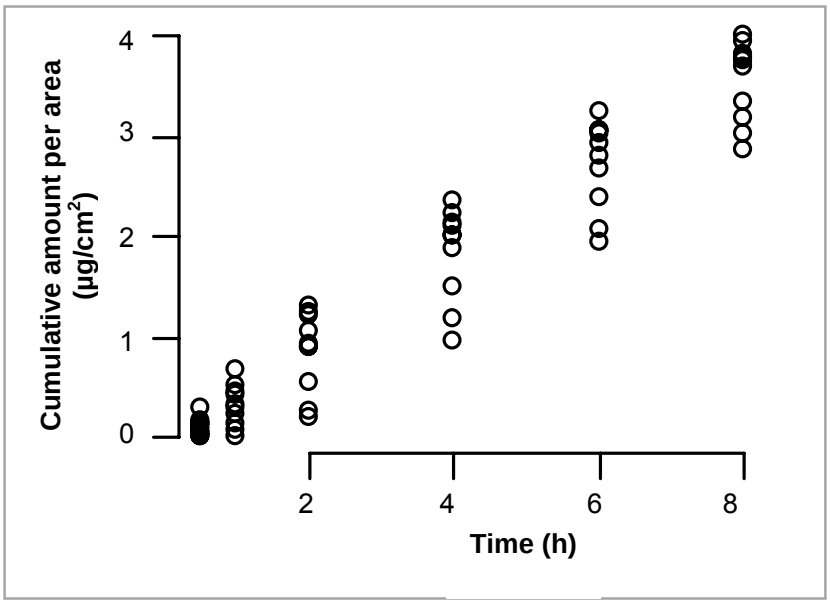

Figure E.2: Cumulative amount per area (µg/cm^2) of tretinoin released from castor oil cream over a period of eight hours

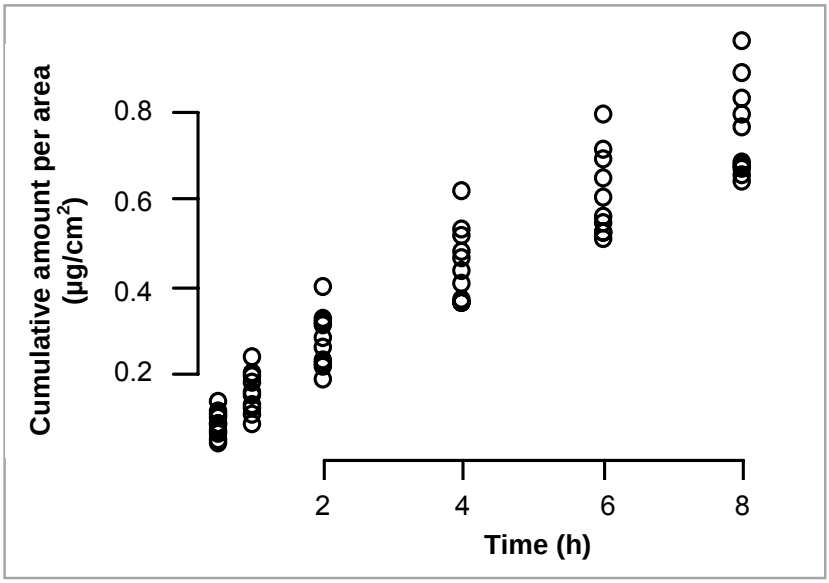

Figure E.3: Cumulative amount per area (µg/cm^2) of tretinoin released from Pheroid™ cream over a period of eight hours

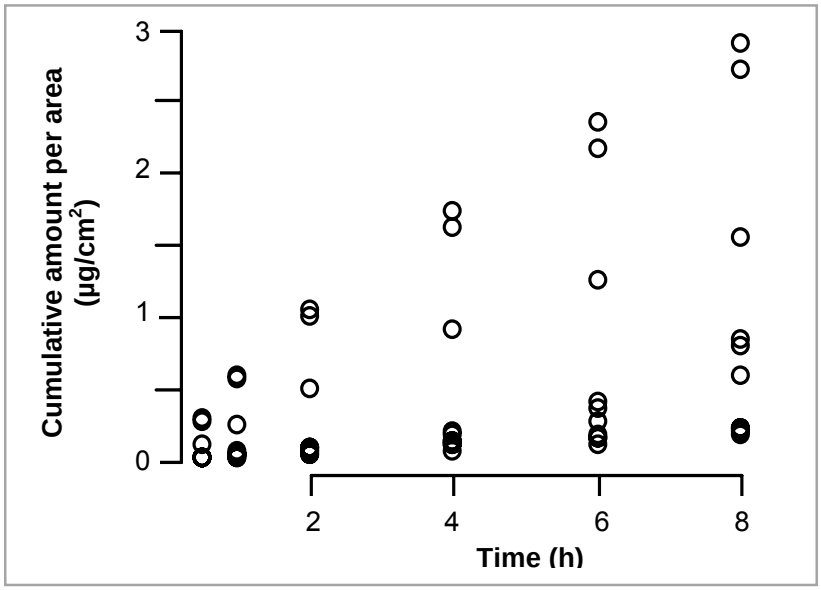

Figure E.4: Cumulative amount per area (µg/cm^2) of tretinoin released from vitamin F cream over a period of eight hours

Flux can be defined as the rate of transfer per unit area of surface (Barry, 2002:506). In order to determine the average flux of tretinoin into the receptor compartments, the average cumulative amount of retinoid that diffused through each cm^2 membrane was plotted against time. The slope of the linear portion of the obtained curve can be interpreted as the average flux. This was observed to extend from two to eight hours. The following three graphs illustrate the average flux obtained as tretinoin was released from castor oil cream (figure E.5), Pheroid™ cream (figure E.6) and vitamin F cream (figure E.7).

