Vir Pa Louis & Ma Jeanette

Chan eil tuil air nach tig traoghadh…

FOREWORD

The aim of this study included the formulation of sodium ascorbyl phosphate, a more stable salt of vitamin C, in cosmeceutical products. The product was formulated in various concentrations, in these cosmeceutical creams and in some formulations use was made of PheroidÊ technology.

This dissertation is presented in the so-called article format. This includes introductory chapters, as well as a full length article for publication in a pharmaceutical journal. The attached appendixes contain the data obtained from the study. The article is to be submitted to Skin Pharmacology and Physiology of which the complete guide for authors is included in Appendix D.

During the two years of working on this project, Iôve grown in leaps and bounds, not only intellectually but also spiritually and emotionally. Iôve learned that not all roads down the research path are paved and wide, but that a lot of hard work, dedication, self-discipline and endurance are needed. My ever-burning passion for knowledge and understanding more of my field of study has intensified along with the immense effort needed to complete this dissertation and I look forward to an exciting career ahead in research and the ever-broadening of my horizons.

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ABSTRACT

People and especially women are forever searching for new and improved ways to alter the appearance of their skin. Skin can be prematurely aged by various environmental factors, including prolonged exposure to ultraviolet (UV) light (Brannon, 2007). The aging of skin is amongst other factors facilitated by the degradation of collagen in the connective tissue (Uitto, 1993:299-314) (as quoted by Fisher *et al.*, 1997:1420).

Vitamin C and its derivatives are known to have anti-oxidant, as well as collagen forming properties (Gibbon *et al.,* 2005:82). Vitamin C is a water-soluble compound and highly unstable. By using vitamin C-salts or esters such as sodium ascorbyl phosphate the absorption of the API (active pharmaceutical ingredient) can be improved, when formulated in topical preparations. This is because of the esters being more stable and lipophilic than the original vitamin (Austria *et al.,* 1997:795).

Transdermal drug delivery has many advantages including bypassing hepatic metabolism and a reduction in side effects (Kydonieus *et al.,* 2000:3). The main problem encountered with transdermal drug delivery is the barrier function of the skin, which assists the body in keeping foreign bodies, infections and UVR (ultraviolet radiation) out and it helps keeping water and other vital substances in the body (Rushmer et *al.,* 1966:343). According to Shindo *et al.* (1994:123) there are significant amounts of natural vitamin C found in both the epidermis, as well as the dermis. The concentrations of vitamin C in the epidermis is however higher than in the dermis.

Penetration of an API may be increased by making use of physical or chemical penetration enhancers. A relatively new carrier medium, used in this study, is PheroidÊ technology. The principle of action for this method of enhancement rests on the use of vesicular structures with no phospholipids or cholesterol (Grobler *et al.,* 2008:283).

Ten different creams were formulated during this study. These formulations included various concentrations of the API, varying polarities; and either PheroidÊ or non-PheroidÊ formulas. Concentrations ranged between 1 and 3% for the formulations. A 1%, 2% and 3% cream was formulated in both PheroidÊ and non-PheroidÊ batches. Furthermore, a 2% cream with more liquid paraffin in the formula, as well as a 2% cream with less liquid paraffin in the formula were formulated (also in both PheroidÊ and non-PheroidÊ batches) in order to determine the effect

of varying polarities of the formulations on the release and penetration of the API. Two placebo formulations were also prepared in order to determine the concentration of natural vitamin C found in the skin, which should be compensated for during diffusion studies.

Furthermore, the aqueous solubility of the active ingredient was determined to be 6.14 mg/ml and the octanol-water partition coefficient (log P) of the drug was found to be -0.005. This indicated that the drug would struggle to penetrate the skin, because of the fact that it is not soluble in both oil and water, but penetration could be improved by the fact that the drug is so highly water-soluble (Naik *et al.,* 2000:321).

Diffusion studies (where polytetrafluoroethylene membranes were used) were done in order to determine if the API was released from the formulation. The membrane release studies were performed over a 6 h period and it was observed that the 2% non-Pheroid$^{\hat{E}}$ cream, with less liquid paraffin in the formula, was the formulation with the highest average percentage released (2.008%) after the 6 h. Secondly was the 1% PheroidÊ formula. It had release of 1.940% after 6 h. It was thought that the higher polarity in the 2% formulation would prevent the highly water soluble API from releasing from the formulation. The polarity of the cream was higher due to the increased amount of water in the formulation (Mitsui, 1997:343). Because of the high percentage of unionised species (99.37%) of the API, a certain degree of release was however expected (Barry, 2002:511).

The vertical Franz cell diffusion studies, performed over 12 h, proved the 2% non-Pheroid formulation to be the cream with the highest average concentration (3.761 µg/cm^2) diffused. The 1% non-PheroidÊ formulation (3.555 µg/cm^2) was the formulation closest to the 2% non-PheroidÊ formulationôs value. The high diffusion rates of the formulations can be attributed to the 99.37% unionised species of the API. According to Barry (2002:511) the unionised species of an API is usually lipid soluble and can pass readily across the stratum corneum. Furthermore, it seemed that the formulations which contained a lower concentration of the API performed greater than the formulations with higher concentrations of the active. This could be because of increased stability of the formulations, with lower concentrations of the API.

The formulation with the highest average concentration of vitamin C in the stratum corneum-epidermis (0.457 µg/ml) was the 2% PheroidÊ formulation with less liquid paraffin in the formula. This formulation showed a higher polarity because of the higher amount of water in the formulation (Mitsui, 1997:343). According to Bickers (2010:22) this could have led to the

largely unionised APIôs penetration into the stratum corneum-epidermis, as this oil soluble species of the API have an affinity for the lipid rich membrane.

The formulation with the highest average concentration of vitamin C in the epidermis-dermis was the 1% PheroidÊ formulation, with an average value of 0.656 µg/ml followed by the 2% PheroidÊ formula with a concentration of 0.530 µg/ml. This could be because of the API being entrapped in the PheroidÊ and thus having an improved lipid solubility (Grobler *et al.,* 2008:297).

According to the experimental data the 1% PheroidÊ cream was the formulation which performed the best overall during the experiments. It was the formulation with the second highest average percentage (1.940%) API released, after a period of 6 h, after the membrane release studies and the 4th highest concentration (3.057 µg/cm^2) of API, after skin diffusion over 12 h. This formulation was also found to be the cream which penetrated the epidermis-dermis (target site) the best to yield an average API concentration of 0.656 µg/ml, which could be ascribed to the PheroidÊ in the formulation. PheroidÊ encapsulated the API molecules and helped increase the penetration of the drug through the stratum corneum and into the dermis (Grobler *et al.* 2008:297).

Keywords: Sodium ascorbyl phosphate, photo-ageing, transdermal diffusion, Pheroid^{Ê}, formulation

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UITTREKSEL

Mense en veral dames is ewig opsoek na nuwe en verbeterde maniere om die voorkoms van hul vel te verander. Vel kan deur verskeie omgewingsfaktore voortydig verouder word. Een van hierdie faktore is langdurige blootstelling aan ultraviolet (UV) lig (Brannon, 2007). Die veroudering van vel word onder andere deur die afbraak van kollageen in die bindweefsel, gefasiliteer (Uitto, 1993:299-314) (aangehaal deur Fisher *et al.*, 1997:1420).

Vitamien C en sy derivate is bekend vir hul anti-oksidant, sowel as kollageenvormende eienskappe (Gibbon *et al.,* 2005:82). Vitamien C is ón wateroplosbare verbinding en is hoogs onstabiel. Die absorpsie van die geneesmiddel kan egter verbeter word deur gebruik te maak van vitamien C-soute of esters soos natriumaskorbielfosfaat, wanneer dit in topikale preparate geformuleer word. Dit kan toegeskryf word aan die verbeterde stabiliteit van die esters, sowel as hul verhoogde lipofilisiteit, vergeleke met die oorspronklike vitamien (Austria *et al.,* 1997:795).

Transdermale geneesmiddelaflewering het verskeie voordele, onder andere om die hepatiese metabolisme uit te sluit en om newe-effekte te verminder (Kydonieus *et al.,* 2000:3). Die versperringsfunksie van die vel is die grootste problem tydens die transdermale aflewering van geneesmiddels; aangesien dit die liggaam help om vreemde voorwerpe, infeksies en UVB (ultravioletbestraling) buite te hou en water en ander noodsaaklike stowwe binne te hou (Rushmer *et al.,* 1966:343). Volgens Shindo *et al.* (1994:123) is daar ón beduidende hoeveelheid vitamien C in beide die epidermis sowel as die dermis. Die konsentrasie vitamien C in die epidermis is egter hoër in vergelyking met die dermis.

Om die penetrasie van farmaseuties aktiewe bestandele (FAB) te verbeter, word daar van fisiese- of chemiese penetrasiebevorderaars gebruik gemaak. PheroidÊ tegnologie is ón relatief nuwe chemiese penetrasiebevorderaar wat gebruik was tydens hierdie studie. Die beginsel van werking vir die metode van bevordering berus op die gebruik van vesikulêre strukture, sonder enige fosfolipiede of cholesterol (Grobler *et al.,* 2008:283).

Tien verskillende rome is tydens hierdie studie geformuleer. Die formulerings het uit verskeie konsentrasies van die FAB, wisselende polariteite en PheroidÊ sowel as nie-PheroidÊ formules bestaan. Die konsentrasies van die FAB het gewissel tussen 1 en 3%. ón 1%, 2% en 3% room is in beide PheroidÊ en nie-PheroidÊ preparate geformuleer. Daarbenewens, is ón 2% room met meer vloeibare paraffien in die formule, sowel as ón 2% room met minder vloeibare paraffien in die formule ook geformuleer (weereens in beide ón PheroidÊ en nie-PheroidÊ formulering) om te bepaal wat die effek van die wisselende polariteite van die formulerings op die vrystelling en

penetrasie van die FAB sou wees. Twee plasebo formulerings is ook voorberei, om vas te stel wat die konsentrasie van natuurlike vitamien C in die vel is, om sodoende daarvoor te kon kompenseer tydens die diffusiestudies.

Die wateroplosbaarheid van die FAB is ook bepaal en het 6.14 mg/ml opgelewer. Die oktanol-waterverdelingskoëffisiënt (log P) was volgens bepaling -0.005. Dit dui daarop dat die FAB sou sukkel om die vel te penetreer as gevolg van die feit dat dit nie in beide olie en water oplosbaar is nie, maar die absorpsie kan moontlik verbeter word a.g.v die hoë wateroplosbaarheid van die middel (Naik *et al.,* 2000:321).

Diffusiestudies (waartydens poli-tertrafluroetileenmembrane (PTFE) gebruik is) is uitgevoer om vas te stel of die FAB vanuit die formulerings vrygestel word. Die membraanstudies is oor ón tydperk van 6 h voltooi en die bevinding was dat die 2% nie-PheroidÊ -room met minder vloeibare paraffien in die formule, die formulering was met die hoogste gemiddelde persentasie vrygestel (2.008%), na die 6 h. Die formulering met die 2de hoogste vrystelling van die FAB was die 1% PheroidÊ formule. Hierdie formulering het ón vrystellingstempo van 1.940% na 6 h gehad. Dit is aanvanklik aanvaar dat die hoër polariteit van die 2% formulering die vrystelling van die hoogs wateroplosbare FAB sou verhinder. Die polariteit van die room was hoër as gevolg van die verhoogde hoeveelheid water in die formulering (Mitsui, 1997:343). ón Mate van vrystelling van die FAB vanuit die formulering was egter verwag, as gevolg van die hoë persentasie ongeïoniseerde spesie (99.37%) van die FAB (Barry, 2002:511).

Die vertikale Franz sel-diffusiestudies wat oor 12 h plaasgevind het, het die 2% nie-PheroidÊ - formulering aangewys as die room met die hoogste gemiddelde kumulatiewe konsentrasie (3.761 µg/cm^2) wat gediffundeer het. Die 1% nie-PheroidÊ formulering (3.555 µg/cm^2) was die formulering wat die 2de hoogste konsentrasie van die FAB getoon het na die diffusiestudies. Die hoë diffusietempo van die formulerings kan toegeskryf word aan die 99.37% ongeïoniseerde spesie van die FAB. Volgens Barry (2002:511) is die FAB se ongeïoniseerde spesie gewoonlik lipiedoplosbaar en kan dit geredelik oor die stratum korneum beweeg. Dit was duidelik dat die formulerings met die laer FAB konsentrasies beter gevaar het as die formulerings met die hoër hoeveelhede van die aktief. Dit kan toegeskryf word aan die verhoogde stabiliteit van die formulerings by laer FAB konsentrasies.

Die 2% PheroidÊ formulering met minder vloeibare paraffien in die formule was die formulering met die hoogste gemiddelde konsentrasie vitamien C in die stratum korneum-epidermis (0.457 µg/ml). Danksy die verhoogde hoeveelheid water in die room het die formulering met minder vloeibare paraffien in die formule ón hoër polariteit gehad (Mitsui,

1997:343). Volgens Bichers (2010:22) kon dit moontlik gelei het tot die penetrasie van die grootliks ongeïoniseerde FAB tot in die stratum korneum-epidermis, juis omdat die lipiedoplosbare spesie van die FAB ón hoër affiniteit het vir die lipiedryke membraan.

Die formulering met die hoogste gemiddelde konsentrasie vitamien C in die epidermis-dermis was die 1% PheroidÊ formulering, met ón gemiddelde konsentrasie waarde van 0.656 µg/ml, gevolg deur die 2% PheroidÊ formule met ón konsentrasie van 0.530 µg/ml. Dit kan moontlik veroorsaak word deur die PheroidÊ wat die FAB omhul en sodoende die lipied oplosbaarheid daarvan verbeter (Grobler *et al.,* 2008:297).

Na aanleiding van die eksperimentele data was die 1% PheroidÊ room die formulering wat die beste algeheel presteer het, gedurende die eksperimente. Dit was tydens die membraanstudies die formulering met die 2de hoogste gemiddelde persentasie FAB vrygestel (1.940%) na ón tydsduur van 6 h en die formulering met die 4de hoogste konsentrasie FAB (3.057 µg/cm^2) na die 12 h lange diffusiestudies. Die formulering was ook die room wat die epidermis-dermis (teiken area) die beste gepenetreer het en ón gemiddelde FAB konsentrasie van 0.656 µg/ml gelewer het. Dit kan toegeskryf word aan die PheroidÊ in die formulering wat die FAB molekules omhul het en dus gehelp het om die penetrasie van die middel deur die stratum corneum, tot in die dermis te bevorder (Grobler *et al.,* 2008:297).

Sleutelwoorde: natriumaskorbielfosfaat, foto-veroudering, transdermale diffusie, PheroidÊ, formulering

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Over the ages, one of the most sought-after answers to a medical question was that of reversing the signs of photo-aging. Xerosis, rhytids, dyschromia, scars, seborrhoeic keratoses and localised adiposity are some of the skin changes observed with prolonged exposure to ultraviolet (UV) light (Flynn & Coleman, 2000:280). Changes in connective tissue such as collagens, elastin and other proteins found in both bone and connective tissue are evident in photo-aged skin. Collagen has a very important role to play in keeping the skin looking younger. It aids the skin in keeping it strong and resilient against the onslaughts of the environment (Uitto, 1993:299-314) (as quoted by Fisher *et al.*, 1997:1420).

Vitamin C and its derivatives have long been proved to increase the synthesis of collagen in the skin. It has been noted that the collagen concentrations in the skin can be increased by as much as eight-fold with prolonged exposure of the skin to vitamin C (Sharma *et al.,* 2008:2049). Furthermore, vitamin C is a potent anti-oxidant. It is however only found in small concentrations in the skin, because of poor transport from the gastrointestinal tract. The topical application of this vitamin is thus the preferred method to increase its presence in the skin; and more specifically, in the dermis (Staloff, 2010:5).

Various topical drug treatments for photo-aging have been promoted over the years. Some of the advantages of topical drug delivery include bypassing the first-pass (hepatic) metabolism, reduction in the presence of side effects and it is a convenient, non-invasive method of drug administration (Kydonieus *et al.,* 2000:3). Additionally, should there be a problem with toxicity, the drug can be removed relatively easily and effectively (Roberts *et al.,* 2002:90). Problems with transdermal drug delivery can be encountered because of the barrier function of the skin (Naik *et al.,* 2000:319).

Some of the factors influencing the permeation of a drug through the skin include the drugs molecular size, partition coefficient, dissociation constant (pKa value) and its aqueous solubility. Other than these factors, the skins hydration, age and condition are also important (Barry, 2002:509).

In order to enhance the penetration of a drug through the skin, either chemical or physical penetration enhancers can be used (Mathur *et al.,* 2010:173). Physical penetration enhancers work on the principle of providing a drug reservoir on the skin surface, from which the needed levels of the drug can be obtained. Some of the physical ways in which to enhance penetration of drugs through the skin include:

- electroporation (Bang *et al.,* 1999:1);
- iontophoresis (Bang *et al.,* 1999:1) and
- ultrasound (Mathur *et al.,* 2010:173).

Chemical penetration enhancers are, according to Barry (2002:509), compounds which reversibly reduce or change the barrier function of the stratum corneum. Pheroid$^{\hat{E}}$ technology is one of the innovative ways to chemically enhance the penetration of an API (active pharmaceutical ingredient) through the skin (Grobler, 2004:4). It is used as a carrier medium to enhance the absorption of compounds, as well as the efficacy of the APIs, whilst making use of a submicron emulsion type formulation (Grobler *et al.,* 2008:284).

1.2 Aim and objectives of the study

The aim of this study was to determine the extent of topical delivery of sodium ascorbyl phosphate, from different topical formulations, for the treatment of photo-aged skin. The objectives of this study thus included the following:

- Development and validation of a HPLC (high performance liquid chromatography) method for the determination of the concentrations of the API in the formulations.
- Determination of both the aqueous solubility and partition coefficient of the API.
- Formulation of various creams, including Pheroid$^{\hat{E}}$ and non-Pheroid$^{\hat{E}}$ formulations, at five different concentrations and polarities, containing the API.
- Formulation of two placebo creams (Pheroid$^{\hat{E}}$ and non-Pheroid$^{\hat{E}}$) to determine the amount of endogenous vitamin C in the skin.
- Determining the release of the API from the various formulations, by making use of membrane release studies.
- Determining if the API did diffuse through the skin after the application of different formulations.

- Determining whether the API reached the target site (dermis) and diffused into the skin after the different formulations were applied.

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CHAPTER 2

TRANSDERMAL DELIVERY OF ASCORBIC ACID

2.1 Introduction

Skin aging, because of sun damage or photo-aging, is one of the cosmeceutical industriesô main focus points. People want to look younger than their chronological age and are forever searching for the newest wonder product on the market. Photo-aging is caused by prolonged exposure to the harsh ultraviolet radiation (UVR) caused by the sun (Cunningham, 2000:14). In countries for example South Africa, with a warmer climate, photo-damage is a big concern. Photo-damage can occur from a very young age and is associated with dryness, fine and course wrinkles, various neoplasms (both benign and malignant), pigmentation and overall older appearance than chronological age (Fisher *et al.,* 1997:1420).

Darr *et al.* (1992:247-253) (as quoted by Thiele *et al*, 2000:168) proposed that topically applied ascorbic acid can only be effective, in the treatment of photo-damage, when formulated in a high concentration. Concentrations lower than 20% (m/m) of the active proved to be the most stable in topical formulations, whilst still being able to penetrate the skin sufficiently (Zussman *et al.* 2010:516). This helps ensure that the ascorbic acid reaches the target site, namely the dermis. An appropriate vehicle for the transport of the ascorbic acid is also needed. Colloidal carrier systems are one of these vehicles used to protect compounds against degradation (Kristi & Volk, 2003:181). Ascorbic acid is a water-soluble vitamin, which is highly unstable when exposed to heat and or light. Because of its solubility, it is very poorly absorbed through the skin. Through using more stable and lipophilic vitamin C esters, the absorption of the compound, when used in transdermal products, may increase. Some of these esters include palmitates, succinyls and phosphates (Austria *et al.,* 1997:795).

2.2 Photo-aging

Premature skin aging or photo-aging is caused by prolonged exposure to UVR and is characterised by wrinkles, loss of skin tone and altered pigmentation. These symptoms are caused by the impact of the UVR on the collagen in the connective tissue. Collagen fibrils have an important function in the dermis of the skin. They are needed in order to provide the skin with the necessary strength and resiliency, to keep skin looking younger. Type 1 collagen is the

main component in the extracellular matrix in the dermis. Other components of the matrix include type 3 collagen, proteoglycans, fibronectin and elastin (Uitto, 1993:299-314) (as quoted by Fisher *et al.*, 1997:1420). Metalloproteinase, a proteolytic enzyme, acts as an intercessor for the degradation of collagen. Collagenase is another protease utilised in the hydrolysation process of fibrillar collagen. Once the collagen has been split, it is broken down further by gelatinases and stromelysins (Fisher *et al.,* 1997:1421).

Table 2.1: Glogau classification of photo-aging (Adapted from Brannon, 2009).

Group	Classification	Typical age	Description	Skin characteristics
I	Mild	28-35 years	No wrinkles	Early photo-aging: mild pigment changes, no keratosis, minimal wrinkles, minimal or no make-up
II	Moderate	35-50 years	Wrinkles in motion	Early to moderate photo-aging: early brown spots visible, keratosis palpable but not visible, parallel smile lines begin to appear, wears some foundation
III	Advanced	50-65 years	Wrinkles at rest	Advanced photo-aging: obvious discolorations, visible capillaries (telangiectasias), visible keratosis, wears heavier foundation always
IV	Severe	65-75 years	Only wrinkles	Severe photo-aging: yellow-grey skin colour, prior skin malignancies, wrinkles throughout - no normal skin, cannot wear makeup because it cakes and cracks

Besides the degradation of collagen, dermal damage caused by sun exposure is also manifested as the accumulation of abnormal elastin-containing material (Lavker, 1995:123-135). UVR can also cause cancer, immune suppression and of course sunburn. Immune suppression and sunburn are caused by excessive exposure to the sun, whereas skin cancer and photo-aging is a result of accumulated damage caused by repeated exposure (Cooper *et al.,* 1992:8500). Other signs of photo-damage include dryness of the skin, rough texture and an overall older appearance than the chronological age (Cunningham, 2000:16). Skin, chronologically aged, protected from the sun, however, appears smooth and unblemished, even though it is thin and has reduced elasticity. An objective measure for the severity of photo-aging is the Glogau classification

system, as seen in Table 2.1. It is used by practitioners to decide on the best possible treatment for photo-aged skin (Brannon, 2006).

Various cosmeceutical products can be used for the treatment of ageing. Some of these products include moisturisers, retinoids, hormones and vitamins, such as vitamin D, -C and -E. Furthermore, miscellaneous agents such as alpha-hydroxy acids, hydroquinnones, alpha-interferon, minerals, hyaluronic acid, natural cartilage polysaccharides and minoxidil can also be used in the fight against ageing (Cunningham, 2000:24).

2.3 Sodium ascorbyl phosphate

Sodium ascorbyl phosphate is a water-soluble, derivative of vitamin C, with inter alia antioxidant action. It is most commonly found in dog rose (*Rosa canina*) fruits, kiwi fruits (*actinidia*) and West-Indian cherry (*Malphigiapunicifolia*) (Khaiat, 2000:101). Other uses for this vitamin include:

- protection from UV damage and resulting photo-ageing,
- healing of scar tissue,
- overall improvement of skin tone,
- formation of collagen,
- synthesis of neurotransmitters, steroid hormones, carnitine, and
- tyrosine degradation and conversion of cholesterol to bile acids.

Vitamin C is also an anti-inflammatory agent, which degrades and eliminates histamine, has immune-stimulating activity and helps maintain the integrity and elasticity of the extracellular matrix (Thiele *et al.,* 2000:146).

2.3.1 Physical properties

Sodium ascorbyl phosphate is also known as L-ascorbic-2-monophosphate or tri-sodium salt. It is a white to light-yellow coloured crystalline powder or colourless crystals with a sharp, acidic taste. As mentioned above, vitamin C salts gradually darken in colour upon exposure to light. It is a stable derivative of vitamin C and is 64.0% soluble in water, 13.2% in glycerol and 1.6% in propylene glycol. As an effective water-soluble anti-oxidant, which is stable in cosmetic

formulations, it is the perfect completion to vitamin E acetate, which is the common oil-soluble equivalent. The structure of sodium ascorbyl phosphate is shown in Figure 2.1.

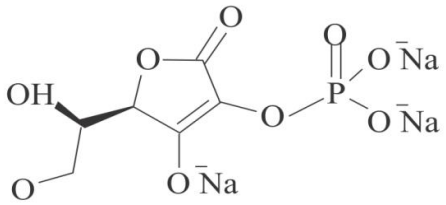

Figure 2.1: Chemical structure of sodium ascorbyl phosphate (Adapted from Ġpiclin *et al.,* 2002).

2.3.2 Mechanism of action

Ascorbic acid is essential in a number of hydroxylation reactions throughout the body. In one of the reactions the amino acids, proline and lysine are transformed into hydroxyproline and hydroxylysine. These hydroxyl (OH)-amino acids play a pivotal role in the improvement of overall skin tone, as they provide the tertiary structure needed to give stability to collagen. The formation of collagen can in many ways be seen as ascorbic acidôs most important role, as collagen is a major component of all connective body tissue including bone matrix, cartilage and dentine (Gibbon *et al.,* 2005:82). Ascorbate aids in the clearance of various free radicals. Some of these include singlet oxygen, thiyl radicals, hydroxyl radicals, superoxide anion radicals and water-soluble peroxyl radicals. The formation of dehydroascorbate, via the ascorbyl radical, is one of the processes in the oxidation of ascorbate. The dehydroascorbate can be recycled back to ascorbate in the presence of thiols (Figure 2.2) or irreversibly decomposes to the unstable diketogulonic acid (Thiele *et al.,* 2000:147). Vitamin C is found in both the dermis and epidermis of human skin. The epidermis does, however, contain approximately five times more vitamin C than the dermis (Shindo *et al.,* 1994:123). This can be attributed to the fact that the epidermis is more directly exposed to the environment and therefore has a higher demand for antioxidant protection (Thiele *et al.,* 2000:149). The lower vitamin C levels in the dermis also indicate the use thereof for collagen regulation and elastin biosynthesis (Davidson *et al.,* 1997:349).

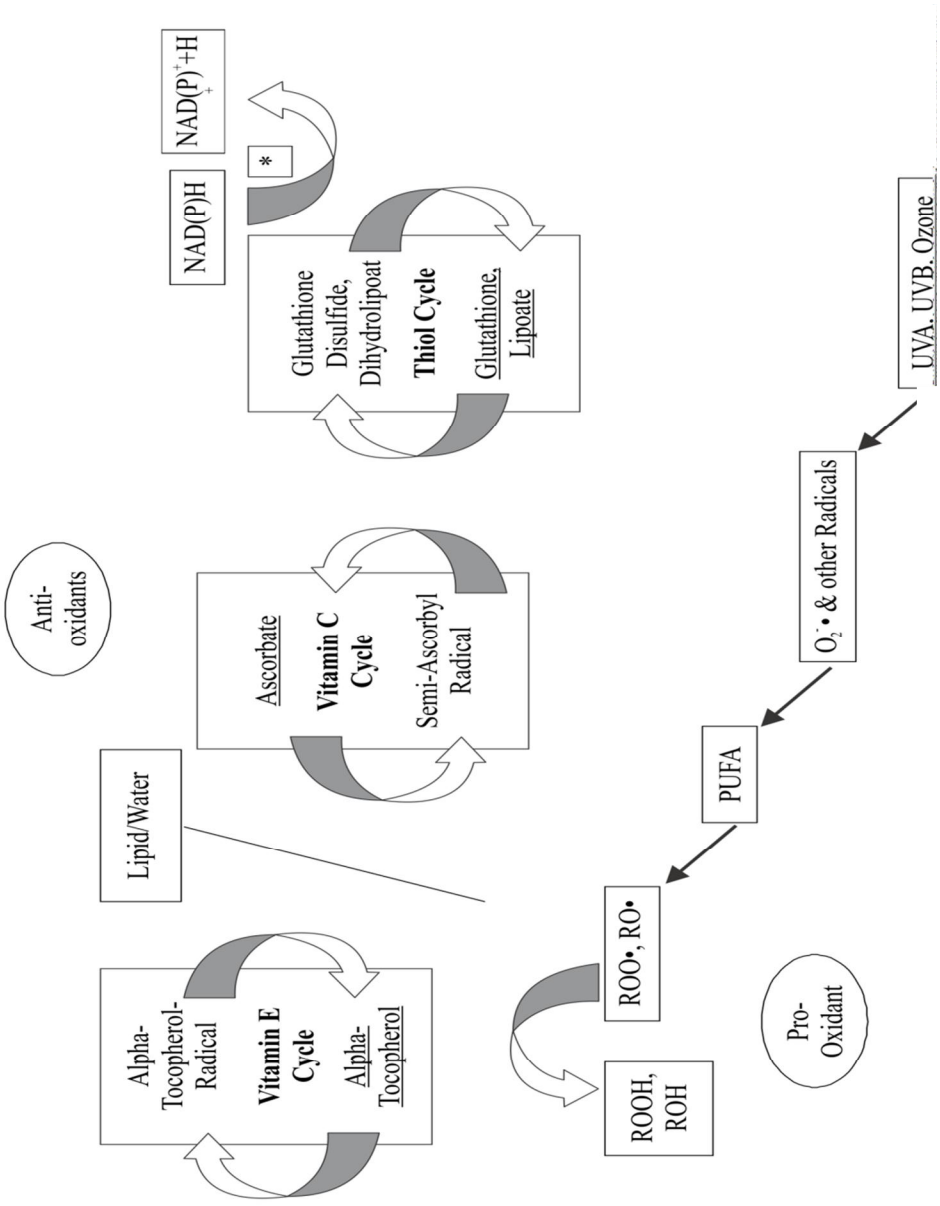

Figure 2.2: Activation of the antioxidant network, by environmental oxidative stressors: superoxide anion radical (O_2^{-}•); polyunsaturated fatty acids (PUFA); lipid(per)oxy radicals (ROO• and RO•); as well as lipidhydro(per)oxides (ROOH and ROH) (Adapted from Thiele *et al.,* 2000:148).

2.3.3 Functions in the human body

Vitamin C is needed in the synthesis of steroid hormones, carnitine and neurotransmitters. It also plays a pivotal role in the conversion of cholesterol to bile acids, as well as the degradation

of tyrosine. The formation of collagen is another very important, if not the most important role of ascorbic acid in the human body. Ascorbic acid also has anti-oxidant action and is necessary in the transformation of amino acids (Gibbon *et al.,* 2005:82).

2.3.4 Therapeutic uses

Vitamin C can decrease the duration and severity of the common cold, if used in doses higher than 1000 mg daily. Intestinal iron absorption is improved, if used in conjunction with vitamin C supplementation. A dose of 200 mg three times daily may also be used in the treatment of methemoglobinaemia (a disease which is marked by the presence of methemoglobin in the blood, resulting in cyanosis). Wound healing is furthermore accelerated by simultaneous use of vitamin C. Other uses for vitamin C include: asthma, osteogenesis imperfecta (autosomal dominant collagen disease, resulting from defective biosynthesis of collagen type 1 and characterised by brittle and easily fractured bones) and acne (Gibbon *et al.,* 2005:82).

2.3.5 Adverse reactions

According to Gibbon *et al.,* (2005:82) mega doses of vitamin C (2-3 g/day) can lead to osmotic diarrhoea, gastrointestinal disturbances and false negative results on occult blood tests. Extremely high doses can also cause cystine, oxalate or urate stones in the urinary tract. Mega doses should be avoided in iron overload states, renal disorders and glucose-6-phosphate-dehydrogenase (G6PD) deficiency, a genetic disorder which causes red blood cells to break down prematurely. Absorption decreases to 50% or even less, with a single oral dose in excess of 1 g. Smokers may require an additional 35 mg/day, compared to non-smokers. Other conditions which require increased amounts of vitamin C include:

- pregnancy and lactation,
- alcoholism,
- hyperthyroidism,
- chronic infections or burns,
- the use of street APIôs or regular aspirin,
- stress as associated with surgical procedures and

- ❖ prolonged administration of certain APIôs including tetracyclines, oral contraceptives and salicylates.

2.4 Anatomy and function of human skin

2.4.1 Structure of the skin

According to Rushmer *et al.* (1966:343) human skin comprises an area of 15 000 to 20 000 cm^2 in most adults and has a varying thickness of between 1.5 and 4.0 mm. The approximate weight of the entire human skin, one of the largest organs of the body, is 2 kg. Skin consists of three layers: the epidermis, dermis and subcutaneous tissue, as seen in Figure 2.3.

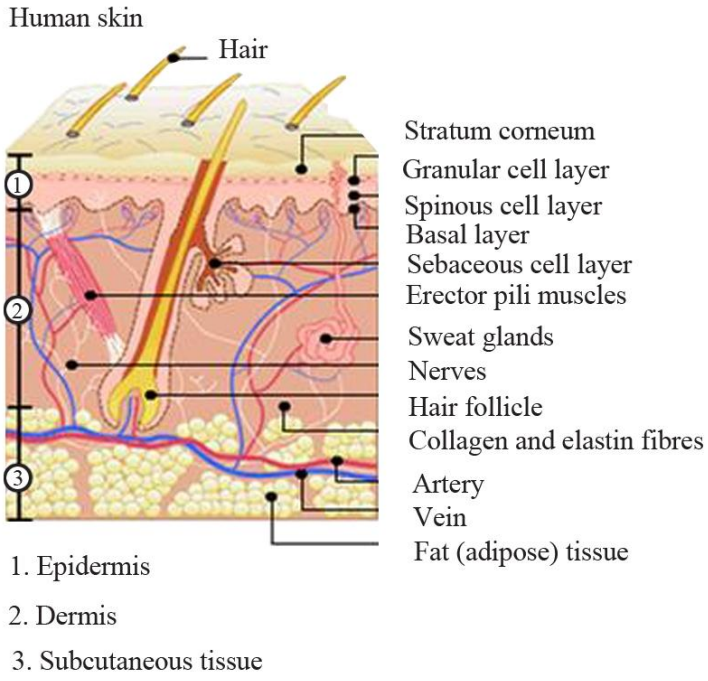

Figure 2.3: Diagram of skin structure (Adapted from Skincare, 2009).

2.4.1.1 Epidermis

The epidermis can be divided into two major parts, namely, the living cells of the Malpighian layer (which can be divided into several strata) and the dead cells of the stratum corneum, also known as the horny layer. It ranges in thickness, from 0.06 to 0.10 mm and is much thicker on the soles of the feet and the palms of the hands. The barrier layer of skin is progressively formed by the differentiation of the viable cells, or keratinocytes, of the epidermis (Franz & Lehman,

2000:17). Some of the structural and biochemical changes taking place during the differentiation of these cells, from inner to outer epidermis include:

- Mitotic activity loss
- Modification of cell membrane and cell surface antigens, as well as receptors
- Synthesis of new organelles
- Synthesis of new lipids, structural and enzymatic proteins
- Total change in cell build, as cells lose their water content, flatten and increase in width (Mitsui, 1997:14).

Some of the specialised cells found in the epidermis include: Merkelôs cells (of which the function is not clearly known), Langerhans cells, responsible for the defence of the immune system in skin and melanocytes (needed for pigmentation). The epidermis consists of five cell layers or strata: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale.

2.4.1.1.1 Stratum corneum

This is the final product of epidermal cell differentiation and is made up of 15 to 20 cell layers all over the body, except the soles of the feet and palms of the hands (Odland & Holbrook, 1974:415). Organelles, including mitochondria and microsomes, as well as nuclei in the granular cells are broken down and the cell envelope is formed. These ôemptiedô cells or corneocytes are then filled with keratin and filaggrin protein (Kydonieus *et al.,* 2000:21). Lipids, organised into bilayers, fill up the intracellular space and comprise around 14% of the stratum corneum. It does, however, have very few phospholipids. This layer also has a low water percentage, but can take up to five times its weight in water, in an aqueous environment (Foldvari, 2000:417). The skinôs impermeability is enhanced by the hydrophobic nature of the stratum corneum. Additionally, the desmosomes found in the stratum corneum, make for a mechanically stronger layer, as they are modified and overlap at their edges (Kligman & Christophers, 1963:702).

2.4.1.1.2 Stratum lucidum

This cell layer is only present in thick skin, such as that of the palms of the hands and soles of the feet. It helps prevent both the loss of water, and the absorption thereof; and reduces the friction and shear forces between the stratum corneum and stratum granulosum (Brannon, 2009).

2.4.1.1.3 Stratum granulosum

The cells in this layer are without nuclei and are characterised by dark clumps of cytoplasmic material. They are known as basophilic granules or keratohyalin granules. Further morphological changes take place and the cells become even flatter and wider. Proteins to be found in the keratohyalin granules of this layer include: pro-filaggrin, involucrin, loricin and small proline-rich proteins, which will all become a component of the thickened envelope of the stratum corneum (Menon, 2002:5).

2.4.1.1.4 Stratum spinosum

Landmann (1988:1) stated that as the cells move up through the strata, they assume a polyhedral shape and appear prickly. This is because of the dehydration, which takes place when the cells start to change shape. The dehydration also makes the cells pull away from each other, except where attached by desmosomes. Keratin filaments become more prominent and a new organelle also makes its appearance in this layer. The lamellar granule is an organelle with a diameter between 0.2-0.3 µm, which contains significantly higher amounts of lipids. Formation of the barrier of the skin is initiated by the issuing of these lipids into the intercellular space of the next strata, the granular layer.

2.4.1.1.5 Stratum basale

This is the bottom layer of keratinocytes in the epidermis. The function of the basal layer is the renewing of the epidermal cells. There is only one row of undifferentiated columnar stem cells in this layer, which divide very frequently. After differentiation, half of the cells move to the next layer to start the maturation process. The cells, which stay behind in the basal layer, divide over and over again, in order to replenish the basal layer (Madison *et al.*, 1987:714). Melanocytes are found in the basal layer. These are cells which produce the pigment melanin, which is necessary for skin colour. The main function of these melanocytes is, however, the

filtering of UVR from sunlight, which can of course damage deoxyribonucleic acid (DNA) or cause harmful effects, such as skin cancer (Porter, 2010).

2.4.1.2 Dermis

This is a thick layer of fibrous and elastic tissue, needed for the skinôs strength and flexibility. The elastic tissue is made mostly of fibrillin, elastin and collagen. Various specialised cells and structures form part of the dermis. Hair follicles are situated in the dermis, as are sebaceous (oil), apocrine (scent) and eccrine (sweat) glands. Blood vessels and nerves are also found in this layer and the latter are needed in order to transmit sensations of pain, temperature and pressure. Essential nutrients for the skin are provided by the blood vessels and they also serve as a temperature-regulating mechanism (Walters & Robert, 2002:11). Blood vessels are dilated by heat and thus allow large volumes of blood to circulate near the skin surface where the heat can be released. Cold facilitates the constriction of vessels, thus, retaining the bodyôs heat. Eccrine glands produce sweat in response to heat and stress; and as the sweat evaporate off the skin, the body is cooled down. The apocrine glands are specialised sweat glands found in the armpits and the genital area. These glands secrete an oily sweat, which produces a characteristic odour when digested by the bacteria on the skin. Sebaceous glands are responsible for sebum secretion into the hair follicles. The sebum acts as a barrier against foreign objects and helps keep skin soft and moisturised. Hair follicles not only produce the various types of hair found throughout the body, they also contain stem cells, capable of re-growing damaged epidermis (Walters & Robert, 2002:12).