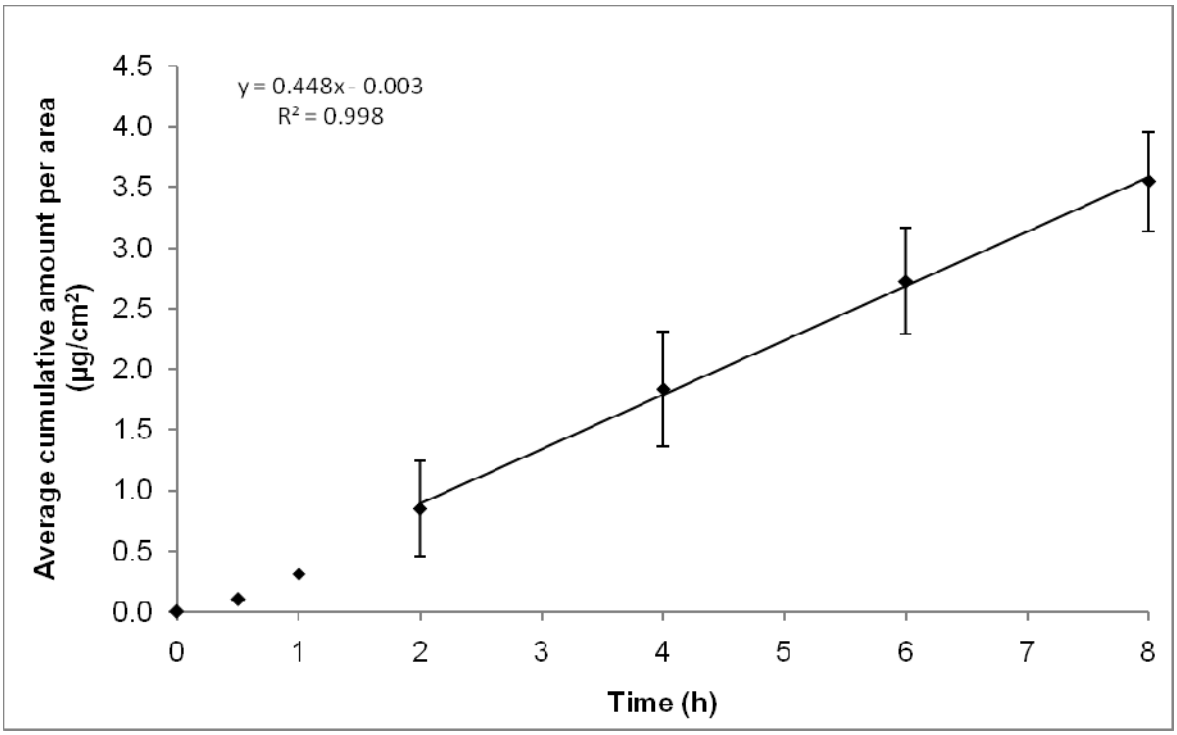

Figure E.5: Average cumulative amount per area (µg/cm^2) of tretinoin released from castor oil cream as a function of time

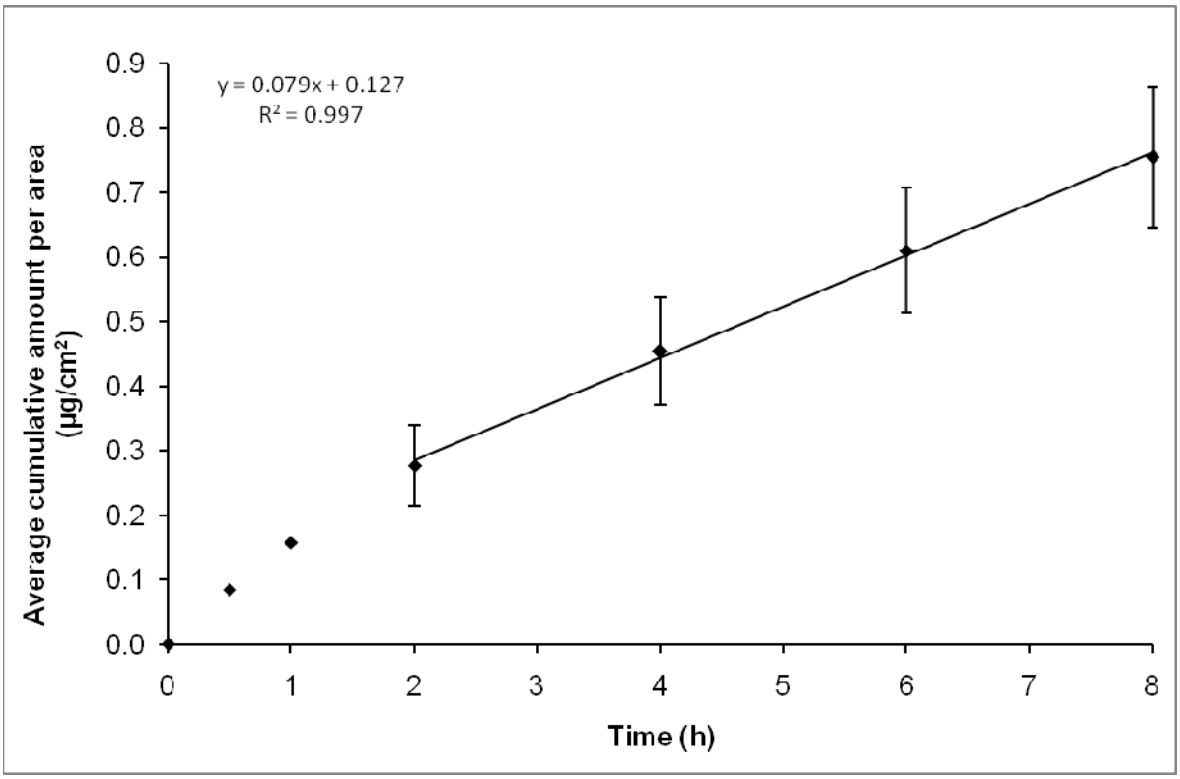

Figure E.6: Average cumulative amount per area (µg/cm^2) of tretinoin released from Pheroid™ cream as a function of time

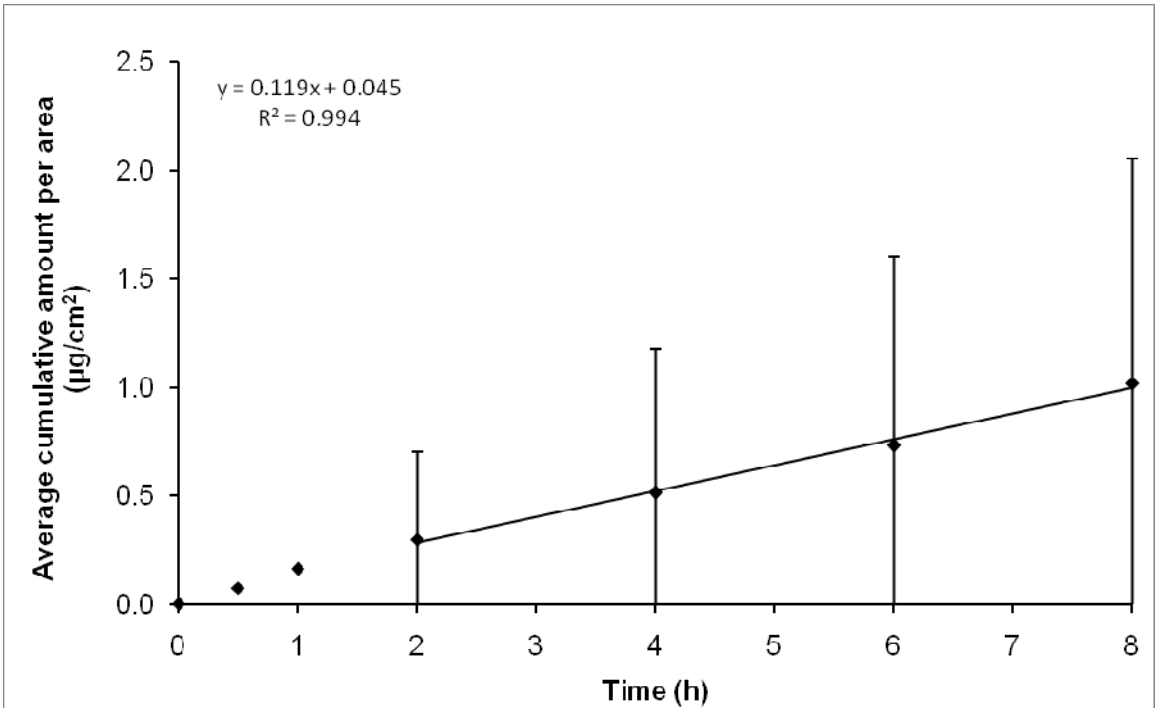

Figure E.7: Average cumulative amount per area (µg/cm^2) of tretinoin released from vitamin F cream as a function of time

The castor oil cream (figure E.5) showed the highest average flux (0.448 µg/cm^2.h), followed by vitamin F cream (figure E.7) with an average flux value of 0.199 µg/cm^2.h. The smallest average flux was obtained by the Pheroid™ cream (figure E.6), namely 0.079 µg/cm^2.h. Castor oil cream, therefore, depicted the best tretinoin release.