2.4.1.3 Subcutaneous tissue

This is also known as the fat layer. It is needed in order to insulate the body from extreme temperatures, acts as an energy storage area and provides the needed mechanical protection or padding against trauma. Fat cells are kept together by fibrous tissue and vary in thickness in different parts of the body. The main function of this layer is to carry the vascular and neural systems for the skin, as well as anchor the skin to the underlying muscle (Porter, 2010).

2.4.2 Function of the skin

As previously mentioned, one of the main functions of skin is that of a physical barrier against infection, chemicals, particles, UVR, etc. It is also needed in order to prevent water and other

vital substances from exiting the body. Furthermore, it acts as a casing for the internal structures, safeguarding it against the external environment (Rushmer *et al.,* 1966:343). Other functions of human skin include: temperature control (Foldvari, 2000:417), sensation (such as pain, touch and temperature), various immunological reactions (wound-healing and antigen presentation) and vitamin D synthesis and conversion of pro-hormones (Menon, 2002:4).

2.5 Transdermal API delivery

The application of medicaments to the skin can be dated back thousands of years. The ancient Greeks applied a mixture of olive oil, water and lead oxide as a balm for the skin. Because olive oil acts as an occlusive barrier it can moisturise the skin, whilst the lead oxide has astringent properties (Morrow *et al.,* 2007:36). Until the mid-20th century, topical preparations were only prescribed for skin diseases. It was only then that physicians observed less frequent angina attacks amongst munitions workers, working with nitroglycerin (El-Kattan *et al.,* 2000:426). Today the majority of preparations applied to the skin, are aimed at delivering the API for local and not systemic action. Preparations tend to be simple semi-solids such as ointments, creams and gels (Morrow *et al.,* 2007:36).

2.5.1 Advantages and disadvantages of transdermal API delivery

There are three main targets of topical and transdermal delivery, namely the skin surface, the epidermis or dermis and the systemic circulation. When considering the delivery of cosmetics, the surface of the skin is targeted, whereas the targeting of the various layers of the skin is known as topical API delivery. In cases where the systemic circulation is the end destination, transdermal delivery is sometimes considered because of some advantages it offers.

2.5.1.1 Advantages

Some of the advantages of transdermal API delivery include:

- A relatively large and accessible surface area is available for absorption (Naik *et al.,* 2000:319).
- Bypassing of the first-pass (hepatic) metabolism takes place.
- It is a non-invasive, convenient means of API delivery.
- There is a marked reduction in dosing frequency.

- Sustained API delivery is facilitated.
- Reduced side effects are obtained, because of the relatively low doses provided.
- It is an alternative route for patients who cannot take drugs orally (Kydonieus *et al.,* 2000:3).
- In the event of toxicity the API can be easily removed (Roberts *et al.,* 2002:90).

2.5.1.2 Disadvantages

The limitations of transdermal API delivery can be summed up in two main problems, i.e.: difficulty of permeation through human skin and skin reactions.

2.5.1.2.1 Permeation through the skin

- The stratum corneum is the main rate limiting factor in the diffusion of APIôs across the skin (Roberts *et al.,* 2002:92).
- Daily doses of 2 to 3 mg/day or less are preferred for transdermal delivery. This means that very few drugs actually permeate the skin in sufficient amounts to deliver a therapeutic dose.
- Drugs which permeate the skin best include drugs with low molecular weights, low melting points and moderate water and oil solubility (Franz *et al.,* 1991:342).
- Chemical, as well as physical methods for enhancing permeation are available. A chemical enhancer alters the barrier function of the skin, in order to allow certain drugs to permeate the skin faster, or increases the concentration of the drug in the skin (Morrow *et al.,* 2007:44). Some of the physical enhancing methods include: iontophoresis, electroporation, phonophoresis, ultrasound, etc. (Morrow *et al.,* 2007:49).

2.5.1.2.2 Skin reactions

- Adverse skin reactions such as irritant and contact allergic dermatitis are possible with the transdermal delivery of drugs.
- Chemical, as well as physical penetration enhancers can enhance the risk of skin irritation, while drugs themselves can also cause severe skin reactions.

- There are several commercial drugs which act as irritants; these include: captopril, prazosin, diclofenac and retinoic acid (Kydonieus *et al.,* 2000:9).

2.5.2 Pathways of transdermal penetration

There are three possible pathways for the permeation of transdermal drugs through the stratum corneum (Figure 2.4). They include the transcellular route, the intercellular lipid route and via skin appendages or transappendageal route. An API will, in most cases, make use of a combination of these routes in order to permeate the stratum corneum effectively (Roberts *et al.,* 2002:96).

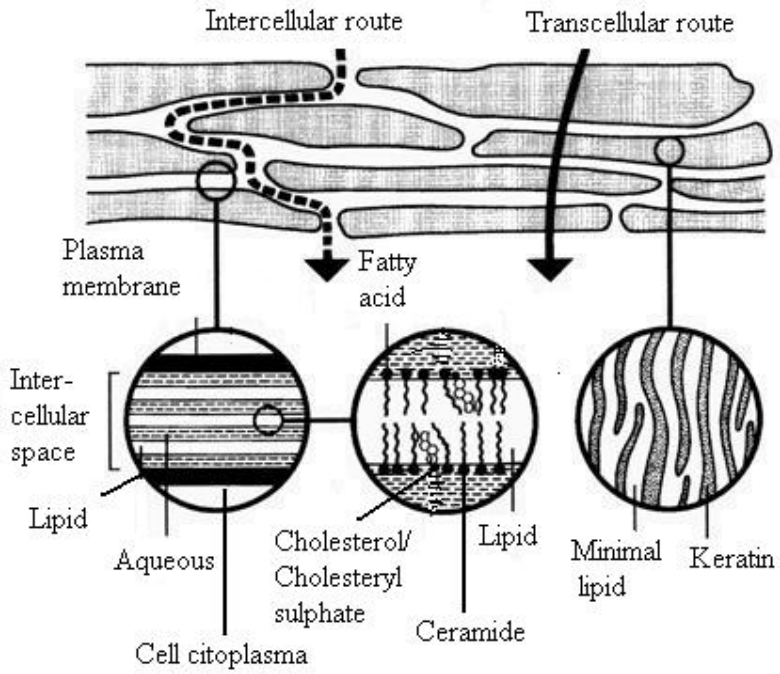

Figure 2.4: Diagrammatic representation of the stratum corneum and the intercellular and transcellular routes of penetration (Adapted from Mathur *et al*., 2010).

2.5.2.1 Diffusion via the transcellular route

In order for an API to diffuse via this particular pathway, it has to be able to partition between both lipophilic and hydrophilic compartments. Firstly, the API must partition into, and diffuse through the aqueous environment of the corneocytes. Secondly, it has to partition into the surrounding lipid envelope and again in and out of the multiple lipid bilayers between the

corneocytes. The physicochemical make-up of a certain API will determine whether or not the API will make use of this particular pathway (Scheuplein, 1978:173).

2.5.2.2 Diffusion via the intercellular route

According to Potts and Francoeur (1990:3871), diffusion through the continuous lipid matrix forms part of the intercellular pathway. There are two problems with this route. The first being the ôbrick and mortarô model of the stratum corneum. The diffusional pathway around keratinocytes is much more complex than that of the relatively direct path of the transcellular route. Previous studies have shown that water travels approximately 50 times further via this route, compared to the transcellular route. Secondly, as with the transcellular route, the API must be able to partition through both aqueous and lipid domains. Small, uncharged molecules usually penetrate the skin, using this pathway (Mansoor, 2003:302).

2.5.2.3 Diffusion through the appendageal route

Skin appendages act as a direct channel across the stratum corneum. The surface area occupied by appendages such as hair follicles or sweat ducts are relatively small though, and thus the surface available for direct API contact is limited. Sweat ducts contain an aqueous salt solution, which, whilst being favourable for hydrophilic APIôs, can cause a problem as it travels against the diffusion pathway when secreted. Sebaceous glands on the other hand are filled with a lipid rich sebum, which can of course cause problems for hydrophilic APIós such as ascorbic acid (Morrow *et al.,* 2007:38).

2.5.3 Properties influencing the permeation through skin

The primary factors influencing bioavailability, therapeutic inequivalence and bioinequivalence of topical products, as stated by Surber & Davis (2002:432), are listed below.

2.5.3.1 Biological properties

2.5.3.1.1 Skin age

There are little data to prove that skin of the elderly or children is more permeable than that of adults. Children are, however, more susceptible to the toxic effects of APIôs, because of their greater surface area per unit body weight (Barry, 2002:509).

2.5.3.1.2 Skin condition

In diseased or damaged skin, where loss of stratum corneum is present, permeability is increased according to Barry (2002:509), whereas calloused or thickened skin should decrease the penetration of APIôs.

2.5.3.1.3 Blood flow

The transepidermal resorption process, (tapping into the cutaneous microcirculation) brings APIôs into the underlying tissues or the systemic circulation. Part of the API accumulates in the superficial dermis and diffuses into deeper parts of the skin. Under these conditions, cutaneous blood flow can modify the concentration levels and the accumulation of substances in the dermis or deeper parts of the skin (Surber & Davis, 2002:433).

2.5.3.1.4 Regional skin sites

The thickness and nature of the stratum corneum is an important factor to consider in the permeation of APIôs. Permeability depends both on the intrinsic resistance to permeation per unit thickness of stratum corneum and the overall thickness of the tissue (Barry, 2002:510).

2.5.3.1.5 Species differences

Differences in horny layer thickness, sweat glands and hair follicle densities are some of the factors to be considered when using mammalian skins. The capillary blood supply and the sweating ability differ between humans and common laboratory animals. These factors influence the routes of penetration and the resistance to permeation (Barry, 2002:510).

2.5.3.1.6 Skin metabolism

There is significant metabolic activity in the skin and it covers a broad range of reductive, hydrolytic, oxidative and conjugative reactions. This can of course influence the therapeutic efficacy of topically applied APIôs (Surber & Davis, 2002:436).

2.5.3.2 Physicochemical properties

Some of the physicochemical factors influencing permeation through the skin include: skin hydration, temperature, pH, the diffusion coefficient, the partition coefficient and the molecular size of particles.

2.5.3.2.1 Skin hydration

Hydration of the stratum corneum appears to increase transdermal delivery of both hydrophilic and lipophilic APIôs as stated by Williams and Barry (2004:606). The water content of the human stratum corneum is usually between 15 and 20% of the tissueôs dry weight. This is of course affected by external factors such as humidity. Saturation of the stratum corneum allows it to reach the same water content as the underlying epidermal tissue.

2.5.3.2.2 Temperature

Partitioning is not usually affected by temperature, but in certain membrane containing structures, which are subject to phase changes on heating or cooling, there may be a simultaneous effect on the diffusion (Barry, 2002:511).

2.5.3.2.3 pH, pKa and ionised and unionised forms

Normal human skin has a pH of 4-6. The pH of topical solutions affects the extent of dissociation of ionisable API molecules and therefore their thermodynamic activity, partitioning and skin permeation (Surber & Davis 2002:432). The appropriate pH value, at which to perform the diffusion studies, was found to be 5.5. The dissociation constant (pKa) of sodium ascorbyl phosphate was determined to be 7.7. This indicated that the percentage unionised species, at a pH of 5.5 and pKa of 7.7 were 99.37% and the ionised species were 0.63%. According to Mansoor (2003:302) API mainly penetrate the hydrophobic biological membranes in the unionised or un-dissociated form. One can therefore predict whether an API will penetrate and absorb efficiently based on the pH of the environment and the pKa or partition coefficient of the API. Following this statement, it is expected that the sodium ascorbyl phosphate should be able to penetrate the skin effectively.

2.5.3.2.4 Partition coefficient

According to Williams (2003:35) when establishing the flux of a compound through the stratum corneum, the partition coefficient (K) must be taken into consideration. If the membrane is the only source of resistance to the diffusion of the API, the partition coefficient becomes very important. An octanol-water partition coefficient (log P) between 1 and 3 indicates the ability of an API to dissolve in both oil and water (Robert & Walters, 1998). This will of course ensure that the API will penetrate the skin relatively fast. The partition coefficient of sodium ascorbyl

phosphate is < -4 at 20 °C and in solutions of between pH 9 and 10. This indicates that the API is not soluble in either oil or water and might have difficulty penetrating the skin. However, as the pH of a solution or formulation increases, the percentage ionised species becomes higher. This then inadvertently leads to a reduction in the partition coefficient (Donnelly *et al.* 2012:215). It was thus expected that the decreased pH value (pH 5.5) at which the experiments were performed, would lead to a decrease in the ionised percentage and a subsequent increase in the partitioning coefficient.

2.5.3.2.5 Molecular size

Small molecules penetrate faster than large ones, as stated by Barry (2002:512). The precise effect of the size of the permeating molecules on the flux can, however, only be determined if the effect of size could be separated from the resultant change in solubility properties, which is difficult to do, because of the major role the partition coefficient plays. For molecules to be able to penetrate the stratum corneum, a molecular mass of less than 600 Da is required (Barry 2002:512). The molecular mass of sodium ascorbyl phosphate is 322.05 Da which points to the ability of the API to penetrate the stratum corneum.

2.5.3.2.6 Aqueous solubility

Considering the intercellular permeation pathway of the skin, it is evident that lipophilic molecules tend to cross the skin faster than hydrophilic molecules (Williams, 2003:37). Although lipophilicity is an important trait for any API applied transdermally, some degree of aqueous solubility is also needed in order for the API to be formulated in an aqueous formulation (Williams, 2003:37). Sodium ascorbyl phosphate is hydrophilic in nature. This indicates that it should have difficulty passing through the largely lipophilic barrier of the skin, but should be released from the formulation with relative ease. The aqueous solubility of sodium ascorbyl phosphate is 789 g/l at 20 °C in solutions with pH values of between 9 and 10. According to Naik *et al.* (2000:319) for an API to be able to permeate the skin, it should have an aqueous solubility of 1 mg/ml or more. This indicates that the active ingredient should be able to penetrate the skin sufficiently. The diffusion studies were however performed at a much lower pH of 5.5 and according to Donnelly (2012:215), a decrease in the pH of a formulation will also lead to a decrease in the ionised species of the API. It was thus expected that the lower pH of the

formulations would cause the APIôs aqueous solubility to decrease, as the ionised percentage of an API is the water soluble species (Mansoor, 2003:302).

2.5.3.2.7 Polarity

According to Guang and Goa (2010:75) the polarity of an API is indicated by the log P value of the API. In order to determine the polarity of the formulation needed, for it to be able to push the API into the stratum corneum, the difference in polarity between the stratum corneum and the API must be determined. Hydrophilic and lipophilic excipients, as well as surfactants from formulations are likely to differentiate into the stratum corneum and thereby alter the distribution of the API between the stratum corneum and the vehicle (Guang & Goa, 2010:75). The polarity of several of the formulations was altered by increasing the water and decreasing the liquid paraffin concentrations in the formulas. Increased amounts of water would insure that the formulations became more polar and the oil soluble unionised species of the API differentiated more easily from the formulation into the lipophilic surrounding of the skin (Bickers, 2010:22). Other formulations were altered by increasing the amount of liquid paraffin and decreasing the amount of water in the formula, making it less polar. It was expected that the decreased polarity of the cream would make it the preferred environment for the API and thus hinder the diffusion of the API from the cream into the skin.

2.5.3.2.8 Polarity gap

Considering the polarity of a formulation, it is important to take note of the so called ñpolarity gapò which exists between the formulation and the API. The polarity gap can be determined by Equation 2.1 (Wiechers, 2004:177).

Penetrant polarity gap = Penetrant polarity ï stratum corneum polarity **Equation 2.1**

According to Wiechers (2004:177) the polarity gap is formed because of the higher affinity that more lipid soluble APIôs have for the stratum corneum, than for the less lipophilic formulation it is in. The API therefore prefers the hydrophobic environment of the stratum corneum and this causes a driving force for the partitioning of the API into the stratum corneum. The larger the difference between the polarity of the API and the polarity of the formulation is, the bigger the force for driving the API into the stratum corneum.

2.5.3.3 Basic mathematical principles in skin permeation

2.5.3.3.1 Fick's law of diffusion

Watkins and Brain (2002:62) stipulated that a good understanding of the basic mathematical principles of membrane transport is needed in order to appreciate how membrane barriers fulfil their function. Fickôs first law, one of the most basic diffusion equations, is based on the theoretical description of heat transfer and conductance. Transport of molecules across any membrane, including the skin, occurs via passive diffusion. The flow is related to the velocity of molecular movement, as well as the concentration of the molecules. The equation for Fickôs first law is related in Equation 2.2.

$(J=K.D\,\Delta C)/l$ **Equation 2.2**

Where:

J = flux (µg/cm^2.h)

K = partition coefficient

D = diffusion coefficient (cm^2/h)

æC = concentration difference (µg/cm^3)

l = membrane thickness (cm)

Fickôs second law of diffusion stipulates the rate of change in concentration, with time at a given point in a system to the rate of change in concentration gradient at that point.

2.5.4 Penetration enhancement

The ability of an API to penetrate the skin in sufficient quantities in order to achieve a therapeutic effect, is the key factor in the successful employment of a dermatological API used for systemic delivery. Penetration enhancers may act by one or more of the following mechanisms:

- disruption of the highly ordered structure of stratum corneum lipid;
- interaction with intercellular protein; and
- improved partitioning of the API, co-enhancer, or solvent into the stratum corneum.

Penetration enhancers are divided into chemical and physical penetration enhancers (Mathur *et al.,* 2010:173).

2.5.4.1 Physical penetration enhancers

Some of the techniques used for the physical enhancement of API penetration through the skin include: electroporation, iontophoresis and ultrasound.

2.5.4.1.1 Electroporation

This method involves the application of high voltage pulses (100 V), in order to induce skin perturbation. This is normally done over very short time periods. The increase in skin permeability is suggested to be caused by the generation of transient pores (Bang *et al.,* 1999:1).

2.5.4.1.2 Iontophoresis

The enhancement of permeation through the skin is achieved by the application of a low-level electric current. It is applied either directly to the skin or indirectly via the dosage form (Bang *et al.,* 1999:1).

2.5.4.1.3 Ultrasound

With this technique the permeation is enhanced by use of ultrasonic energy. This is used in order to improve the transdermal delivery of solutes; either all together or through pre-treatment. Gaseous cavities form within the intercellular lipids on exposure to ultrasound, resulting in disruption of the stratum corneum (Mathur *et al.,* 2010:174).

2.5.4.2 Chemical penetration enhancers

According to Barry (1983:160), compounds which reversibly reduce the barrier function of the stratum corneum are known as chemical penetration enhancers. An ideal penetration enhancer should comply with the following standards:

- It should be non-toxic, non-allergenic and non-irritant.
- It must be cosmetically acceptable in terms of odour, colour, taste and texture.
- It should be inexpensive.

- ❖ The compound must be able to formulate easily into semi-solids, aerosols and skin adhesives.
- ❖ Barrier integrity should recover after the compound is removed from the skin.
- ❖ It should be physicochemically compatible and stable with the other components of the formulation.
- ❖ The compound must be a suitable solvent for the API used.
- ❖ No pharmacological action should be elicited in the body.
- ❖ It must facilitate enhanced API absorption, but not promote the loss of endogenous substances from the body.
- ❖ It must have a fast-acting and reproducible effect.