Table E.1 summarises the statistical data of the drug release studies. Castor oil cream showed the highest release with 0.763 % tretinoin released after eight hours, when compared to vitamin F cream (0.219 %) and Pheroid™ cream (0.162 %).

Table E.1: Results obtained from studies of tretinoin release over a period of eight hours

Formulation	Average % released	Average flux (µg/cm^2.h)	Average cumulative conc. (µg/ml)	Median flux (µg/cm^2.h)	Median cumulative conc. (µg/ml)
Castor oil cream	0.763	0.448 ± 0.032	1.907	0.465	2.010
Pheroid™ cream	0.162	0.079 ± 0.0096	0.405	0.084	0.338
Vitamin F cream	0.219	0.119 ± 0.108	0.548	0.083	0.367

The three box-plots in figure E.8 were drawn using the respective flux values of tretinoin released from the three cream formulations. Castor oil cream presented a nearly symmetrical distribution. Its average and median flux values were, therefore, approximately the same, as could be observed in table E.1. The Pheroid™ cream showed a smaller and slightly asymmetrical distribution. Its average and median flux values did not differ significantly. Vitamin F cream depicted a wide distribution, as had been observed in figure E.4. Its distribution was skewed to the right, resulting in a significant difference between the average flux (0.119 ± 0.108 µg/cm^2.h) and the median flux (0.083 µg/cm^2.h). In this case the median was a better indicator of flux than the average, because the skewed distribution did not affect the median (Gerber *et al.,* 2008:190).

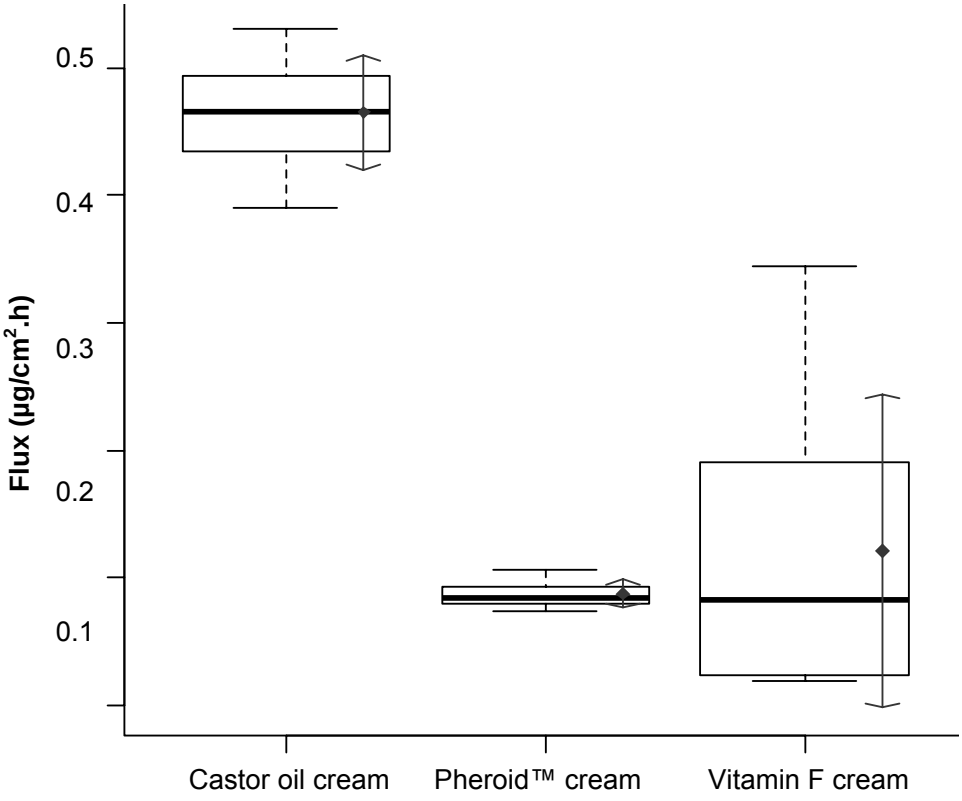

Figure E.8: Box-plots of the flux values (µg/cm^2.h) for tretinoin in the different cream formulations. The median flux is illustrated by the thick black lines and the average flux by the diamond shapes in the boxes

A p-value of 0.001 was calculated by means of a Kruskal-Wallis test. This p-value indicated that there was at least one significant difference between the median fluxes of the three tretinoin creams. In order to determine which creams were different from the others, multiple comparison tests were conducted. P-values of 0.0006 and 0.0003 were obtained when castor oil was compared to Pheroid™ cream and vitamin F cream respectively, indicating that the median flux of castor oil cream was significantly different from the other two creams. When Pheroid™ cream

and vitamin F cream were compared, a p-value of 1.0000 resulted. The median fluxes of Pheroid™ cream and vitamin F cream were, therefore, not significantly different. The difference and similarity between the median fluxes could be observed in figure E.8.

Aside from tretinoin, some tretinoin metabolites were also present in the receptor compartments. The receptor phase contained 0.018 % metabolites from the vitamin F cream. In the case of castor oil cream, an average metabolite percentage of 0.014 % was detected, whereas no metabolites resulted from Pheroid™ creams. It could be possible that the Pheroid™ cream protected tretinoin from degradation.

E.2.5.2 Adapalene creams

Figures E.9 to E.11 illustrate the cumulative amounts of adapalene released into ten receptor compartments over a period of eight hours. The distribution width of the data points of the three creams was approximately the same, thereby implying that there was no significant variation in measurement between the creams.

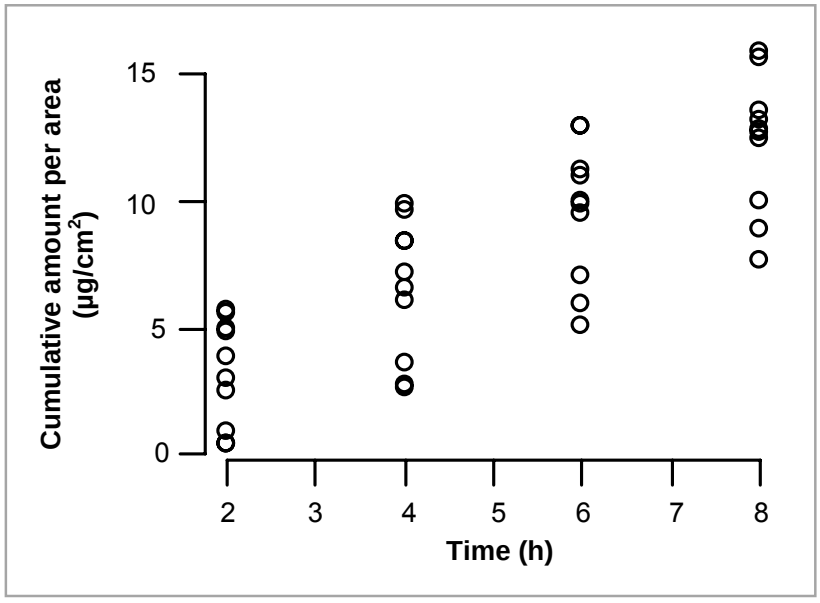

Figure E.9: Cumulative amount per area (µg/cm^2) of adapalene released from castor oil cream during eight hours

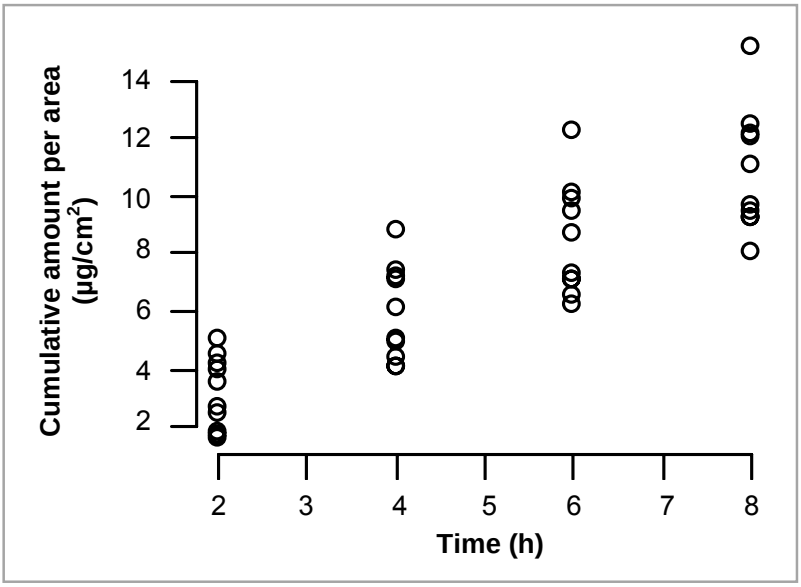

Figure E.10: Cumulative amount per area (µg/cm^2) of adapalene released from Pheroid™ cream during eight hours

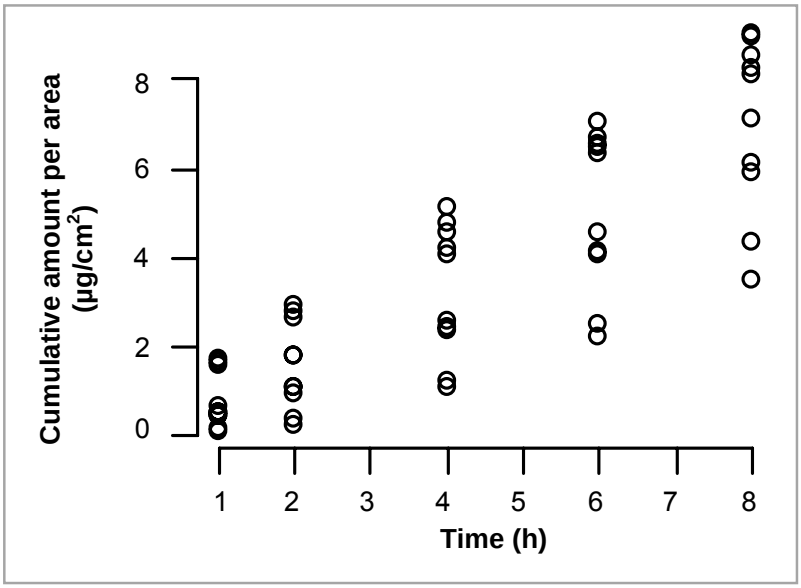

Figure E.11: Cumulative amount per area (µg/cm^2) of adapalene released from vitamin F cream during eight hours

Average flux values were determined by calculating the slope of the linear portions of figures E.12 to E.14. Adapalene in the castor oil cream (figure E.12) presented the highest average flux (1.5106 µg/cm^2.h). The average flux value for the Pheroid™ cream was 1.289 µg/cm^2.h (figure E.13), whereas the flux for the vitamin F cream (figure E.14) was the smallest (0.902 µg/cm^2.h).

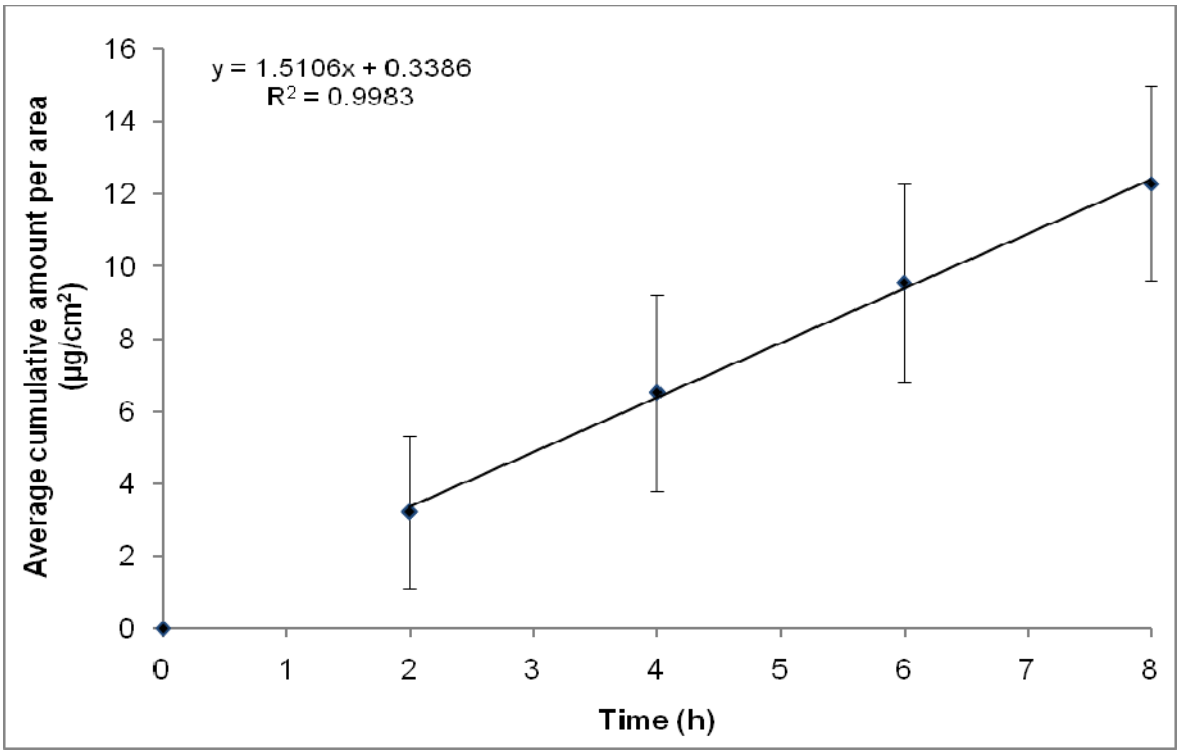

Figure E.12: Average cumulative amount per area (µg/cm^2) of adapalene released from castor oil cream as a function of time