2.5.4.2.1 Azone

This highly lipophilic material has a log P of approximately 6.2 and is soluble in and compatible with most organic solvents. It is most effective at low concentrations, between 0.1 and 5.0%. Partitioning into a bilayer lipid, in order to disrupt the packing arrangement, is the method by which permeation is enhanced (Hoogstrate *et al.,* 1991:38).

2.5.4.2.2 Water

Increased hydration of the skin enhances permeability according to Mathur *et al.* (2010:180). It is a well known fact that the stratum corneum swells upon emersion in water and can absorb up to ten times the dry weight. The water is bound within the intracellular keratin and permeation increases rapidly at first and slows down to a steady-state after a period of time (Roberts *et al.,* 2002:140).

2.5.4.2.3 Vesicular structures

These structures or their components interact with stratum corneum lipids to enhance skin permeability and are usually composed of amphiphilic molecules. According to Williams and Barry (2004:614) previous studies have used phospholipids as vesicles (liposomes) to carry compounds into and through the skin. Furthermore phospholipids can occlude the skin, and thereby increase permeability. By interacting with the stratum corneum a collapse of the stratum corneum structure is brought on, allowing permeant into the vehicle. Other chemical penetration

enhancers include: pyrrolidones, alcohols, surfactants, fatty acids, dimethylsuphoxide, terpenes, etc.

2.5.4.3 Penetration enhancement by removal of the stratum corneum

2.5.4.3.1 Microneedle-based devices

This technique involves the puncturing of the skin, using micron-sized needles. These devices have been proven to penetrate the stratum corneum, as well as the viable epidermis without affecting or reaching nerve fibres or blood vessels (Henry *et al.,* 1998:923).

2.5.4.3.2 Skin abrasion

The upper layers of skin are removed or disrupted. The physicochemical properties of an API will not limit the delivery of the API when making use of this method. It has been used to control and optimise the delivery of a vitamin C vaccine, as well as other APIôs, in previous studies according to Lee *et al.* (2003:1118).

2.6 Pheroid™ technology

The PheroidÊ system comprises a submicron emulsion type formulation. The manipulation of this stable structure, within a system, in terms of morphology, size, structure and function is one of the advantages of the PheroidÊ system. It predominantly consists of plant and essential fatty acids and is used to deliver pharmacologically active compounds (Grobler, 2004:4).

2.6.1 Pheroid™ technology for transdermal delivery

One of the biggest problems faced when applying cosmeceuticals, is insufficient delivery. PheroidÊ technology can enhance the efficacy and/or absorption of active ingredients and various other compounds. Studies have also shown that a major improvement in charge, control of size and the hydrophilic-lipophilic characteristics of formulations are possible with the PheroidÊ technology (Grobler *et al.,* 2008:284).

2.6.2 Structural characteristics of Pheroid™

The structure comprises a colloidal system which contains stable lipid-based submicron- and micron-sized structures, homogeneously distributed in a dispersion medium. As mentioned

previously, the dispersed phase can be manipulated in terms of morphology, structure, size and function (Grobler *et al.,* 2008:285). In typical colloidal systems particle size is usually between 1-100 nm in diameter, whereas the various types of PheroidÊ are formulated to have a diameter of between 200 nm and 2 µm. The required amount and size of the active compound to be entrapped, the delivery rate and the administration route, are all factors to be taken into consideration when deciding on the type and diameter of the PheroidÊ. Reduction in side effects and enhancement of the efficacy of the active compounds are some of the reasons for using colloidal systems as carriers of APIôs or cosmetics. These colloidal systems can be classified as:

- Network colloids that have two phases forming an inter-penetrating network, as is found in polymer matrices.
- Simple colloids where a clear distinction between the dispersed phase and dispersion medium is found, as in oil-in-water (o/w) or water-in-oil (w/o) emulsions.
- Multiple colloids in which three phases co-exist ï two finely divided dispersed phases, such as in multiple emulsions of water-oil-water (w/o/w) or oil-water-oil (o/w/o).

2.6.3 Functional characteristics of Pheroid™

Various processes are involved in the delivery of active compounds to skin. These processes depend on the target of delivery ï the tissue as well as the cell type. In the perfect product, each of these processes will be optimised. Delivery of an active compound to the viable epidermis will involve at least the following: pliable system design and versatility, entrapment efficiency (EE), penetration efficiency, uptake of PheroidÊ and entrapped compounds by cells, as well as metabolism, targeting and distribution.

2.6.3.1 Pliable system design and versatility of Pheroid™

According to Grobler *et al.* (2008:293), versatility is one of the characteristics of an acceptable carrier system, because of the different target sites in the body for pharmaceutical and cosmetic applications. Due to the use of gas as well as the pliable pegylated tails added to the fatty acids, exceedingly elastic structures are formed. The pegylation serves to stabilise the PheroidÊ and maintain their interior spaces.

2.6.3.2 Entrapment efficiency of Pheroid™

The EE can be expressed as the percentage of the initial amount of compound added to the formulation that is entrapped (Equation 2.3):

$$EE\% = \frac{\text{Amount of entrapped compound}}{\text{Total amount of compound initially used}} \times 100 \qquad \textbf{Equation 2.3}$$

An entrapment efficiency of more than 90% is needed for products in development. Due to the elastic design of the PheroidÊ the number of colloidal particles per volume can be increased or decreased to suit the required concentration of active compound. Various factors influence the internal entrapment volume, including the size of the vesicles as well as the concentration and character of fatty acids. Other factors include: presence of charged molecules and the hydration medium (Grobler *et al.,* 2008:294).

2.6.3.3 Penetration efficiency of Pheroid™

The percentage of active compound delivered to skin is enhanced by entrapment in PheroidÊ . The efficiency of penetration can be measured in a number of ways including a comparative investigation. In this instance the enhancement caused by the carrier is determined by comparing it to an existing commercial product (Grobler *et al.,* 2008:297).

2.6.3.4 Uptake of Pheroid™ and entrapped compounds by cells

PheroidÊ uptake is influenced by the formulation and by the mechanism of uptake by the cells. The permeation of the formulation is determined by the following factors:

- morphology of the PheroidÊ ,
- size of the PheroidÊ ,
- molecular geometry of the fatty acids themselves,
- concentration and ratios of the various fatty acids,
- hydration medium,
- pH of the preparation,
- character and concentration of the active ingredient or API,
- state of the PheroidÊ (either gel or fluid state),

- presence of charge-changing molecules and
- presence of molecules which influence the electrostatic environment (Grobler *et al.,* 2008:299).

2.6.3.5 Metabolism, targeting and distribution of Pheroid™

The type and extent of the fatty acid modifications influence the distribution of PheroidÊ . Depending on the composition of the formulation, they are metabolised in either the mitochondria or the peroxisomes of the cell, resulting in the release of the active compound.

2.6.4 Therapeutic efficacy of Pheroid™

The effect of a delivery system should be measured by its contribution to therapeutic efficacy, according to Grobler *et al.* (2008:300). Formulation of active compounds in PheroidÊ has been shown to increase the efficacy of a number of such active compounds. The effect of essential oils on the skin is enhanced by entrapment in PheroidÊ .

2.6.5 Possible applications of Pheroid™ technology in cosmetics

Mitosis of the stem and transit amplifying cells which are primarily situated in the stratum basale of the epidermis are involved in the proliferation process. Proliferation of these cells can be directly influenced by diffusion of soluble factors from the underlying dermis or by transport of topically applied molecules through the cohesive stratum corneum. Retinoids have been formulated with PheroidÊ and are specifically used for the treatment of skin eruption and enhancement of healthy and glowing skin (Grobler *et al.,* 2008:308).

2.6.6 Advantages of Pheroid™ technology as a transdermal API delivery system

The basic fundamentals of the system, as stated by Grobler *et al.* (2008:308), prove that it differs substantially from conventional macromolecular carriers such as liposomal delivery systems. Some of the advantages of PheroidÊ include:

- Cytokine studies demonstrated that it elicits no immune responses in man.
- It consists mainly of essential fatty acids, a natural and essential ingredient of the body.
- A variety of types are formulated, depending on the composition and method of manufacturing.

- ❖ It can be manipulated in terms of size, charge, lipid composition and membrane packing.
- ❖ A specific protein family in cell membranes is responsible for the binding and uptake of essential fatty acids. This ensures that the PheroidÊ interacts with the cell membrane and follows the endosome sorting mechanism resulting in penetration and delivery.
- ❖ Different combinations of fatty acids and/or other added molecules are used to target PheroidÊ at sub-cellular level to some extent.
- ❖ Since it is part of the natural biochemical pathways, the PheroidÊ causes no cytotoxicity and assists with maintenance of the cell membrane (Grobler *et al.,* 2008:308).

2.6.7 Conclusion regarding Pheroid™

Most delivery systems can be classified structurally as colloids. The therapeutic profiles of the delivered compounds are determined by the surface properties and the interfacial interactions of the colloids with the biological environment. An understanding of these therapeutic profiles enables one to manipulate the colloidal systems. The PheroidÊ system consists mainly of essential fatty acids. These fatty acids are the main components of the delivery system and are also innate components of the skin. Essential fatty acids assist in the normalisation of the physiological micro-environment, which leads to added anti-inflammatory action, suppression of epidermal hyper-proliferation, fast and effective API delivery and normalising of the water barrier of the skin. PheroidÊ is a safe, versatile, effective and inexpensive delivery system. The stability of the PheroidÊ system has also been proved for PheroidÊ -based commercialised products (Grobler *et al.,* 2008:308).

2.7 Summary

Skin ageing is one of the oldest cosmetic problems facing the modern woman. Aged skin is characterised by a loss in elasticity, altered pigmentation, wrinkles, dry skin and rough texture, among other factors. This is widely due to depletion of collagen in the connective tissue of the skin matrix. The accumulation of abnormal elastin containing material plays another major role in the aged look of skin, damaged by sun exposure. Cancer, immune suppression and sunburn are some of the other effects that prolonged UVR exposure can have. One way in which to measure objectively the severity of photo-aging is the Glogau classification system, described previously.

The use of vitamin C as an anti-aging product lies in the fact that the formation of collagen is one of this vitaminôs most important functions within the body. Furthermore, it is also an anti-oxidant and is essential in the transformation of amino acids. These amino acids play a pivotal role in the improvement of overall skin tone, as they provide the tertiary structure needed to give stability to collagen.

Due to various factors, such as the hepatic first-pass metabolism APIôs administered orally are not always as effective as an API administered alternatively. Therefore, the skin can be used as an alternative route for administration. Some of the advantages of the transdermal delivery of APIôs are the reduced side effects encountered, as well as this being a non-invasive, convenient means of API delivery. One of the ways in which to enhance the penetration of an API when administered transdermally, is the use of PheroidÊ technology. Use is made of vesicular structures with no cholesterol or phospholipids in order to enhance the penetration of certain APIôs. The aim of this study included the formulation of various sodium ascorbyl phosphate containing creams, some with PheroidÊ vesicles, for the treatment of aged skin; the development and validation of a HPLC method for the quantitative determination of the API within the formulations; the determination of the aqueous solubility, as well as the partition coefficient of sodium ascorbyl phosphate and determining whether the active ingredient diffused into the skin.

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CHAPTER 3

ARTICLE FOR THE PUBLICATION IN SKIN PHARMACOLOGY AND PHYSIOLOGY

This chapter was written in article format for publication in Skin Pharmacology and Physiology. The complete authors guide is given in Appendix D and therefore the following text it is written in concise US English. Following the guidelines given in the authorôs guide, no formatting was done for this chapter. The only formatting applied to this chapter was the justification of the document, in order to keep it as neat as possible.

Topical delivery of sodium ascorbyl phosphate in cosmeceutical products

Lonette Wallis, Minja Gerber*, Jaques Lubbe, Jeanetta du Plessis

* Corresponding author. Unit for Drug Research and Development, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa.
Tel.: +2718 299 2328; fax: +2718 293 5219. E-mail address: Minja.Gerber@nwu.ac.za.

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Abstract

Vitamin C and its derivatives have long been known to have an effect on collagen formation in the skin, as well as being an anti-oxidant, to be used in the fight against aging skin. One of these derivates is sodium ascorbyl phosphate, a more stable salt of vitamin C. **Purpose:** The main aim of this study was to determine whether sodium ascorbyl phosphate could effectively be delivered transdermally by utilizing transdermal diffusion studies on twelve different formulations. These formulations included varying concentrations of the active pharmaceutical ingredient (API), different polarities of the carrier and both PheroidÊ and non-PheroidÊ creams. **Procedures:** In order to determine the transdermal diffusion of the API, diffusion studies and tape-stripping techniques were used. **Results:** The API was found to penetrate both the epidermis, as well as the dermis depending on the concentration of the API in the formulation, the polarity of the carrier medium and the absence or presence of PheroidÊ in the formulation. The formulations with lower concentrations of the API (1%) and PheroidÊ in the formula, which were in a carrier with a higher polarity than the skin, penetrated the skin best. **Conclusion:** It was possible to formulate sodium ascorbyl phosphate in a cream formulation, from which the API was effectively released and which penetrated the skin sufficiently.

1 Introduction

Photo-aging is one of the biggest skin problems facing people and especially women today. New and innovative ways of improving the appearance of skin are always on the horizon, but it is getting increasingly harder because of especially environmental factors such as prolonged exposure to harsh ultraviolet radiation (UVR) [1]. Prematurely aged skin caused by exposure to UVR is characterized by amongst others wrinkles, loss of skin tone and altered pigmentation. These symptoms are caused by the impact of UVR on the collagen in the connective tissue, causing the degradation of the collagen [2]. Collagen is very important in keeping the skin strong and resilient, playing a major role in keeping skin looking younger.

Vitamin C is a water-soluble compound, highly unstable and it is known for its ability to improve the overall skin tone, as well as for its collagen-forming properties [3]. Sodium ascorbyl phosphate is a derivative of vitamin C which has improved stability, because of the addition of a phosphate group in the second position of the cyclic ring, which protects it from oxidation [4]. Vitamin C is found in both the dermis and epidermis [5]. Lower levels of the

vitamin in the dermis indicate the use thereof for collagen regulation as well as elastin biosynthesis [6].

Transdermal delivery of active pharmaceutical ingredients (APIs) has become increasingly popular due to its various advantages. Some of these advantages include bypassing the hepatic metabolism and a reduction in the number of side effects [7]. Another advantage of this delivery system is the relatively large and accessible surface area which is available for absorption [8]. One of the main problems encountered with transdermal delivery is, however, the barrier function of the skin. It assists the body in keeping water and other vital substances inside the body and keeping harmful UVR and foreign bodies out [9]. The physicochemical properties of APIs can assist them in overcoming this barrier which the skin provides. For an API to be able to penetrate the skin effectively it should be relatively lipophilic, but it should also have a certain degree of aqueous solubility, in order to be formulated in an aqueous formulation [10]. Using a suitable carrier medium such as PheroidÊ technology could improve the lipid solubility of a highly water soluble API such as sodium ascorbyl phosphate [11]. The PheroidÊ consists of vesicular structures that contain no phospholipids or cholesterol, but are compiled of customized essential fatty acids. These fatty acids are similar to those found in the human body [11] and together with the nitric oxide, found in the PheroidÊ , comprise a vesicular structure which is able to transport both hydrophilic and hydrophobic drugs [11].

During this study, ten different creams were formulated. These included five PheroidÊ and five non-PheroidÊ creams at varying concentrations and polarities. Two placebo formulations were produced; one as a PheroidÊ and one as a non-PheroidÊ cream. Membrane diffusion studies were performed for a period of 6 h and Franz diffusion studies for 12 h for each of the formulations. Tape-stripping techniques were implemented to determine the epidermal, as well as dermal diffusion of the API.

2 Materials and methods

2.1 Materials

The API together with Cremophor® A6 and Cremophor® A25 were sourced from BASF Chem Trade GmbH (Burgbernheim, Germany). The following ingredients were obtained from Merck Chemicals (Wadeville, South Africa) and used in formulating the semisolid products namely, liquid paraffin, methyl paraben, propyl paraben, Tween-80, cetyl alcohol and propylene glycol. The water used throughout the experimental work, as well as in the formulations, was purified water obtained from a Milli-Q® water purification system (Milipore, Milford, USA). Potassium orthophosphate crystals and sodium hydroxide pearls were required in preparing the phosphate

buffered solution and received from Merck Laboratory Supplies (Midrand, South Africa). High performance liquid chromatography (HPLC) analytical grade methanol and phosphoric acid (85%), both obtained from Merck Laboratory Supplies (Midrand, South Africa) were used as mobile phase.

2.2 Sample analysis

2.2.1 HPLC analysis of the API for diffusion studies

A validated HPLC method was developed in conjunction with the personnel from the Analytical Technology Laboratory at the North-West University, Potchefstroom Campus, South Africa. This method was used in the analysis of the samples obtained from the skin diffusion studies as well as in the analysis of the samples obtained from the tape stripping experiments.

The instrumentation used included an Agilent® 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA). The instrument was equipped with an Agilent® 1200 pump, ultraviolet (UV) detector, autosampler injection mechanism and Chemstation Rev.A.06.02 software for data acquisition and analysis. A RESTEK Ultra C_{18} column, (250 x 4.6 mm) with a 5 µm particle size was used, during the analysis of the samples. The mobile phase used consisted of a mixture of 1 ml phosphoric acid (85%) and HPLC grade methanol (95:5) in 1000 ml HPLC grade water. The runtime was set at 4 min, with a flow rate of 1 ml/min and a 25 µl injection volume. The API was detected at 270 nm and eluted at 2.8 min. A constant temperature of 25 ºC was obtained throughout the environment in which the analyses were done.

2.3 Preparation of the API-containing semisolid formulations

Twelve creams were formulated in total, each containing sodium ascorbyl phosphate as the API. Three different creams containing 1%, 2% and 3% API, respectively, were formulated. Each one of these creams was formulated as both a PheroidÊ and non-PheroidÊ formulation. Furthermore, a cream containing 2% of the API and a lower concentration of liquid paraffin, as well as a cream containing 2% of the API and a higher concentration of the liquid paraffin were formulated. These creams were formulated as both a PheroidÊ and non-PheroidÊ formulation. Two placebo formulations (containing no API) were produced as well. In order to clearly distinguish between the formulations, a numbering system was implemented. The formulations were referred to as follow: 1% PheroidÊ cream **(1)**, 1% cream **(2)**, 2% PheroidÊ cream **(3)**, 2% cream **(4)**, 3% PheroidÊ cream **(5)**, 3% cream **(6)**, 2% PheroidÊ cream with more paraffin liquid in the formula **(7)**, 2% cream with more paraffin liquid in the formula **(8),** 2% PheroidÊ cream with less paraffin liquid in the formula **(9)** and 2% cream with less paraffin liquid in the formula **(10)**.

2.3.1 Composition of formulations

The ingredients which were used in the semisolid formulations each had a specific function. These ingredients included sodium ascorbyl phosphate (API), methyl and propyl paraben (preservatives), liquid paraffin (emollient), cetyl alcohol (thickening agent), Tween-80 (surfactant) and Cremophor® A6 and A25, both solubilizing agents. The propylene glycol was added as an emulsifier.

2.3.2 General method for preparation of a cream

Table 1: Composition of the various cream formulations **(1)-(12)**

The API was dissolved in 25% of the total amount of distilled water to be added to the formulation, whilst the remaining water was heated to a temperature of 80 °C. Liquid paraffin, Cremophor® A6, Cremophor® A25 and cetyl alcohol mixed together, formed phase A which was also heated to a temperature of 80 °C. The pre-heated distilled water was added to phase A whilst homogenizing the mixture at 13 500 rpm. After allowing this mixture to cool to an approximate temperature of 50 °C, the API (dissolved in distilled water) was added and the formulation was once again homogenized. Tween-80, propylene glycol, methyl and propyl paraben were mixed (phase B) and added to phase A. The emulsion was allowed to cool to room temperature of approximately 25 °C, whilst still being homogenized. The specific composition of each formulation is given in Table 1.