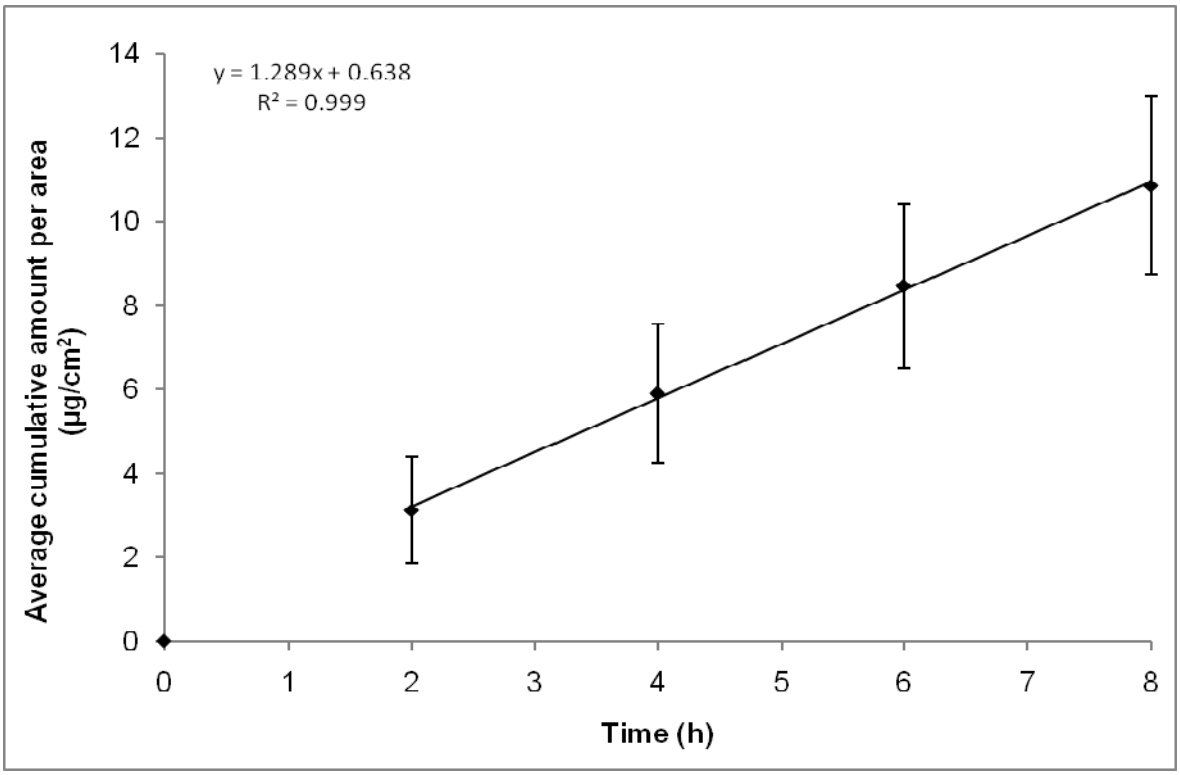

Figure E.13: Average cumulative amount per area (µg/cm^2) of adapalene released from Pheroid™ cream as a function of time

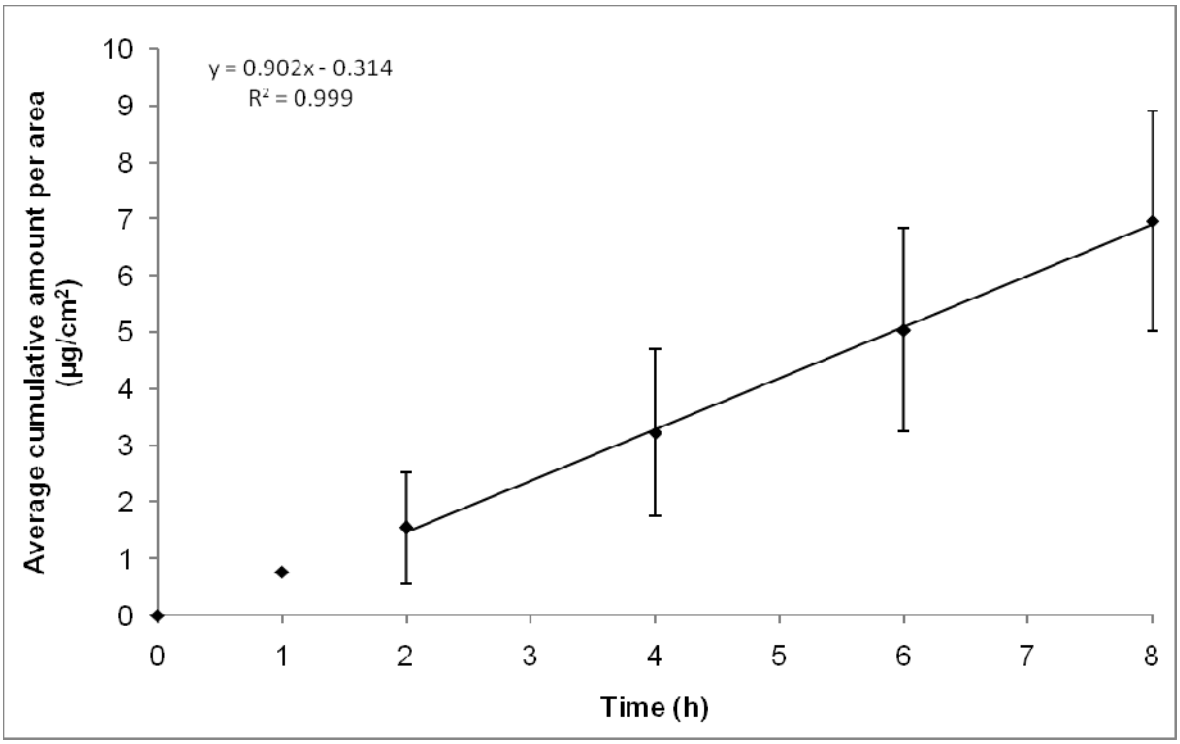

Figure E.14: Average cumulative amount per area (µg/cm^2) of adapalene released from vitamin F cream as a function of time

Data obtained from the release studies are summarised in table E.2. Castor oil cream released the most adapalene (0.660 %), followed by Pheroid™ cream (0.584 %) and vitamin F cream (0.374 %).

Table E.2: Results obtained from studies of adapalene release over a period of eight hours

Formulation	Average % released	Average flux (µg/cm^2.h)	Average cumulative conc. (µg/ml)	Median flux (µg/cm^2.h)	Median cumulative conc. (µg/ml)
Castor oil cream	0.660	1.51 ± 0.15	6.605	1.51	6.852
Pheroid™ cream	0.584	1.29 ± 0.18	5.842	1.27	5.579
Vitamin F cream	0.374	0.90 ± 0.20	3.746	0.92	4.080

Flux values were used to draw the box plots in figure E.15. The distribution of data points for castor oil cream was slightly asymmetrical, and the median flux was the same as the average flux. Pheroid™ cream had a distribution that was slightly skewed to the left. The average flux was higher than the median flux due to the presence of an outlier. The data distribution of the vitamin F cream was approximately symmetrical. The low minimum values rendered the average flux slightly lower than the median flux.