2.4 Franz cell diffusion experiments

2.4.1 Skin preparation for diffusion studies

Skin collected from female Caucasian patients, after having abdominoplastic surgery done, was used during this study. Ethical approval for obtaining and utilizing the donated skin was granted by the Research Ethics Committee of the North-West University under the reference number NWU-00114-11-A5 (Issue date: 2011-08-25 / Expiry date: 2016-08-24). Informed consent was obtained from the patients as well as the surgeon before obtaining the skin; and the patientsô anonymity was guaranteed. Within 24 h after the surgery had been done, the skin was frozen at a temperature of -20 ± 2 °C. Full thickness skin without stretch marks or other scarring was obtained using a Zimmer® Electric Dermatome and used during the experiments. Only the subcutaneous fat layer was removed during preparation and the skin had an approximate thickness of 200-400 µm. Hereafter the skin was placed on Whatman® filter paper with the stratum corneum facing upwards. The skin was cut into circles with a diameter of approximately

15 mm and covered with aluminum foil after which it was placed in a sealed plastic bag (Ziploc®) and stored at a temperature of approximately -20 °C. The skin samples were kept under these conditions until use and were thawed prior to commencement of the experiments.

2.4.2 Donor phase preparation for diffusion studies

The various cream formulations were used as the donor phases during the diffusion studies (see Section 2.3.3-2.3.6). An individual study comprising 12 diffusion cells was done for each of the ten formulations, as well as the two placebo creams. According to [5] vitamin C is found in both the epidermis and dermis of the skin, although the concentration of the vitamin in the epidermis is approximately five times that of the dermis. It was thus necessary to determine the amount of intrinsic vitamin C diffused when using the control samples in order to compensate for its effect on the diffusion data of the active products.

2.4.3 Receptor phase preparation for diffusion studies

Pure Milli-Q® water was used as the receptor phase during the diffusion studies. The pH of the water was determined at 5.5 and prior to use, the water was heated to a temperature of 37 °C.

2.4.4 Franz cell diffusion studies

Amber Franz diffusion cells with a receptor capacity of approximately 2 ml and a diffusion area of 1.075 cm^2 were used in the study. Amber cells were used in order to prevent the oxidation of the API-containing formulations. Ten diffusion studies were performed, one for each of the ten cream formulations and twelve diffusion cells were used per study. A complete diffusion study was done for each of the two placebo creams. The cells comprised a donor compartment and a receptor compartment. Full thickness skin samples were mounted between the donor and receptor compartments with the stratum corneum facing upwards. The cells were sealed using Dow Corning® vacuum grease to secure the donor and receptor compartments after which the complete cell was secured with a horseshoe clamp to prevent any form of leakage. A magnetic stirrer was inserted into the receptor compartment before full assembly of the cells in order to ensure homogeneity during the study. The donor compartment was filled with 1 ml of the API-containing formulations and the receptor compartment with 2 ml of the Milli-Q® water (temperature of 37 °C) after which the donor compartment was sealed using a piece of Parafilm® to prevent the evaporation of any compounds. Care was taken to ensure that no air bubbles were formed during the filling of the receptor compartment. In order to maintain a constant temperature mimicking the bodyôs own thermal regulation system, the complete Franz cells were placed in a water bath at a temperature of 37 °C. This would ensure that the surface of the skin remained at a constant temperature of approximately 32 °C. The entire content of the receptor

compartment was extracted after 12 h and placed in HPLC vials; and the API concentration in the sample determined through HPLC analysis.

2.4.4.1 Franz cell membrane diffusion experiments

The same method as described in Section 2.4.4 was used for the membrane diffusion studies. The only exception was the use of polytetrafluorethylene (PTFE) membranes, instead of full thickness skin. The extraction times differed as the aim of the membrane diffusion studies was to determine whether the API was release from each of the formulations. Extractions were made after 1, 2, 3, 4, 5 and 6 h. After each extraction the receptor compartment was immediately refilled with 2 ml pre-heated Milli-Q® water (32 °C). Analyses of the samples were performed using HPLC.

2.4.4.2 Franz cell skin diffusion experiments

The same method as described in Section 2.4.4 was used during skin diffusion studies. Initially, extractions were made hourly up until 12 h, but the generated data were not measurable because of the low API concentrations and subsequently only one extraction was made after 12 h. The samples were analyzed by means of HPLC.

2.4.5 Tape-stripping

Tape-stripping removes the entire epidermis, including the stratum corneum, according to [12]. It is an effective and simple way in which to analyze the efficacy of transdermal products [13]. After each diffusion study the skin used during the experiment was carefully removed from the Franz diffusion cell and the stratum corneum of each skin sample removed using 3M Scotch® MagicÊ tape. Tape-strips were cut into sections big enough to cover the diffusion area. The first tape-strip was discarded in each case as it could contain possible residue from the formulation on the skin surface. Each skin sample was tape-stripped fifteen times in order to remove the entire stratum corneum in the diffusion area. The glistening of the viable epidermis indicated the removal of the entire stratum corneum. The tape-strips were then placed into a poly-top filled with 5 ml of Milli-Q® water and kept at a temperature of 4 °C over a period of 8 h, after which the samples were analyzed through HPLC.

2.4.6 Statistical analysis

Statistical analysis was performed using SPSS (version 18) and the R statistical package (version 2.13); and was summarized in the form of averages, medians and standard deviations. Furthermore, graphical representation of the data was presented using box-plots and all statistical tests were performed at a 5% level of significance. As previously stated, there is a certain amount of vitamin C naturally found in the skin [5]. A t-test as well as a non-parametric Mann

Whitney U-test was done in order to determine whether the data from the experimental work should be corrected to compensate for the concentration of intrinsic vitamin C found in the skin. Two approaches were followed in the statistical analysis of the experimental data. The bootstrap method entailed the correction of the expected (average) concentration of each formulation with the expected concentrations of the respective placebo formulation. Only one average corrected value could thus be determined for each formulation and, hence, this unconventional method was employed in order to determine a 95% confidence interval for a test statistic that measured the difference between the PheroidÊ and non-PheroidÊ corrected values [14].

As previously stated a non-parametric Mann Whitney U-test and a standard t-test were done to again determine the difference between the corrected PheroidÊ and non-PheroidÊ concentrations. Average concentrations of both the PheroidÊ and non-PheroidÊ placebos were calculated and the experimental concentration of each formulation was then corrected by subtracting the respective placebo from the value. Relevant p-values were calculated and the smaller the p-value, the more significant the statistical difference [15] was.

3 Results and discussion

3.1 Franz cell diffusion experiments

3.1.1 Membrane diffusion studies

Table 2: Diffusion data of various formulations **(1)-(10)**

A membrane release study was performed for each of the ten cream formulations containing the API. The data for the membrane release study are given in Table 2.

When comparing the average % API released during the membrane release studies after 6 h, it was evident that **(10)** was the cream with the highest release rate. It was followed by **(1)** with a percentage of (1.940%), **(4)** (1.813%), **(8)** (1.677%), **(5)** (1.384%), **(2)** (1.259%), **(6)** (0.685%), **(7)** (0.683%), **(9)** (0.594%) and lastly **(3)** (0.278%).

3.1.1.1 The effect of Pheroid™ on API release

A comparison between the PheroidÊ and non-PheroidÊ formulations proved the non-PheroidÊ creams (**(2)**, **(4)**, **(6)**, **(8)**, **(10)**) to be more efficient in releasing the API from the formulation. This could be as a result of the hydrophilic nature of the creams (because of the lower oil concentrations in the formula) and the oil soluble unionized species of the APIs affinity for the less polar environment of the membrane [16].

3.1.1.2 The effect of the polarity of the carrier medium on API release

Small non-polar and relatively lipophilic APIs penetrate the skin best and highly polar compounds less [17]. The unionized (oil-soluble) species of the API were determined to be 99.37%, which indicated that the drug should thus be able to cross the skin. No significant difference was evident when comparing the formulations with higher polarities and the formulations with lower polarities. Both **(9)** and **(10)** had a higher polarity, because of a part of the liquid paraffin in the formula being substituted by water. The higher polarity of **(10)** helped improve the release of the API from the cream, because of the unionized species of the API being lipid-soluble and having a higher affinity for a less polar environment [18]. **(9)** was however, not improved by the higher polarity of its formulation as is evident in the fact that **(6)**, **(7)** and **(9)** showed only a slight difference in the percentage API released from the formulation.

3.1.1.3 The effect of concentration

Differing concentrations of the API also had an impact on the release of the API from the formulation. The lower the concentration of sodium ascorbyl phosphate in a formulation is, the better its stability will be [19]. Concentrations of lower than 20% (m/m) of the API in formulations proved to form more stable creams which could lead to improved release of the API. **(1)**, **(3)**, and **(4)** each showed a higher release rate when compared to the formulations with higher concentrations of the API such as **(5)** and **(6)**.

3.1.2 Skin diffusion studies

3.1.2.1 Determination of the intrinsic vitamin C that diffused by making use of placebo formulations

The data obtained during the diffusion studies are given in Table 2. Both of the placebo creams used in two individual diffusion studies proved to succeed in extracting a certain amount of the intrinsic vitamin C found in the skin [5]. In the case of the PheroidÊ placebo the average concentration diffused after a period of 12 h was 1.715 µg/cm^2 and for the non-PheroidÊ cream the average concentration diffused after the study was 1.867 µg/cm. This average placebo effect obtained during the two placebo diffusion studies was deducted from each of the API-containing formulations in order to obtain an actual percentage diffused value for each formulation.

3.1.2.2 The effect of Pheroid™ on transdermal diffusion

(2), **(4)** and **(6)** penetrated the skin most effectively, with the average concentration for **(4)** being only slightly higher than that of the other two formulations after 12 h. As with the membrane diffusion studies, the non-PheroidÊ formulations proved to have a higher diffusion rate than the PheroidÊ formulations. Bickers stated that the lipid-soluble unionized species of an API would

be more likely to have an affinity for a less polar surrounding, as in the case of the non-PheroidÊ creams where the API preferred the more lipophilic environment of the skin [16]. The API was thus released from the formulations and penetrated the skin, because of the difference in polarity between the skin and the formulations [18].

3.1.2.3 The effect of the polarity of the carrier medium transdermal diffusion

The average concentration values of API that diffused after 12 h for each of the formulations, ranged between 0.021 µg/cm^2 for **(7)** and 3.601 µg/cm^2 for **(4)**. Comparing the formulations with higher amounts of liquid paraffin (**(7)** and **(8)**) with the formulations with less liquid paraffin in the formula (**(9)** and **(10)**); the more polar creams proved to penetrate the skin more efficiently. Because of an increased amount of water in **(9)** and **(10)** the polarity of these formulations was higher than that of **(7)** and **(8)**. This increase in polarity could be the reason for the improved penetration of the API into the skin, as the API would have a higher affinity for the less polar surrounding of the stratum corneum and would thus partition from the formulation into the skin.

3.1.2.4 The effect of API concentration on transdermal diffusion

As with the membrane diffusion studies, the formulations with a lower API concentration in the formula proved to penetrate the skin better than those with higher amounts of the API [19]. This could be as a result of the increased stability of the formulation at lower concentrations of the API. According to [4] the increased stability of the formulations could be attributed to the chemical structure of the API. A phosphate group in the second position in the cyclic ring protected the enediol system from oxidation, making the API more stable than regular vitamin C [4].

3.1.3 Results obtained after tape-stripping of the skin

3.1.3.1 Stratum corneum-epidermis

3.1.3.2 The effect of Pheroid™ on transdermal delivery

Contrary to what was expected, the non-PheroidÊ formulations proved to penetrate the stratum corneum-epidermis more effectively on average, than the PheroidÊ formulations. As previously explained, this could be due to the 99.37% unionized species of the API which is more likely to be lipid-soluble and thus have a higher affinity for the lipophilic stratum corneum [18]. All of the tape-stripping data are given in Table 2.

3.1.3.3 The effect of the polarity of the carrier medium on transdermal delivery

The formulations with the higher polarities proved to penetrate the skin best as **(9)** was the formulation which penetrated the stratum corneum most effectively with an average

concentration of 0.457 µg/ml. It was followed by **(10)**, with an average concentration of 0.259 µg/ml. The higher polarity of these two formulas, because of the higher amount of water in the formulations, could be the reason for their observed penetration [18]. Formulations **(7)** and **(8)** were significantly less effective at penetrating the stratum corneum; **(7)** had an average concentration of 0.246 µg/ml and **(8)** a much lower average of 0.130 µg/ml.

3.1.3.4 The effect of concentration of API on transdermal delivery

When comparing **(1)** and **(2)** (both containing 1% API) with **(3)** and **(4)** (both containing 2% API) it was evident that the formulations with a lower concentration of the API in the cream penetrated the stratum corneum better. Formulation **(1)** had an average concentration of 0.217 µg/ml and **(2)** had an average of 0.407 µg/ml. The average concentration values for **(3)** and **(4)** were 0.127 µg/ml and 0.217 µg/ml, respectively. Increased stability could be due to the fact that the API (when formulated in formulations with concentrations lower than 20% m/m of the API) penetrated the skin more significantly [19].

3.1.4. Epidermis-dermis

3.1.4.1 The effect of Pheroid™ on transdermal delivery

Formulation (**2**) with an average concentration of 0.226 µg/ml was the only non-PheroidÊ formulation to penetrate the epidermis-dermis, whilst all of the PheroidÊ creams effectively penetrated this layer of skin. The formulation which released the highest average concentration of API found in the epidermis-dermis was **(1)** (0.656 µg/ml), followed by **(3)** (0.530 µg/ml). From this it was clear that the API was encapsulated by the PheroidÊ which improved its lipid solubility, causing it to penetrate the skin more effectively and enhancing its absorption [11].

3.1.4.2 The effect of the polarity of the carrier medium on transdermal delivery

The formula with the higher amount of liquid paraffin i.e., **(7)** (0.297 µg/ml) penetrated the skin only slightly more effectively than the formulation with the higher polarity i.e., **(9)** (0.231 µg/ml). This could possibly be because of the bigger the ñpolarity gapò between the stratum corneum and the formulation, the greater the driving force for the penetration of the API into the skin [18].

3.1.4.3 The effect of API concentration on transdermal delivery

As with the penetration through the skin and into the stratum corneum-epidermis, the lower the concentration of API in the formula, the better the penetration was [19].

3.2 Statistical analysis for diffusion studies

Figure 1: Box-plot representations of the concentration values ($\varepsilon g/cm^2$) after skin diffusion: **A)** before the correction of endogenous vitamin C (data are superimposed on the graph as filled black dots) and **B)** bootstrap corrected concentrations (*more liquid paraffin; **less liquid paraffin in the formulations)

Table 3: Statistical comparison between PheroidÊ and non-PheroidÊ formulations, using a standard t-test, Mann Whitney U-tests and 95% bootstrap confidence intervals

No significant difference was observed between the two placebo groups, which can clearly be seen in Figure 1 A) which is a box-plot representation of the data obtained from the bootstrap method. Using a more conventional statistical method, it was once again clear from the t-test as well as the Mann Whitney p-values (Table 3: Comparison of placebo formulations) that there was no significant difference between the PheroidÊ and non-PheroidÊ placebo formulations. The average placebo concentrations were, however, corrected in order to present corrected summary statistics of the experimental groups.

3.2.1 Conventional method

Summary statistics as well as the p-values, for the PheroidÊ versus non-PheroidÊ comparisons were obtained. These values are presented in Table 3. From the data it was clear that a statistical significant difference was observed between **(5)** and **(6)**. This was determined by the small p-value of 0.001 for the two formulations. The smaller the p-value of compared data, the bigger the significance in the difference between the data is [15]. When the differences between **(3)** and **(4)** ($p = 0.079$) as well as **(9)** and **(10)** ($p = 0.036$), respectively, were examined, no significant statistical difference was encountered at a 5% significance level. Determining the significance on a 10% level, however, a slight difference between both **(3)** and **(4)**; and **(9)** and **(10),** could be established. Experimentation with a larger number of cells per study should validate these findings. Formulations **(7)** and **(8)** also had a statistically significant difference (t-test: 0.017; Mann Whitney: 0.038) when using the conventional method, but no significant statistical difference was observed with the bootstrap method.

3.2.2 Bootstrap method

In Figure 1 B) the distributions of the corrected bootstrap averages for each of the formulations are given. As with the conventional method, a significant difference could be observed for the 3% creams (**(5)** and **(6)**). The statistical significance between the PheroidÊ and non-PheroidÊ

formulations was determined by making use of 95% bootstrap percentile confidence intervals. These calculated confidence intervals are given in Table 3. If the value zero (0) is included in the confidence interval, as in the case of **(1)** and **(2)** ([-2.89:1.12]), it indicates an insignificant statistical difference between the relating formulations (PheroidÊ and non-PheroidÊ). If zero is excluded from the confidence interval data, as in the case of formulations **(5)** and **(6) (**[-5.71:-2.16]) a significant difference exists between the two compared formulations. The confidence intervals are given to state the existence of a significant statistical difference, when using the bootstrap method, as p-values are given when using the more conventional statistical method.

As with the conventional method, **(3)**, **(4)**, **(9)** and **(10)** produced confidence intervals for which the upper bounds were close to zero. This suggested, as in the case of the conventional analysis, that a repetition of the study is required with larger numbers.

The bootstrap confidence interval for **(7)** and **(8)** was [-4.10:0.18] which included zero. This, contradictory to the data obtained with the conventional method, proved that there was no significant statistical difference between these two formulations and the findings could once again be correlated by doing a study consisting of a larger number of data points.

4 Conclusion

After the membrane release studies of the ten different formulations, formula **(10)** was determined to be the cream with the highest average percentage released after 6 h (2.008%). This formulation had a higher polarity because of the substitution of some of the liquid paraffin in its formula, with water. This higher polarity could have been the cause of the release of mainly unionized and oil-soluble APIs from the formulation. Lipid soluble APIs generally prefer a lipophilic environment such as the skin, or in this case the membrane [20] and this could be the reason for the API being released from the hydrophilic formulation. The cream with the second highest release rate was **(1)** with an average percentage diffused of 1.940% after 6 h. The overall average API concentrations found in the samples after the membrane release studies were much higher than the average concentrations found after the diffusion studies. A possible reason for this could be the less intricate structure of the membrane when compared to the skin [20].

Formulations **(2)**, **(4)** and **(6)** were the formulations which penetrated the skin most effectively during the skin diffusion studies. The average concentrations of these creams ranged between 3.761 and 3.344 $\mu g/cm^2$ with **(4)** being the cream with the highest average concentration after 12 h. All of the abovementioned formulations were non-PheroidÊ creams. A possible reason for the diffusion of the API could be that the unionized species of a drug are usually lipid-soluble

and can pass readily across the stratum corneum [20]. The unionized species of the API were determined to be 99.37%.

Considering the penetration of the API into the stratum corneum, the non-PheroidÊ creams proved to perform better. Due to the hydrophilic nature of these formulations, the unionized oil-soluble species of the API had a higher affinity for the lipid rich membrane of the stratum corneum [16]. Formulation **(9)** was the formulation with the highest API concentration in the stratum corneum-epidermis (0.457 µg/ml) followed by **(10)** with a concentration of 0.259 µg/ml. The only non-PheroidÊ formulation to penetrate the epidermis-dermis was **(2)** (0.226 µg/ml). Formulation **(1)** depicted the highest concentration API in the epidermis-dermis (0.656 µg/ml) compared to its closest competitor **(3)** (0.530 µg/ml). The formulations with lower API concentrations penetrated both the stratum corneum-epidermis and epidermis-dermis to a higher extent, when compared to the formulations with higher concentrations of API. This could be as a result of the improved stability of the formulations when the API was used in lower concentrations [19]. It was thus evident that the API could reach the target site (dermis), when formulated in low API concentrations in PheroidÊ formulations (less than 2% m/m).

Formulation **(1)** was the formulation which performed the best overall during the experiments. It had a low concentration (1%) of the API which was encapsulated by PheroidÊ , improving its penetration into the skin [11]. Once again it was evident that the lower API concentration was more effectively released from the formulations, as well as diffused through the skin, when compared to the other formulations [19]. Regarding the polarity of the formulation, it was relatively neutral when compared to the formulations with more or less liquid paraffin in the formula, but it still released the API effectively. This could have been, as previously stated, because the lipid-soluble unionized species of the API preferred a less polar surrounding such as the skin [18].

Conflicts of interest

All the experimental work was carried out with financial support from the National Research Foundation (NRF) and the Unit for Drug Research and Development of the North-West University, Potchefstroom Campus, South Africa. The authors wish to express their gratitude towards Dr. Gerhard Koekemoer for his assistance in the analysis of the statistical data. The authors have no declaration of interest to report.

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Tables

Table 1: Composition of the various cream formulations **(1)-(12)**

	Concentration % (m/m)											
	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**	**(8)**	**(9)**	**(10)**	**(11)**	**(12)**
Sodium ascorbyl phosphate	1	1	2	2	3	3	2	2	2	2	0	0
Pheroid™	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
A:												
Liquid paraffin	12	12	12	12	12	12	18	18	6	6	12	12
Cetyl alcohol	7	7	7	7	7	7	7	7	7	7	7	7
Cremophor® A6	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Cremophor® A25	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
B:												
Propylene glycol	8	8	8	8	8	8	8	8	8	8	8	8
Tween 80	4	4	4	4	4	4	4	4	4	4	4	4
Methyl paraben	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Propyl paraben	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C:												
H_2O to	100	100	100	100	100	100	100	100	100	100	100	100

Table 2: Diffusion data for the various formulations **(1)-(10)**

Formulations	Release studies		Diffusion studies				Tape-stripping		
	Ave. %released after 6 h (%)	**Ave. cumulative conc. after 6 h (µg/cm^2)**	**Ave. %diffused after 12 h**	**Ave. conc. after 12h [(µg/cm^2)]**	**Corrected ave. conc. after 12h (µg/cm^2)**	**n**	**Ave. conc. stratum corneum-epidermis (µg/ml)**	**n**	**Conc. epidermis-dermis (µg/ml)**
1% PheroidÊ cream **(1)**	1.940	360.696	0.026	4.772	3.057	3	0.217	10	0.656
1% cream **(2)**	1.259	234.157	0.029	5.422	3.555	7	0.407	10	0.226
2% PheroidÊ cream **(3)**	0.278	103.250	0.012	4.486	2.771	12	0.127	12	0.530
2% cream **(4)**	1.813	674.067	0.015	5.628	3.761	11	0.217	11	0
3% PheroidÊ cream **(5)**	1.384	772.185	0.007	2.511	0.796	11	0.115	6	0.220
3% cream **(6)**	0.685	382.192	0.014	5.211	3.344	11	0.247	11	0
2% PheroidÊ cream with more liquid paraffin **(7)**	0.683	253.922	0.005	1.895	0.108	8	0.247	4	0.297
2% cream with more liquid paraffin **(8)**	1.677	623.818	0.008	2.920	1.053	12	0.130	12	0
2% PheroidÊ cream with less liquid paraffin **(9)**	0.594	220.814	0.009	3.208	1.493	10	0.457	6	0.231
2% cream with less liquid paraffin **(10)**	2.008	746.738	0.012	4.432	2.565	12	0.259	12	0

Table 3: : Statistical comparison between PheroidÊ and non-PheroidÊ formulations, using a standard t-test, Mann Whitney U-tests and 95% bootstrap confidence intervals

	t-test	Mann-Whitney	95% Bootstrap Confidence Intervals
1% (1 & 2)	0.349	0.580	[-2.89:1.12]
2% (3 & 4)	0.079	0.097	[-3.61:0.49]
3% (5 & 6)	0.001	0.000	[-5.71:-2.16]
2% more liquid paraffin (7 & 8)	0.017	0.038	[-4.10:0.18]
2% less liquid paraffin (9 & 10)	0.036	0.065	[-4.06:0.22]
Placebo (11 & 12)	0.703	0.806	NA

Figure legends

Figure 1: Box-plot representations of the concentration values (εg/cm^2) after skin diffusion: **A)** before the correction of endogenous vitamin C (data are superimposed on the graph as filled black dots) and **B)** bootstrap corrected concentrations (*more liquid paraffin; **less liquid paraffin in the formulations)

Figures

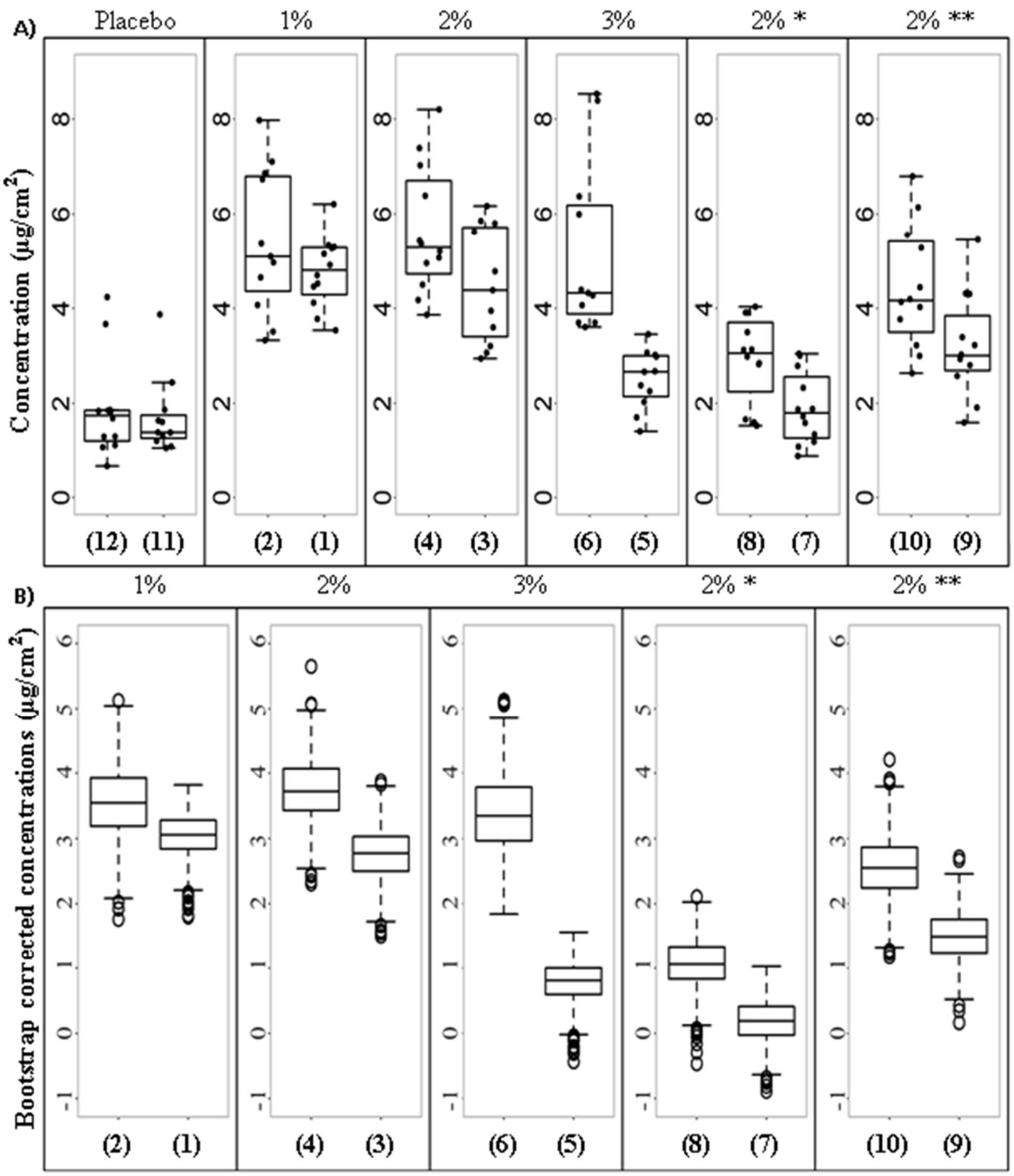

Figure 1: Box-plot representations of the concentration values (ε g/cm^2) after skin diffusion: **A)** before the correction of endogenous vitamin C (data are superimposed on the graph as filled black dots) and **B)** bootstrap corrected concentrations (*more liquid paraffin; **less liquid paraffin in the formulations)

CHAPTER 4

FINAL CONCLUSIONS AND FUTURE PROSPECTS

Sodium ascorbyl phosphate, used in topical formulations, is a new approach to the age old question of how to assist photo-damaged skin in the recovery process (Mitsui, 1997:150). Sodium ascorbyl phosphate is a water-soluble and more stable salt of vitamin C and is very important in the formation of collagen in the skin, protection against UV light and healing of scar tissue (Thiele *et al.,* 2000:146).

The aim of this study was to determine the extent of topical delivery of sodium ascorbyl phosphate, from different topical formulations, for the treatment of photo-aged skin. The objectives of this study thus included the following:

1. Development and validation of a HPLC method for the determination of the concentrations of the API in the formulations.
2. Determination of both the aqueous solubility and partition coefficient of the API.
3. Formulation of various creams, including Pheroid$^{\hat{E}}$ and non-Pheroid$^{\hat{E}}$ formulations at five different concentrations and polarities containing the API.
1. Formulation of two placebo creams (Pheroid$^{\hat{E}}$ and non-Pheroid$^{\hat{E}}$) to determine the amount of endogenous vitamin C in the skin.
2. Determining the release of the API from the various formulations by making use of membrane release studies.
3. Determining if the API did diffuse through the skin after the application of different formulations.
4. Determining whether the API researched the target site (dermis) and have diffused into the skin after the different formulations were applied.

A validated HPLC method was developed, in conjunction with the personnel from the Analytical Technology Laboratory of the North West Universityõs Potchefstroom Campus. This method was used in the analysis of all of the experimental data.

The physicochemical characteristics of the API determined during the study included the partition coefficient, as well as its aqueous solubility. The aqueous solubility of the API was

found to be 6.14 mg/ml and the log P was -0.005. An aqueous solubility of 1 mg/ml or more is needed in order for the API to permeate the skin according to Naik *et al.* (2000:319). With a value of 6.14 mg/ml for aqueous solubility, the sodium ascorbyl phosphate should be able to penetrate the skin with relative ease. A partition coefficient of -0.005 indicated that the API was soluble in neither oil nor water and should struggle to penetrate the skin effectively (Williams, 2003:35).

The creams formulated included creams with 1% API in the formula, a cream with a 2% concentration of the API and creams with a 3% concentration of the API in the formula. Each of these creams were formulated as both a Pheroid$^{\hat{E}}$ and non-Pheroid$^{\hat{E}}$ cream. Furthermore, a 2% cream with less of the paraffin liquid in the formula, and a 2% cream with more liquid paraffin in the formula was formulated. Once again, each of these creams were formulated as both a Pheroid$^{\hat{E}}$, and a non-Pheroid$^{\hat{E}}$ formulation.

Two placebo creams were also formulated in order to determine with individual diffusion studies, the effect of the natural vitamin C found in the skin (Shindo *et al.,* 1994:123), on the diffusion data. The average vitamin C concentration found in the PheroidÊ placeboôs sample after diffusion was 1.715 µg/cm^2 and for the non-PheroidÊ formulations was 1.867 µg/cm^2. This indicated that the placebo formulations were able to push some of the natural occurring vitamin C in the skin through into the receptor cell solution.

Diffusion studies (where polytetrafluoroethylene membranes were used) were done in order to determine if the API was released from the formulation. Membrane release studies determined that the API was indeed released from all of the abovementioned API formulations. The formulation with the highest API concentration released was the 2% non-Pheroid$^{\hat{E}}$ cream with less paraffin liquid in the formula (2.008%). The 1% PheroidÊ formulation was the cream with the 2nd highest release rate, at 1.940% released after 6 h. The high release rate of the 2% non-PheroidÊ cream can possibly be attributed to the fact that artificial membranes are not as complex as human skin, thus making penetration easier (Barry, 2002:518). All of the formulations released the API, with the lowest percentage released equal to 0.278%. Contrary to what was expected, the higher polarity of the cream did not prevent the API from releasing. The lower liquid paraffin concentration contributed to the formulation having a higher polarity (Mitsui, 1997:343). The API being water soluble was thought to prefer the highly polarised cream environment, as opposed to the membrane surface. With an unionised species of 99.37%, however, it was expected that there would be a certain degree of release (Barrry, 2002:511). The

API also had small molecules, which would improve its penetration across artificial, as well as biological membranes, as smaller molecules penetrate easier than larger molecules (Barry, 2002:513).

The formulation with the highest average concentration, after 12 h was the 2% non-PheroidÊ formulation (3.761 µg/cm^2), followed by the 1% non-PheroidÊ formulation (3.555 µg/cm^2). Formulations with lower concentrations of the API seemed to perform better than the formulations with higher amounts of the active, as they showed higher concentrations of API penetrating the skin. This could be because of the increased stability of the formulations with lower concentrations of the API. The high diffusion rates of the formulations could be attributed to the 99.37% unionised species of the API. According to Barry (2002:511) the unionised species of an API is usually lipid soluble and can pass readily across the stratum corneum.

Tape stripping was performed to determine the amount of vitamin C in the stratum corneum-epidermis, as well as the epidermis-dermis. The 2% Pheroid^{Ê} formulation with less paraffin liquid, showed the highest API concentration in the stratum corneum-epidermis (0.457 µg/ml). The formulation with the 2nd highest concentration of active in the stratum corneum-epidermis was the 1% non-PheroidÊ formulation (0.407 µg/ml). The formulation with less paraffin liquid in the formula had a higher polarity because of the higher amount of water in the formulation (Mitsui, 1997:343). This could have led to the largely unionised APIøs penetration into the stratum corneum-epidermis as this oil soluble species of the API has an affinity for the lipid rich membrane (Bickers, 2010:22).

The highest concentration of vitamin C in the epidermis-dermis was found after analysis of the skin samples used in the 1% PheroidÊ formulationøs diffusion study. The average value obtained by this formulation was 0.656 µg/ml. This could be because of the API being encapsulated in the PheroidÊ , allowing it to better penetrate the lipid bilayer of the skin (Grobler *et al.* 2008:297).

The 1% Pheroid formulation proved to perform the best overall in the experiments. It was the formulation which penetrated the epidermis-dermis (target site) the best, with an average concentration yield of 0.656 µg/ml and was the formulation with the second highest average percentage diffused (1.940%) after 6 h of membrane release studies.

The following can thus be concluded from this study:

1. The formulations with lower concentrations of the API proved to perform significantly better than the formulations with higher amounts of the API in the formula,
2. Creams with higher polarities released the API better during the membrane release studies, as well as during the diffusion studies,
3. Non-PheroidÊ formulations released higher amounts of the API during the experiments,
4. The 1% PheroidÊ cream was the formulation which performed the best overall during the experimental work.

Future prospects for this study include:

1. Investigation of the reduced efficacy of the Pheroid^{Ê} formulations,
2. The reasons for the lowered stability with higher concentrations of the API in formulations,
3. Furthered research on the effect of different polarities on the release of sodium ascorbyl phosphate from formulations.

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APPENDIX A

VALIDATION OF THE HPLC ANALYTICAL METHOD FOR TRANSDERMAL ANALYSIS

A.1 Purpose of the validation

The aim of the validation process is to ensure that the analytical method used was both reliable and sensitive in the determination of the amount of API found in the transdermal samples. The active ingredient was sodium ascorbyl phosphate.

A.2 Chromatographic conditions

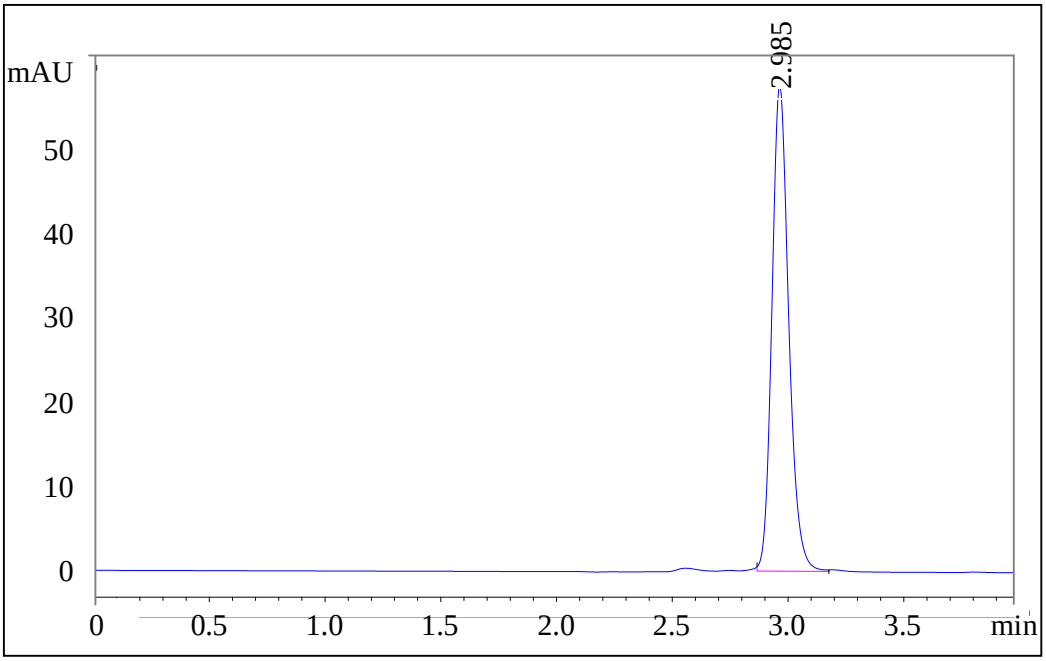

Figure A.1: Chromatogram of sodium ascorbyl phosphate

Analytical instrument: An Agilent® 1200 Series HPLC system was used for the analysis (Agilent Technologies, Palo Alto, CA). The instrument is designed with an Agilent® 1200 pump, diode array detector, autosampler injection mechanism and Chemstation Rev. A.06.02 software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25 °C.

Column:	RESTEK Ultra C_{18} column, (250 x 4.6 mm) with a 5 µm particle size was used.
Mobile phase	A mixture of 1 ml phosphoric acid (85%) in 1000 ml of HPLC water:methanol (95:5) was used as mobile phase.
Solvent:	HPLC water was used, in both the preparation of the standard solutions and the preparation of samples.
Flow rate:	1.0 ml/min
Injection volume:	25 µl
Retention time:	Sodium ascorbyl phosphate eluted at 2.8 min.
Run time:	4 min

A.3 Preparation of standard and samples

A.3.1 Standard preparation

Sodium ascorbyl phosphate (5 mg) was weighed in order to obtain a standard solution of 100% and dissolved. It was made up to volume with HPLC water in a 100 ml volumetric flask. The three different concentrations of this standard solution used for the determination of the accuracy were:

- 0.4 µg/ml
- 4.0 µg/ml
- 20.0 µg/ml

A.3.2 Sample preparation

Sodium ascorbyl phosphate (5 mg) was weighed in a 100 ml volumetric flask and made up to volume with HPLC water, in order to obtain a 100% sample.