A p-value of 0.000 was calculated by means of a Kruskal-Wallis test, which indicated that there was at least one significant difference between the median fluxes of the three adapalene creams. Multiple comparison tests were conducted in order to determine which cream was different from the others. P-values of 0.000018 and 0.019715 were obtained when vitamin F cream was compared to castor oil cream and Pheroid™ cream, respectively. This indicated that the median flux of vitamin F cream was significantly different from the fluxes of the other two creams. When castor oil cream and Pheroid™ cream were compared, a p-value of 0.213975 resulted. The median fluxes of these two creams were, therefore, not significantly different.

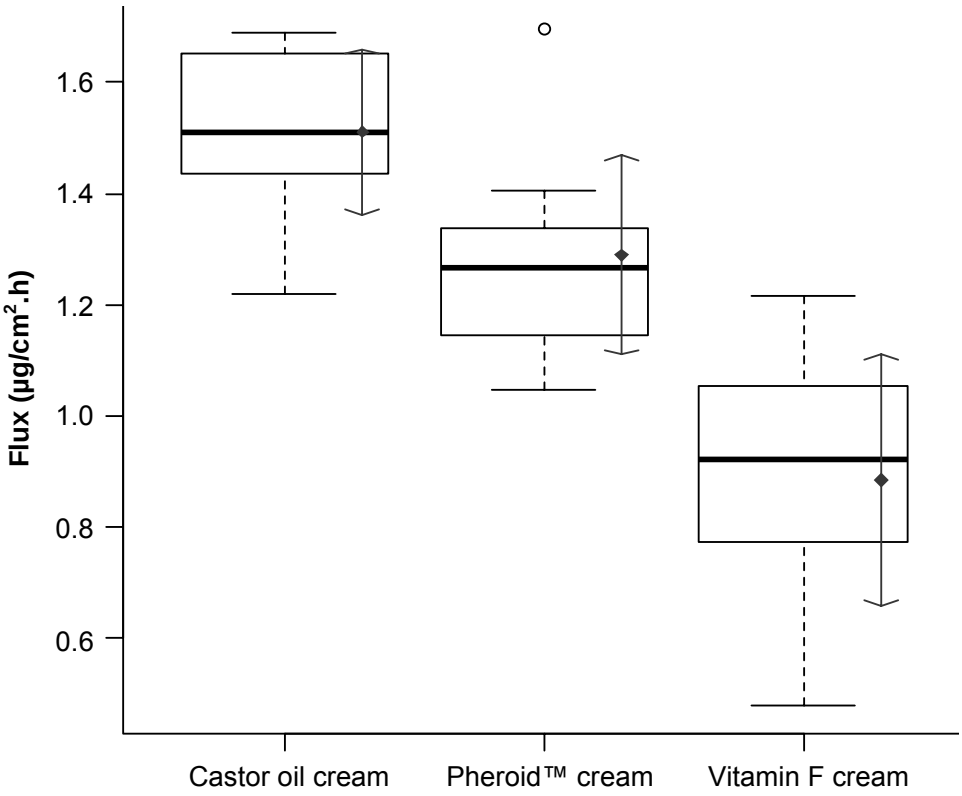

Figure E.15: Box-plots of the flux values (µg/cm^2.h) for adapalene in the different cream formulations. The median flux is illustrated by the thick black lines and the average flux by the diamond shapes in the boxes

E.3 Transdermal diffusion studies

The passive diffusion of tretinoin and adapalene into and across human skin was determined using *in vitro* diffusion cells with full thickness skin as rate-limiting membrane.

E.3.1 Skin preparation

Abdominal skin was obtained from Caucasian female patients after cosmetic abdominoplastic surgery (Leveque *et al.*, 2004:324) and frozen at -20 °C. Fresh skin can be stored at -20 °C for up to 466 days with no change in permeability (OECD, 2004:17). The Research Ethics Committee of the North-West University provided ethical approval to acquire and utilise the skin (reference number 04D08). Informed consent was obtained from all donors beforehand.

Full thickness skin (i.e. the stratum corneum, viable epidermis and dermis) was used for the diffusion studies. For this purpose, adipose and connective tissue layers were removed with a scalpel. This was done carefully in order to keep the skin layers intact. The skin was punched into circles (approximately 15 mm in diameter), placed between Whatman® filter papers, wrapped with aluminium foil and frozen at -20 °C.

Before diffusion studies were conducted, the skin circles were thawed at room temperature and examined for any defects.

E.3.2 Receptor phase preparation

The receptor fluid must have adequate capacity to solubilise the test substance. Due to the insolubility of tretinoin and adapalene in phosphate buffered solution (PBS), a mixture of equal parts of PBS and ethanol was used as receptor phase (Manconi *et al*., 2002:246; OECD, 2004:13, 15).

Crystallisation of the receptor phase occurred when stored at 4 °C. Therefore, fresh receptor phase was prepared daily before commencement of diffusion studies. No crystallisation, however, occurred when PBS alone was stored at 4 °C.

E.3.3 Franz cell diffusion

Vertical Franz diffusion cells were assembled as described in section E.2.2, with full thickness skin used instead of silicone membranes. Each skin circle was placed between the two compartments with the stratum corneum facing towards the donor compartment. The water bath maintained a temperature of 37 °C, thereby ensuring a skin surface temperature of 32 °C (Williams, 2003:62). A sample of 1 ml cream was placed in each donor compartment and made contact with the whole diffusion area of the skin.

The entire receptor volume was withdrawn after 20, 40, 60, 80, 100 and 120 minutes and after 4, 6, 8, 10 and 12 hours. After withdrawal, the receptor compartment was immediately refilled

with 2 ml receptor phase at 37 °C to maintain sink conditions. Samples obtained from receptor compartments were analysed by means of HPLC for retinoid content (see section E.3.5).

Six diffusion studies were performed, i.e. one study per cream formulation. Ten cells were used for each study. In order to prevent photo-isomerisation, amber Franz cells were used for studies involving tretinoin creams.

E.3.4 Tape stripping

After the 12 hour samples had been withdrawn from the receptor compartments, the Franz cells were removed from the water bath and disassembled. Parafilm® was stapled to hardboard and the skin circles pinned onto the Parafilm® with the stratum corneum facing upwards. The diffusion area of each skin circle, clearly marked by an indent made by the two cell compartments, was carefully patted clean with tissue paper.

3M Scotch® Magic™ tape was cut into pieces large enough to cover only the diffusion area. Tape stripping was done according to the method described by Pellet *et al*. (1997:94). Sixteen strips of tape were used for each skin circle. Due to the probability of a cream residue on the surface of the skin, the first tape strip was discarded. The fifteen remaining tape strips were used to separate the epidermis from the dermis. After discarding the non-diffusion area of the skin, the diffusion area was cut into small pieces. In order to extract the retinoids, the skin cuttings and tape strips were placed in separate vials and soaked in a mixture of ethanol and THF with a ratio of 9:1. These vials were stored at 4 °C for approximately eight hours. The skin and tape samples were centrifuged and the supernatants were analysed by means of HPLC (section E.3.5).

E.3.5 HPLC analysis

Samples were analysed using the HPLC methods described in section E.2.3. A standard solution was used to draw a standard curve before analysing samples. The standard was reanalysed after every 20 sample runs for control purposes. Injection volumes of 100 µl and 50 µl were used for sample solutions and standard solutions, respectively.

For analysis of the sample solutions withdrawn from receptor compartments, standards were prepared by dissolving 2 mg of the appropriate retinoid in 5 ml THF and adding this solution to 45 ml of the receptor phase.

Other standards were used to compare samples obtained from skin cuttings and tape strips. These samples were prepared by dissolving 2 mg of the appropriate retinoid in 5 ml THF, which was then added to 45 ml ethanol.

E.3.6 Results and discussion

It was desired that the retinoids remain in the skin and not reach the systemic circulation, as the target for retinoid therapy is located in the dermis. No retinoids were detected in any receptor compartments, as was anticipated. This could be attributed to the high lipophilicity of tretinoin and adapalene, as indicated by their high log P values of 6.30 and 8.04, respectively (Syracuse Research Corporation, 2010; Valiveti *et al*., 2008:14).

The epidermal samples (tape strips) contained no retinoids. The rate and extend of transdermal diffusion of very lipophilic drugs can be limited by the hydrophilicity of the viable epidermis and dermis (ICPS, 2006:23). It was, therefore, expected that the epidermis, specifically the stratum corneum, would retain the largest amount of retinoids, because of the lipophilic nature of the stratum corneum (ICPS, 2006:23). The absence of retinoids in the epidermis might have been due to the significantly low drug release of the creams which led to low concentrations of retinoids being available for diffusion.

Very small amounts of adapalene were present in the dermis (skin cuttings), as shown in table E.3. The castor oil cream and Pheroid™ cream had the same average adapalene concentration in the dermis (1.085 µg/ml) and were superior to the vitamin F formulation (0.531 µg/ml). Tretinoin reached the dermis from castor oil cream only. Therefore, retinoids from these four creams reached the target site. The amount of the applied active ingredient that reached the dermis was significantly low and this was probably due to the low release of retinoids by the creams, as had been observed in Section E.2.

Table E.3: Average concentration of tretinoin and adapalene that penetrated the dermis, together with the percentage of the applied dose that reached the dermis

Formulation	Average concentration (µg/ml)	Percentage of applied dose (%)
Tretinoin creams		
Castor oil cream	0.076	0.0076
Pheroid™ cream	0.000	0.000
Vitamin F cream	0.000	0.000
Adapalene creams		
Castor oil cream	1.085	0.109
Pheroid™ cream	1.085	0.109
Vitamin F cream	0.531	0.053

According to the pH-partition hypothesis, only unionised molecules readily traverse lipid membranes (Barry, 2002:511). The degree of ionisation (see table E.4) of tretinoin and adapalene in the various cream formulations was calculated using the pK_a of tretinoin and

adapalene (6.00 and 4.23, respectively) and the pH of the creams (Brisaert *et al*., 2001:913; Trichard *et al.*, 2008:435).

Table E.4: Percentage of unionised retinoid molecules present in the different creams

Formulation	pH of cream	Percentage unionised retinoid (%)
Tretinoin creams		
Castor oil cream	5.298	83.4
Pheroid™ cream	4.881	92.9
Vitamin F cream	5.212	86.0
Adapalene creams		
Castor oil cream	5.296	7.91
Pheroid™ cream	5.345	7.13
Vitamin F cream	5.245	8.81

Although the small fraction of unionised adapalene could explain this retinoid's significantly small passage into the skin, it does not explain the better penetration of adapalene relative to tretinoin. The amount of tretinoin that reached the dermis was significantly smaller than that of adapalene, although tretinoin was more unionised than adapalene in the same formulation. It must be kept in mind that the applied concentration of adapalene (0.1 %) was four times higher than that of tretinoin (0.025 %). This higher concentration could have contributed to the significantly better penetration of adapalene.

Another factor influencing diffusion is the concentration gradient. The flux of a solute is proportional to the concentration gradient across the entire barrier phase (Barry, 2002:512). Due to the small amounts of tretinoin and adapalene applied to the skin (250 µg and 1000 µg, respectively), their concentration gradients across the skin were possibly suboptimal.

As was observed in Section E.2, the composition of the cream matrices hindered adequate release of the retinoids, which might have led to insufficient concentrations in the skin. As stated earlier, membrane studies do not determine skin absorption (Barry, 2002:518). Nonetheless, the small amounts of tretinoin and adapalene released by the creams influenced the concentration of retinoids available for diffusion and, therefore, lowered the concentration gradient.

Compared to results of the release studies of the tretinoin creams (Table E.3), it was observed that the castor oil cream showed a significantly higher release as well as higher skin penetration than the other two creams.

There was a correlation between the percentage adapalene released (Table E.4) and the percentage that penetrated the dermis. The castor oil and Pheroid™ creams were the two adapalene creams with the highest percentage release, as well as the highest skin penetration.

The obtained distribution profiles of tretinoin and adapalene in the dermis and epidermis necessitate further investigation.

E.4 Conclusion

Castor oil cream released a significantly larger amount of tretinoin than the other creams. Tretinoin metabolites were detected in receptor phases of both castor oil cream (0.018 %) and vitamin F cream (0.014 %). Pheroid™ cream, on the other hand, might protect tretinoin from photodegradation.

The highest concentration adapalene was released from castor oil cream, followed by Pheroid™ cream, although the difference between the two creams was statistically insignificant. Vitamin F released the lowest concentration adapalene. No degradation of adapalene was observed.

Tretinoin from only castor oil cream reached the target site, namely the dermis. Castor oil and Pheroid™ creams were superior to vitamin F cream in terms of diffusion of adapalene into the dermis. The skin distribution profiles of the retinoids were in contrast with expected results, although no retinoid reached the receptor compartments. No retinoid was located in the epidermis, whereas minor amounts were found in the dermis. Reasons for these results might include excessive log P values, insufficient concentration gradients, the percentage of unionised molecules, and the suboptimal release from cream matrices. The latter has a definite influence, as no cream released more than 1 % of its retinoid.

It can be concluded that castor oil cream was the superior formulation in terms of release and skin diffusion of tretinoin. The castor oil and Pheroid™ creams were superior in release and skin penetration of adapalene.

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Appendix F

Guide for authors: International Journal of Pharmaceutics

F.1 Scope of the journal

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

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

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.

F.2 Editorial Policy

The over-riding criteria for publication are originality, high scientific quality and interest to a multidisciplinary audience. Papers not sufficiently substantiated by experimental detail will not be published. Any technical queries will be referred back to the author, although the Editors reserve the right to make alterations in the text without altering the technical content. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur with the submission and that a copy of the final manuscript has been approved by all authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted, the manuscript shall not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher.

Authors must state in a covering letter when submitting papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of

another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

F.3 Submission of Manuscripts

Authors are strongly encouraged to submit their manuscript electronically by using the Elsevier submission site at http://www.elsevier.com/journals.

After registration, authors will be asked to upload their manuscript and associated artwork. Full instructions on how to use the online submission tool are available at the web address listed above.