A.4 Validation parameters

A.4.1 Linearity

The linearity of an analytical method is defined as its ability to obtain results that are directly proportional to the concentration of analyte in the sample. Linear regression analysis was performed by injecting five different concentrations of the API into the HPLC. A 100% standard

solution was prepared and dilutions were made in order to obtain concentrations between 0.51 µg/ml and 51.00 µg/ml. The different concentrations of the samples were injected in duplicate, into the HPLC.

A.4.1.1 Linear regression analysis

The linearity of the active ingredient was determined by performing linear regression analysis on the plot of the peak area ratios, versus concentration (µg/ml). The data can best be described by making use of a linear equation (Equation A.1):

$$y = mx + c \qquad \textbf{Equation A.1}$$

Where:

y = peak area ratios of the different APIôs
m = slope
x = concentration of the different active ingredients in µg/ml
c = y-intercept

A.4.1.1.1 Sodium ascorbyl phosphate

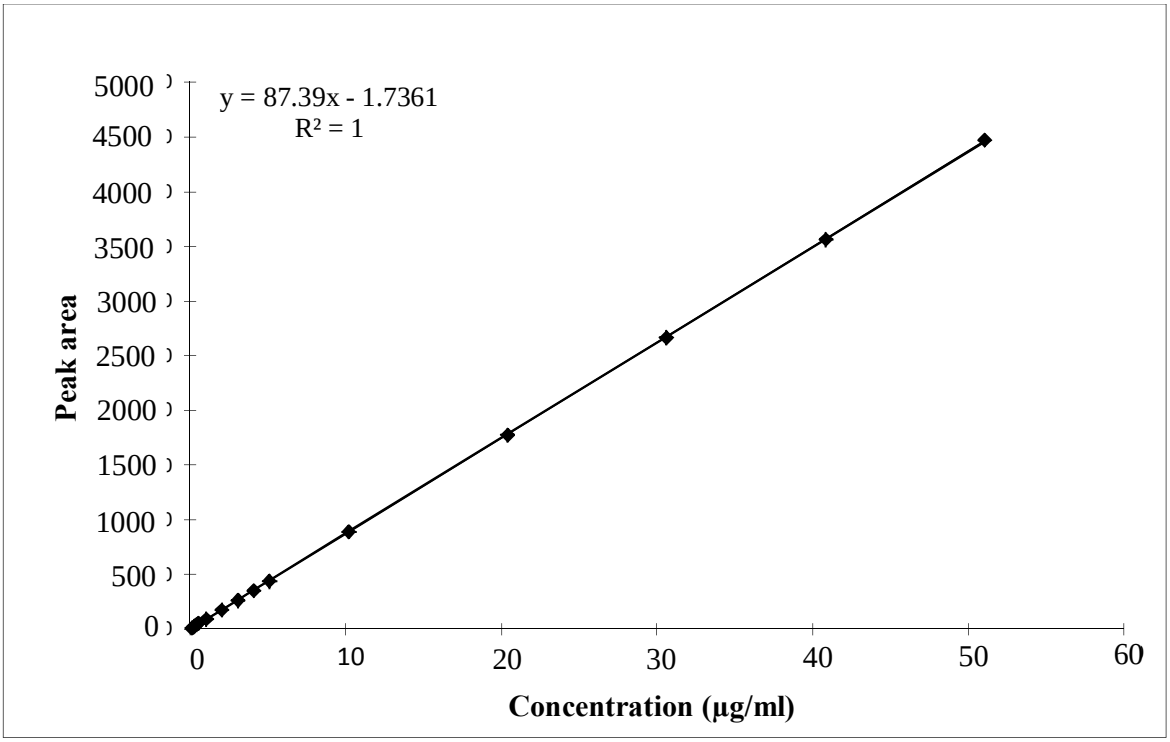

Figure A.2: Linear regression curve of sodium ascorbyl phosphate

According to the International Conference on Harmonisation (ICH, 2005:8) the linearity of analytical methods, is the ability of the specific methods (within a specified range) to obtain test results directly proportional to the concentration of the analyte in the sample solution. It is important that the linearity be determined across the entire range of the analytical procedure. A regression coefficient (r^2) of Ó0.99, obtained from the data, proves linearity.

Table A.1: Peak area values of sodium ascorbyl phosphate

Standard (µg/ml)	Peak area (mAU*s)
0.1	10.3
0.2	20.8
0.3	30.8
0.4	40.1
0.5	50.7
1.0	87.9
2.0	175.4
3.1	262.3
4.1	349.5
5.1	436.2
10.2	886.5
20.4	1773.7
30.6	2662.5
40.8	3559.0
51.0	4469.7
Slope	87.39
y-intercept	-1.73
r^2	1

A regression coefficient of 1 obtained from the data in this study showed a high degree of linearity (r^2 Ó0.99) and thus demonstrated the stability of the analysis system.

A.4.2 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value, or an accepted reference and the value found (USP, 2011:1225). The acceptance criteria for accuracy as stated by the ICH (2005:9) should cover the entire range of the analytical procedure. A minimum of three concentrations across this range should be used in determining the accuracy of the procedure. Typically, a

concentration close to the quantisation limit, one closer to the middle of the range and a concentration at the highest end of the range should be used. The recovery value should be between 98% and 102%. Concentrations used during this study comprised a range from 80-120%.

A 100% standard solution was prepared as described in Section A.3.1. The stock solution was diluted by taking 5 ml and making it up to 50 ml in a volumetric flask. A further 5 ml, from the diluted solution, was taken and once again filled up to 50 ml in order to dilute it even further. An injection volume of 20 µl of both the 0.5 and 5.0 µg/ml was injected, respectively into the HPLC, as was 10 µl of the 50 µg/ml sample. The afore-mentioned samples were all injected in triplicate. The three different concentrations used for the determination of the accuracy were:

- 0.4 µg/ml (80%)
- 4.0 µg/ml (100%)
- 20.0 µg/ml (120%)

A.4.2.1 Accuracy analysis

A.4.2.1.1 Sodium ascorbyl phosphate

Table A.2: Accuracy parameters of sodium ascorbyl phosphate

Concentration spiked(µg/ml)	Peak area 1	Peak area 2	Mean	Recovery (µg/ml)	%
0.4	34.22	33.91	34.07	0.41	102.42
0.4	33.58	33.08	33.43	0.40	100.60
0.4	34.12	33.70	34.91	0.41	101.97
4.0	356.69	355.27	356.98	4.09	99.84
4.0	353.66	358.95	356.31	4.10	99.93
4.0	356.60	355.92	356.26	4.10	99.92
20.0	1786.67	1765.20	1788.94	20.34	99.72
20.0	1767.12	1776.55	1787.84	20.29	99.49
20.0	1764.20	1778.72	1784.46	20.29	99.46
				Mean	100.4
				SD*	1.0
				%RSD**	1.0

* SD refers to standard deviation
** %RSD refers to relative standard deviation

The percentage sodium ascorbyl phosphate recovered ranged between 99.5% and 102.4%. The average recovery was found to be 100.3%. This indicated the accuracy of the procedure as the percentage recovered, fell in the pre-determined range of 80-120% and the preferred recovery rate of 98-102%.

A.4.3 Ruggedness

A.4.3.1 System repeatability

In order to evaluate the repeatability of the peak areas and retention times of the API, a 100% sample was injected six times. It was done on the same day and under the same conditions. Repeatability pertains to the precision of a method, under the same operating procedures over a short period of time (USP, 2011:1225). The peak area and retention times should have a relative standard deviation (RSD) of 2% or less in order to comply with the standards as set by the ICH (2005:10).

A.4.3.1.1 Sodium ascorbyl phosphate

Table A.3: Variations in response (%RSD) of the detection system regarding peak area and retention time of sodium ascorbyl phosphate

Injection	Peak area (mAU*s)	Retention time (min)
1	438.57	2.85
2	415.45	2.99
3	405.43	2.93
4	401.19	2.91
5	400.08	2.94
6	399.70	2.96
Mean	410.00	2.93
SD*	13.84	0.04
%RSD**	3.37	1.48

* SD refers to standard deviation

** %RSD refers to relative standard deviation

The RSD percentage for the peak areas and retention times should be 2% or less. The RSD value of the retention time fell in this category (1.48%), but the peak area value was above the 2% limit, which led to problems with the repeatability of the experiment.

A.4.3.2 System stability

The stability of the system, over a set period of time, is determined by injecting a 100% (10 mg/100 ml) solution under the same chromatographic conditions as previously stated. The experiment was done over a period of 48 h with injections made at: 0, 1, 6, 12, 24, 36 and 48 h.

A.4.3.2.1 Sodium ascorbyl phosphate

Table A.4: Percentage sodium ascorbyl phosphate in solution at each time interval

Time (h)	Peak area (mAU*s)	%
1	5543.78	100.00
2	5537.59	99.89
3	5532.74	99.80
4	5533.78	99.82
5	5529.15	99.74
6	5519.60	99.56
7	5514.36	99.47
Mean	5530.14	99.71
SD*	9.43	0.17
%RSD**	0.17	0.17

* SD refers to standard deviation

** %RSD refers to relative standard deviation

Thus it was clear, from the above data that sodium ascorbyl phosphate was stable over 24 h, in an aqueous solution. The degradation of the solution was less than 2% indicating that the solution was stable over the 24 h period.

A.5 Conclusion

The HPLC method was found to be both reliable and sensitive enough for the determination of the API concentration (sodium ascorbyl phosphate) in aqueous solutions.

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ICH ***see*** International Conference of Harmonisation

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APPENDIX B

FORMULATION OF A COSMECEUTICAL CREAM WITH SODIUM ASCORBYL PHOSPHATE

B.1 Introduction

The word cosmeceutical is generally applied to products that claim to accomplish one or more of the following:

- improve skin tone;
- give more even skin texture;
- increase skin radiance;
- decrease the appearance of skin wrinkling and/or
- enhance anti-aging benefits (Draelos, 2008:628).

The actual definition of a cosmeceutical, as stated by Mitsui (1997:3) is any article of which the intended use comprises the cleaning, beautifying, altering of the appearance of the human body or maintaining the health of skin and hair, provided that the action is not invasive or harsh.

A drug, as defined by the United States Food and Drug Administration (FDA), is any product which can prevent, treat, cure or mitigate the structure or function of the human body (Milikan, 2001:371). One of the major differences between a pharmaceutical drug and a cosmeceutical is the need for the determination of the safety of a pharmaceutical drug. Safety studies for drugs include pharmacokinetics, toxicology, pharmacology, drug-drug interaction, etc. A cosmetic on the other hand, must be safe for intended use, but no further safety regulation is stated by the FDA (Newburger, 2009:449).

In this study various semi-solid formulations containing sodium ascorbyl phosphate were formulated, as well as placebo formulations. The ten sodium ascorbyl phosphate formulations included 5 creams without PheroidÊ and 5 creams with PheroidÊ . Both PheroidÊ and non-PheroidÊ formulations were formulated precisely the same with the exception that the PheroidÊ cream contained PheroidÊ ingredients. The 5 creams consisted of the following formulations: creams with 1% API, 2% API and 3% API in the formulations, as well as 2% creams with more paraffin liquid and 2% creams with less paraffin liquid.

B.2 Development of a product for cosmeceutical use

Before starting with the formulation of a cosmeceutical product, thorough research must be done. Some of the aspects to be investigated include design, manufacturing and marketing. Basic quality requirements pertain to safety, stability, usability and efficacy (Mitsui, 1997:8). It is helpful to follow a type of formulation checklist, as proposed by Barry (2002:532). This ensures the design of a satisfactory formulation and can help to keep the development program on track.

First and foremost is the determination of the disease or condition to be treated. Secondly the site of drug action (skin surface, stratum corneum, viable epidermis, dermis, appendages or systemic circulation) and the receptor site within the target area should be noted. Barry (2002:532) further advises choosing the best drug or pro-drug for the condition to be treated, evaluating the optimal kinetics for the delivery of the specific API and deciding on the formulation to be manufactured. After deciding on the use of penetration enhancers or suitable vehicle ingredients for the formulation, *in vitro*, as well as *in vivo* studies are recommended, whilst bearing in mind the physiochemical behaviour and the stability of the formulation.

B.2.1 Formulation of cosmeceutical products

The development and formulation of a cosmeceutical product is an intrinsic and costly process. Two of the most important principles in the formulation of a cosmetic product are the use of quality ingredients and the performance of proper tests, involving quality and safety (Trefny, 2009).

B.2.2 Pre-formulation

Some of the most important aspects of pre-formulation are the in-depth research to be done on the physical, as well as the chemical properties of a compound. It is important to look at the stability of the API; the stability of the additives; and a suitable pH range for all of the compounds, to ensure maximum use of the cosmeceutical over an extended period of time (Barry, 2002:531). Loss of volatiles, including water, the rheological properties and particle size distribution are also of importance. During the pre-formulation of the cosmeceutical creams, immense care was taken to ensure the stability of the product over a set period of time, because of the stability issues faced, when working with vitamin C. Various pilot studies on the stability of the creams were done and different formulations tried, in order to access the potential problems with each of the formulas. The final formula was decided on after rigorous testing of the pH range (initially between pH 5.0 and 7.4) and the type and quantity of additives in the

formula. The final pH value, at which the experiments were conducted, was determined to be pH 5.5.

B.2.3 Early formulation

Various formulations were tried and tested, changing basic formulas as needed, until the final formulation was decided on. The final formulation was the formula which was most esthetical in nature and did not discolour as quickly as the other formulations.

B.2.4 Final formulation

During the final formulation stage, the thoroughly researched, previously manufactured and modified formula was used to formulate master batches of each of the specified formulations. These bulk formulations were then stored, at room temperature (approximately 25 °C), in amber glass sample jars, for the duration of the experiment.

B.2.5 Preservation of cosmeceutical products

Preservatives are essential for the ensured longevity of cosmeceutical products. They are added in order to prevent the contamination and subsequent degradation of products by suppressing the proliferation of microorganisms (Mitsui, 1997:201). It is important to remember that cosmeceutical products must be especially well equipped to deal with contamination factors, as the esthetic appeal of cosmetics is highly valued. Bacterial, as well as fungal and yeast contamination are common. As concluded by Barry (2002:532) there are many potential sources for contamination of cosmeceutical products. Contamination can take place when working with the raw materials, when manufacturing the water needed for the formulation or even when poor hygiene forms part of the processing and manufacturing environment. When choosing a preservative, it is important to keep, the stability of the additive when exposed to heat, in mind. It must be suitable for prolonged periods of storage and of course be non-toxic and non-sensitising (Barry, 2002:532). Anti-oxidants are also needed in some cases. They tend to stop the oxidation of compounds, once exposed to oxygen. The perfect anti-oxidant should comprise the following traits:

- It should be non-volatile.
- It must be stable and effective over a range of pH values.
- It should be effective, even in small quantities.

- ❖ It should only act as an anti-oxidant, and have no further chemical reaction with any of the other components.
- ❖ Its decomposition products must be non-toxic, colourless, odourless and non-sensitising (Barry, 2002:532).

B.3 Formulation of a cream

B.3.1 Purpose and function of a cream

A cream can be seen as a type of emulsion in which two liquids (that do not mix well, i.e. oil and water) are combined. The dispersed phase is thoroughly dissolved in the dispersion medium, making it into a stable semi-solid. Mitsui (1997:342) stated that the main function of a cream is to maintain moisture balance, whilst also keeping the skin supple through the steady supply of humectants, oils and water. Other uses for cream formulations vary between cleansing of the skin, make-up removal and stimulation of the circulation. Creams are generally classified according to their formulation and ingredients. Furthermore, it is possible to increase or decrease the amount of certain ingredients, such as the water phase or the humectants, in order to vary the consistency of the cream for different uses, skin types, environment, etc. (Mitsui, 1997:343).

B.3.2 Main ingredients of a cream

According to Mitsui (1997:342) the percentage of each of the main ingredients used when formulating a cosmetic cream, depends on whether the formulation is an (o/w) or (w/o) formula. Most cosmetic formulations will however make use of the following main ingredients in varying amounts:

- ❖ Oil phase or oily ingredients,
- ❖ aqueous phase,
- ❖ surfactants,
- ❖ preservatives,
- ❖ chelating agents,
- ❖ perfumes; and of course,
- ❖ APIôs.

Oil phase ingredients can include the following: hydrocarbons (liquid paraffin), fats and oils (olive oil), waxes (bees wax), fatty acids (stearic acid), higher alcohols (stearyl alcohol) and synthetic esters (cholesteryl ester). The water phase can consist of the following: humectants such as glycerine, thickening agents for example xanthan gum, alcohols such as ethanol and purified water. Some of the surfactants or emulsifiers comprise the following: non-ionic surfactants such as glycerine monostearate and anionic (fatty acid soaps) surfactants. APIôs include various vitamins, such as sodium ascorbyl phosphate, amino acids, whitening agents etc. Chelating agents used include ethylenediaminetetraacetic acid (EDTA) (Mitsui, 1997:344).

B.3.3 General method for manufacturing a cream

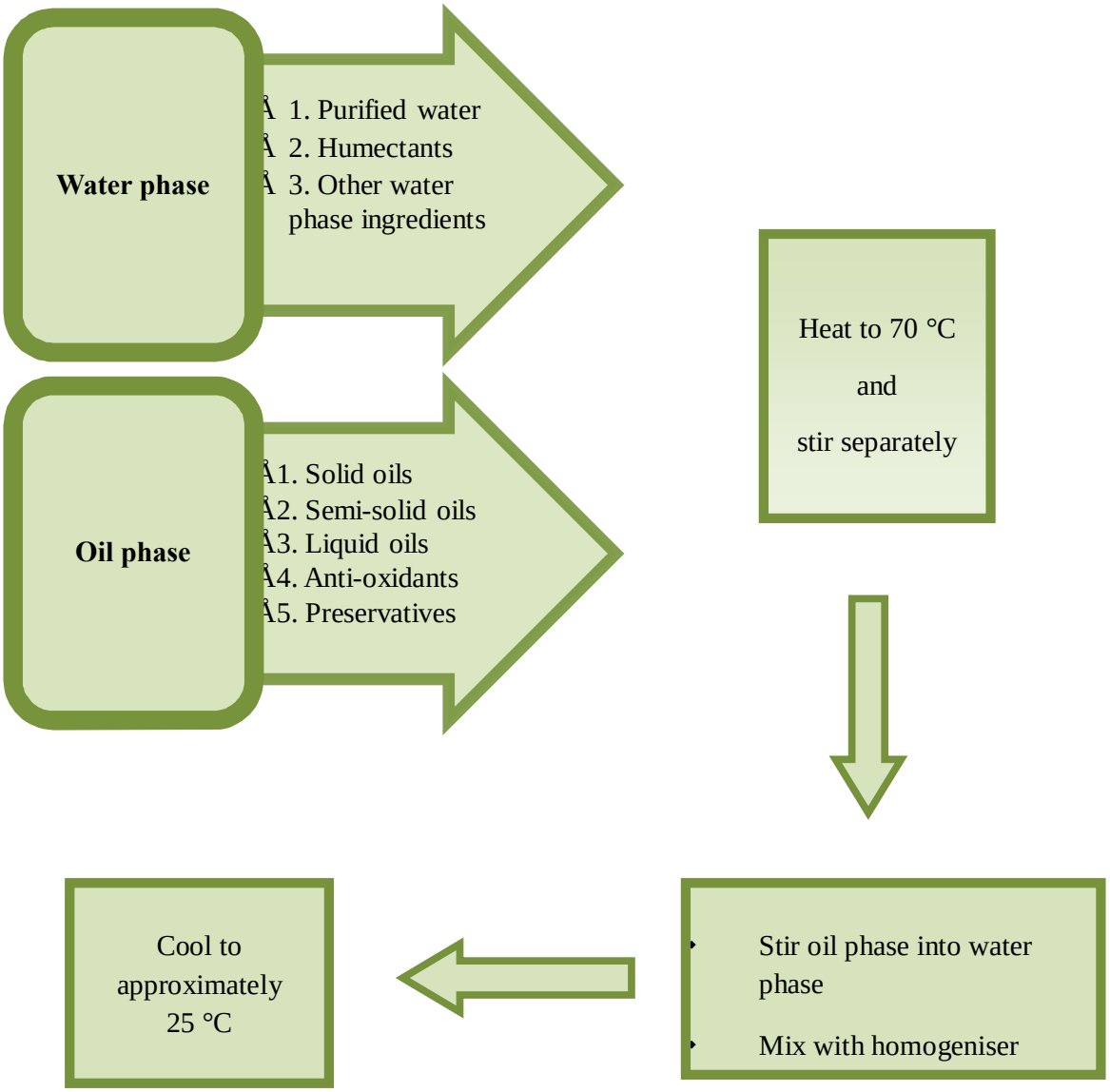

Figure B.1: The general method for manufacturing of a cosmeceutical cream

A basic method for the manufacturing of a cosmeceutical cream, as given by Mitsui (1997:343) is illustrated in Figure B.1. The water, as well as the oil phase ingredients, are mixed and heated separately. Only after being heated to approximately 70 °C, does one stir the oil phase into the water phase. The two phases are then thoroughly mixed by making use of an emulsifying apparatus, such as a homogeniser, and cooled to room temperature (25 °C).