If an author cannot submit their manuscript electronically, then for the initial submission of manuscripts for consideration, hardcopies are sufficient. The original plus two copies, complete with two sets of figures (including originals or duplicates of sufficient quality for clarity of reproduction) and tables, must be submitted in English. All data that would help referees to evaluate the paper should also be supplied. Manuscripts should be typewritten with double spacing and adequate margins on one side of the sheet only (not more than 26 lines per page). All pages should be numbered sequentially. Manuscripts should be sent to one of the following Editors-in-Chief according to the geographical origin of the author. Please include full contact information - corresponding author name, e-mail address, telephone and fax numbers, and full postal address.

After final acceptance for publication, your revised manuscript on disk together with two printed hard copies, should be submitted to the accepting editor. It is important that the file on disk and the printout are identical. Both will then be forwarded by the editor to Elsevier. In-depth guidelines for submitting artwork/illustrations can be found at: http://www.elsevier.com/artworkinstructions.

When the paper is to be published as a Rapid Communication, this should be clearly indicated to the Editor-in-Chief.

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F.4 Manuscript Types

F.4.1 Full Length Manuscripts

The arrangement of full length papers should accord with the following:

F.4.1.1 Title

The full title should not exceed 85 characters including spaces between words.

F.4.1.2 List of Authors

Initial(s) (one given name may be used) followed by the surname of author(s) together with their affiliations. When the work has been carried out at more than one address, the affiliation of each author should be clearly indicated using superscript, lower-case letters. The author to whom correspondence should be directed must be indicated with an asterisk.

F.4.1.3 Affiliation(s)

Name(s) and address(es) of the establishment(s) where the work was done, designated by superscript, lower-case letters where appropriate.

F.4.1.4 Abstract

An Abstract not exceeding 200 words (a single paragraph) should be provided typed on a separate sheet.

F.4.1.5 Keywords

A maximum of 6 keywords or short phrases suitable for indexing should be supplied. If possible keywords should be selected from Index Medicus or Excerpta Medica Index. Authors may also wish to refer to the Subject Index published in *International Journal of Pharmaceutics*, for example, Vol. 287/1-2, pp. 205-219.

F.4.1.6 Corresponding Author

The author to whom correspondence should be directed should be designated with an asterisk (do not include the address unless different from that indicated by the author's affiliation). Telephone, fax and e-mail address of the corresponding author must be provided.

F.4.1.7 Text

The text should be divided into main sections, such as the following: 1. Introduction. 2. Materials and methods. 3. Results. 4. Discussion. Acknowledgements. References, figure legends, tables and figures. These sections must be numbered consecutively as indicated. Subdivisions of a section should also be numbered within that section, for example, 2.1. Materials, 2.2. Relative humidity measurement, 2.3. Sample preparation, etc.

F.4.1.8 Nomenclature

Standard nomenclature should be used throughout; unfamiliar or new terms and arbitrary abbreviations should be defined when first used. Unnecessary or ambiguous abbreviations and symbols are to be avoided. Data should be expressed in SI units.

F.4.1.9 Figure Legends, Table Legends, Footnotes

Figure legends, tables and footnotes should be typed on separate sheets, lines double spaced. Footnotes, to be numbered consecutively in superscript throughout the text, should be used as little as possible.

F.4.1.10 References

See below for full details.

F.4.2 Rapid Communications

(a) These articles should not exceed 1500 words or equivalent space.

(b) Figures should not be included otherwise delay in publication will be incurred.

(c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

F.4.3 Notes

Should be prepared as described for full length manuscripts, except for the following:

(a) The maximum length should be 1500 words, including figures and tables.

(b) Do not subdivide the text into sections. An Abstract and reference list should be included.

F.4.4 Reviews and Mini-Reviews

Suggestions for review articles will be considered by the Editors-in-Chief. "Mini-reviews" of a topic are especially welcome.

F.5 References

F.5.1 Text citation

The Harvard system of citation must be used. References should be cited in the text within parentheses: where several citations are given within a single set of parentheses, they should be arranged in ascending order of year of publication; where more than one reference with the same year of publication is cited, they should be arranged in alphabetical order of the first authors' names. When referring to a work of more than two authors, the name of the first author should be given, followed by et al.

Examples of text citations:

(Gesztes et al., 1988; Chestnut et al., 1989; Legros et al., 1990; Mhando and Li Wan Po, 1990; Korsten et al., 1991; Langerman et al., 1991, 1992a,b; Masters et al., 1991; Bonhomme et al., 1992; Kolli et al., 1992).

(Shaw et al., 1978; Nakano and Arita 1990b; Nakano et al., 1990a,b; Bone et al., 1992)

F.5.2 Reference list

All references cited in the text should be listed at the end of the paper (typed with double spacing) and assembled alphabetically. More than one paper from the same author(s) in the same year must be identified by the letters a b c, etc. placed after the year of publication.

References must consist of names and initials of all authors, year, title of paper, abbreviated title of periodical, and volume and first and last page numbers. 'Personal communication' and 'unpublished data' should be cited in the text only. Papers referred to as 'submitted for publication' must include the name of the journal to which submission has been made. Journal titles should be abbreviated according to the '*List of Serial Title Word Abbreviations*' (available from International Serials Data System, 20, rue Bachaumont, 75002 Paris, France. ISBN 2-904939-02-8).

F.5.2.1 Example of arrangement in the reference list:

Crowe, J.H., Crowe, L.M., Chapman, D., 1984a. Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. Arch Biochem. Biophys., 232, 400-407.

Crowe, J.H., Crowe, L.M., Hoekstra, F.A., 1989. Phase transitions and permeability changes in dry membranes during rehydration. J. Bioenerg. Biomembr., 21, 77-92.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Aurell Wistrom, C., 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem. J., 242, 1-10.

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

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

Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A., Womersley, C., 1984b. Effects of carbohydrates on membrane stability at low water activities. Biochim. Biophys. Acta, 769, 141-150.

F.5.2.2 Examples of presentation for various types of publication:

Langerman, L., Chaimsky, G., Golomb, E., Tverskoy, M., Kook, A.I., Benita, S., 1990. A rabbit model for evaluation of spinal anesthesia: chronic cannulation of the subarachnoid space. Anesth. Analg., 71, 529-535.

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

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

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

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

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

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

F.5.3 Use of Digital Object Identifier (DOI)

The digital object identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document particularly "Articles in press" because they have not yet received their full bibliographic information.

The correct format for citing a DOI is shown as follows: doi:10.1016/j.ijpharm.2005.01.041

F.6 Articles in Special Issues:

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F.7 Figures and Tables

F.7.1 Figures

Line drawings (including graphs) should be drawn in black ink on white paper or on tracing paper with blue or faint grey rulings; graduation will not be reproduced. Lettering should be large enough to permit photographic reduction. If figures are not to be reduced, their format should not exceed 16 x 20 cm. Photographs (or half-tone illustrations) must be of good quality, submitted as black and white prints on glossy paper, and have as much contrast as possible. The magnification of micrographs should be indicated by a scale bar in the figure. Figures should be clearly marked on the reverse side with the number, orientation (top) and author's name; a soft pencil or a felt-tipped pen should be used for marking photographs. The illustrations should be numbered with Arabic numerals. The legends should be typed separately with double spacing.

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