B.4 Formulation of a cream and Pheroid™ cream containing sodium ascorbyl phosphate

Several cream formulations of sodium ascorbyl phosphate were prepared during the study. Some of these creams contained PheroidÊ as part of the formulation, whilst the polarity of other creams was altered by adding or reducing the amount of the oil phase. The final formulations to be used were decided on after comparing the homogeneity, stability over a period of 24 h and overall appearance of the various formulas. The specific ingredients, along with their batch numbers and suppliers are given in Table B.1.

Table B.1: Ingredients used in the selected formulations

Ingredient	Supplier	Batch number
Tween 80	Merck Chemicals	1035460
Liquid paraffin	Merck Chemicals	1034378
Cetyl alcohol	Merck Chemicals	S5513404 018
Cremophor® A 6	BASF Chem Trade GmbH	51635129
Cremophor® A 25	BASF Chem Trade GmbH	12903975LO
Propylene glycol	Merck Chemicals	S5433688 018
Methyl paraben	Merck Chemicals	GBGA0001371
Propyl paraben	Merck Chemicals	GBGA032949
Sodium ascorbyl phosphate	BASF Chem Trade GmbH	S30052

B.4.1 Formula of the sodium ascorbyl phosphate cream

The formulations prepared, included the following:

- Formulations with 1% API;
- Formulations with 2% API;
- Formulations with 3% API;
- Formulations with 2% API and less paraffin liquid in the formula and
- Formulations containing 2% API with more paraffin liquid in the formula.

The exact ingredients used in preparing each formulation are given in Table B.2.

Table B.2: Ingredients of the various formulations (**(1)-(12)**)

	Concentration % (m/m)											
	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**	**(8)**	**(9)**	**(10)**	**(11)**	**(12)**
Sodium ascorbyl phosphate	1	1	2	2	3	3	2	2	2	2	0	0
Pheroid™	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
A:												
Liquid paraffin	12	12	12	12	12	12	18	18	6	6	12	12
Cetyl alcohol	7	7	7	7	7	7	7	7	7	7	7	7
Cremophor® A6	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Cremophor® A25	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
B:												
Propylene glycol	8	8	8	8	8	8	8	8	8	8	8	8
Tween 80	4	4	4	4	4	4	4	4	4	4	4	4
Methyl paraben	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Propyl paraben	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C:												
H_2O to	100	100	100	100	100	100	100	100	100	100	100	100

All of the above formulations, were formulated in both a PheroidÊ and non-PheroidÊ cream. As previously mentioned in some of the formulations, the amount of the oil phase (liquid

paraffin) was altered and was named 2% cream with more paraffin liquid and 2% cream with less paraffin liquid. In two of the formulations (PheroidÊ and non-PheroidÊ) the liquid paraffin was increased to 18% and in two other formulations (PheroidÊ and non-PheroidÊ) the amount of liquid paraffin was decreased to 6%. The effect of varying polarities on the formulations was the motivation for altering the amount of liquid paraffin (oil phase) in the formulations. This was done in order to determine the effect of varying polarities on the so called ñpolarity gapò which exists between a formulation and an API. The bigger the difference in polarity between a formulation and an API is, the bigger the driving force for diffusion into the stratum corneum (Wiechers, 2004:177). The final formula used for the formulations, is given in Table B.3.

Table B.3: Final formula of the formulations

Ingredients	Percentage (m/m)	Activity
A: Liquid paraffin	12.0%	Oil phase of the emulsion
Cetyl alcohol	7.0%	Thickening agent
Cremophor® A 6	1.5%	Solubilising agent
Cremophor® A 25	1.5%	Solubilising agent
B: Propylene glycol	8.0%	Emulsifier
Tween 80	4.0%	Surface active agent
Methyl paraben	0.3%	Preservative
Propyl paraben	0.2%	Preservative
C: dH_2O	to 100.0%	Solvent

B.4.2 Procedure for the preparation of the sodium ascorbyl phosphate cream

Sodium ascorbyl phosphate was dissolved in 25% of the distilled water. The remaining water was heated to 80 °C. The ingredients in phase A were mixed and also heated to 80 °C. It was added to the heated water whilst being homogenised at 13 500 rpm. This mixture was cooled to approximately 50 °C before the sodium ascorbyl phosphate was added, whilst still being homogenised. The ingredients in phase B were mixed and added along with the sodium ascorbyl phosphate; and the emulsion was allowed to cool to room temperature in a water bath.

B.4.3 Procedure for the preparation of the sodium ascorbyl phosphate Pheroid™ cream

The same method was used in preparing the PheroidÊ creams as for the creams that do not contain PheroidÊ . The only difference was that the tocopherol and other PheroidÊ ingredients were added to phase B in the formulations.

B.4.4 Outcomes

Each of the different formulations had a clear white colour and a good consistency. The formulations with more of the liquid paraffin had a slightly lower viscosity than the formulations with less of the liquid paraffin.

B.5 Summary

Various semi-solid formulations containing sodium ascorbyl phosphate was formulated. The formulations included:

- 1% PheroidÊ cream **(1)**;
- 1% Cream **(2)**;
- 2% PheroidÊ cream **(3)**;
- 2% Cream **(4)**;
- 3% PheroidÊ cream **(5)**;
- 3% Cream **(6)**;
- 2% PheroidÊ cream with more paraffin liquid **(7)**;
- 2% Cream with more paraffin liquid **(8)**;
- 2% PheroidÊ cream with less paraffin liquid **(9)**;
- 2% Cream with less paraffin liquid **(10)**;
- Placebo PheroidÊ cream without API **(11)** and
- Placebo cream without API **(12)**.

Each of the abovementioned formulations was prepared in sufficient quantities and stored at room temperature, for the duration of the experiments.

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APPENDIX C

FRANZ CELL DIFFUSION STUDIES

C.1 Introduction

According to Chattaraj *et al.* (1995:119) Franz diffusion cells are a popular way to determine both the diffusion of drugs from transdermal delivery systems and drug release from semi-solid formulations. It was one of the *in vitro* studies used during this study to determine whether the API was indeed released from the formulated creams and if it was subsequently absorbed into the skin and more specifically the dermis. Due to the collagen regulating properties of this layer of the skin, the vitamin C concentrations found here are significantly lower than that of the epidermis (Davidson *et al.,* 1997:349). The formation and upkeep of collagen fibres are a very important aspect in keeping skin looking young; and according to Thiele *et al.* (2000:146) vitamin C is known for its role in the formation of collagen and overall improvement of skin tone. For an API to cross the skin and reach the target site (in this case the dermis) it has to be relatively soluble in both oil and water (Williams, 2003:37). This is because of the fact that lipophilic molecules cross the skin faster when considering the intercellular permeation pathway of the skin, but a certain degree of hydrophilicity is also needed in order to make sure that the API can indeed be formulated in a water-based formulation (Williams, 2003:37). It is thus important to determine both the aqueous solubility as well as the partition coefficient of a drug in order to predict the extent of penetration into the skin.

C.2 Methods

C.2.1 HPLC analysis of sodium ascorbyl phosphate

A validated HPLC method has already been developed in the Analytical Technology Laboratory at the North-West University, Potchefstroom Campus, South Africa (See Appendix A). An Agilent 1200 Series HPLC fitted with an Agilent 1200 pump, autosampler injection mechanism and UV-detector was used (Agilent Technologies, Palo Alto, CA). The apparatus was interfaced with Chemstation Rev. A.06.02 data acquisition and analysis software was used. The column used for the duration of the experiments was a RESTEK Ultra C_{18} (250 x 4.6 mm) silica-based, reversed phase column. The particle size of the column was 5 µm. The mobile phase consisted

of a mixture of 1 ml phosphoric acid (85%) in 1000 ml HPLC grade water. Operating flow was set at 1.0 ml/min and the injection volume at 25 µl. The retention time of sodium ascorbyl phosphate was between 2 and 3 min and the entire running time of the cycle was 4 min. Furthermore, the UV-detector was set at 245 nm for the detection of sodium ascorbyl phosphate. All of the above analyses were performed in a temperature-regulated laboratory at 25 °C. The solvent used was Milli-Q water.

C.2.2 Aqueous solubility

The aqueous solubility of sodium ascorbyl phosphate was determined by preparing an oversaturated solution of the compound in Milli-Q water (pH 5.5). This was decided on after initially dissolving the API in both a PBS buffer (pH 7.4) and phosphate buffer (pH 5.5) in order to determine the pH value at which the drug would be most stable. In both solutions, however, the drug was highly unstable and it was decided that the best approach would be working with the pure Milli-Q water. One other buffer namely tris(hydroxymethyl)aminomethane (TRIS) (pH 7.4) was also tested, but the sodium ascorbyl phosphate was still unstable. The standard pH of the Milli-Q water was determined (5.5) and the percentage unionised species at this pH was found to be 99.37%, indicating that the API would penetrate the mainly hydrophobic membranes of the skin with relative ease. The solution was stirred for 24 h by making use of a magnetic bar. An excess of the compound was added throughout this time in order to ensure the saturation of the solution. The aqueous solubility of sodium ascorbyl phosphate was determined at 37 °C instead of the usual 25 °C. This was done because during diffusion studies, the receptor phase had to be kept at a constant temperature, in order for the temperature of the skin to stay at a regulated 32 °C. Samples of the solution were filtered, diluted and analysed by HPLC. This experiment was done in triplicate.

C.2.3 Octanol-water partition coefficient (log P)

Equal volumes of pre-saturated *n*-octanol and Milli-Q water were stirred for 24 h after which the two phases were allowed to separate. Sodium ascorbyl phosphate (803.6 mg) was dissolved in 10 ml pre-saturated octanol and stirred for a period of 10 min. The pre-saturated water phase (10 ml) was added to the aforementioned solution and stirred for a further 45 min after which it was centrifuged for 30 min at 4 500 rpm. Analysis of the aqueous phase was performed by HPLC. The experiment was performed in fourfold. The log P value was calculated by using the

logarithmic ratio of the concentration in the aqueous phase to the concentration in the *n*-octanol. After determining the aqueous phase, the concentration of sodium ascorbyl phosphate in the oil phase was determined by simply deducting the amount of API in the aqueous phase from the original concentration.

C.2.4 Skin preparation

Before obtaining skin samples, ethical approval was obtained from the Research Ethics Committee of the North-West University, under the title: Application for the use of biological material, obtained from human subjects in experiments and reference number NWU-00114-11-A5 (Issue date: 2011-08-25 / Expiry date: 2016-08-24). The abdominal skin of Caucasian female patients was used during this study. The surgeon was contacted in advance in order to obtain permission from him, before asking the consent of the patient. Patient anonymity was guaranteed, and therefore, the identity of the patients will not be made public. Within 24 h after being surgically removed, the skin was frozen at a temperature of -20 °C. The skin needed to be thawed to room temperature before preparation after which the subcutaneous fat and connective tissue were removed with the use of a Zimmer® Electric Dermatome. Thickness of the dermatome skin was approximately 200-400 µm. The processed skin was placed on Whatman® filter paper with the stratum corneum facing upwards. Circles with a diameter of between 15 and 20 mm were cut out of the skin, placed on a piece of aluminium foil and stored in a re-sealable plastic bag at a temperature of -20 °C until they were used for the diffusion studies.

C.2.5 Diffusion studies

During the diffusion studies vertical Franz diffusion cells were used. Each cell consisted of a donor (top half) and receptor compartment (bottom part). Each cell has a receptor capacity of 2 ml and diffusion area of 1.075 cm^2. Twelve diffusion cells were used per diffusion study. A study was done for each of the formulations containing the API as well as two complete placebo studies for both the PheroidÊ and non-PheroidÊ placebo creams. During the literature study of this project, it was found that a significant amount of vitamin C is present in the skin (Shindo *et al.,* 1994:123). This could lead to a definite difference in the amount of vitamin C actually found in the sample solutions after diffusion with the PheroidÊ as well as non-PheroidÊ formulations. To quantify this, the placebo formulations were included. Formulations containing the API, were placed in the donor compartment, whereas the receptor compartment was filled with 2 ml

Milli-Q water. Prior to each study the receptor phase was heated in a water bath to a temperature of 37 °C. During heating of the receptor phase (to 37 °C - the temperature of blood in the body) the surface of the skin is at a constant temperature of 32 °C (Rachakonda *et al.,* 2008:2697). Skin samples were placed on the receptor compartment with the stratum corneum facing up. The donor compartment was secured on top of the skin. To ensure the complete sealing of the two compartments, the outside of the Franz cell, where the two halves of the cell come together, was covered with Dow Corning® vacuum grease. The fully assembled cells were finally secured with a horseshoe clamp. A magnetic stirring bar was placed in the receptor phase to ensure the continuous stirring during the experiment. Milli-Q water (2 ml) was inserted into the receptor compartment whilst making sure that the compartment was kept free from air bubbles. The donor phase was added to the donor compartment and was topped up throughout the experiment to ensure the complete coverage of the skin with the formulation. To make sure that as little as possible of the donor phase evaporated during the experiment, the donor compartment was sealed with Parafilm®. The diffusion cells were placed in a water bath at a temperature of 37 °C in order to ensure that the receptor solution could stay at 37 °C; maintaining the 32 °C temperature of the skin.

C.2.6 Membrane diffusion

The complete method as described in Section D.2.5 was used during the membrane diffusion studies. One of the small differences was the use of polytetrafluoroethylene (PTFE) membranes instead of skin. Furthermore, because the goal of the membrane study was to determine whether the APIôs were released from the formulations, the extraction times differed from those of the diffusion study. Extractions were made hourly from the receptor solution for 6 h.

C.2.7 Skin diffusion

The method for the diffusion studies as described in Section D.2.5. was used for this experiment. A single extraction was made at 12 h. This was done after previous diffusion studies proved to have no conclusive data, because the API concentrations were too low before a 12 h period had expired. The extracted samples were analysed by HPLC.

C.2.8 Tape stripping

According to Bronaugh and Maibach (2001:222) tape stripping removes the entire epidermis, including the stratum corneum. It is an effective and relatively simple way to analyse the quality and efficacy of transdermal formulations. After topical application of the product, the stratum corneum was removed by using adhesive film. The amount of the applied cosmeceutic product was determined by making use of certain chemical methods (Lademann *et al.,* 2009:317). After each diffusion study the skin used during the experiment was carefully removed from the Franz diffusion cell. It was placed on a piece of Whatman® filter paper and the excess cream was removed with a piece of tissue paper. Small pieces of 3M Scotch® MagicÊ Tape were cut, so as to cover the diffused area. The first piece of tape, used when starting the tape stripping was always discarded to make sure that the surface of the skin was completely clean. Fifteen more strips of adhesive film were used to individually strip the entire diffused area of skin of the stratum corneum. The strips were put into a poly top and immersed in 5 ml Milli-Q water. These vials were kept at a temperature of 4 °C for a period of 8 h. The remainder of the skin was cut into smaller pieces in order to enlarge the surface area and placed in separate poly-tops, which were once again filled with 5 ml Milli-Q water. These skin samples were stored under the same conditions as the tape strips for a period of 8 h. All samples were filtered and analysed by use of HPLC.

C.2.9 Data analysis

C.2.9.1 Release and diffusion data analysis

After completion of the membrane studies, the percentage yield after 6 h was determined for each cell. The yield of each cell was expressed as a percentage of the applied concentration. The average percentage diffused was determined by combining each of the individual values and dividing it by the number of cells used, in the study. Cumulative concentrations were also determined individually for each cell and thereafter used to calculate the average cumulative concentration (µg/cm^2) for each study. This was once again done by dividing the sum of the individual values by the number of cells used per study.

The same method of analysis was followed for the diffusion studies in order to determine a percentage yield for each cell after a period of 12 h. The individual percentages were once again combined and divided by the number of cells used during the study to calculate the average

percentage diffused after 12 h. The same was done to calculate the average concentration (µg/cm^2) after 12 h. In order to calculate the corrected average concentrations, however, placebo studies were done for both the PheroidÊ and non-PheroidÊ formulations. The corrected average concentrations (µg/cm^2) were determined by subtracting the average concentration value (of the API in the receptor) for the PheroidÊ placebo from the average concentrations (of the API in the receptor) of the PheroidÊ formulations and the average concentration of the non-PheroidÊ placebo from the non-PheroidÊ formulations. Furthermore, when plotting the concentration (µg/ml) or total amount of diffused API (per cell), and the percentage API diffused (per cell) a linear correlation was evident for each of the formulations.

C.2.9.2 Statistical data analysis

During the literature study of this project, it was determined that a certain amount of vitamin C is naturally found in human skin. To investigate whether the data should be corrected to account for the concentration of vitamin C found in the skin a t-test and the non-parametric Mann Whitney U-test were performed. The data were corrected and analysed according to a more conventional as well as a bootstrap simulation method. These two approaches are described next.

When using the conventional method, the average concentrations of the PheroidÊ and non-PheroidÊ placebos were calculated. For each experimental case the concentration was corrected by subtracting the average concentration of the respective PheroidÊ and non-PheroidÊ placebo concentrations. The standard t-test and non-parametric Mann Whitney U-test were used to determine if a significant difference existed when the corrected PheroidÊ concentrations were compared to the corrected non-PheroidÊ concentrations. A relevant p-value was calculated for the compared formulations and the smaller the p-value, the more significant the statistical data. The p-value is a probability which has a value ranging from zero to one. The p-value of a specific data group determines the probability that random sampling would lead to a difference between sample means as large (or larger) than observed, if the populations really have the same mean overall (Mendelhall *et al.,* 2009:352).

With the bootstrap method the expected (average) concentration of each experimental formulation, i.e. PheroidÊ and non-PheroidÊ was corrected with the expected concentrations of the PheroidÊ and non-PheroidÊ placebos, respectively. Following this approach of correction,

only one corrected value for each formulation was determined. Hence, to compare the PheroidÊ and non-PheroidÊ groups a statistical simulation method called the bootstrap was employed. A 95% percentile bootstrap confidence interval for a test statistic that measures the difference between the PheroidÊ and non-PheroidÊ corrected values was obtained. If these bootstrap confidence intervals exclude zero, it can be concluded that a significant difference exists between the two study groups. A description of the test statistic used, as well as a brief discussion of the bootstrap methodology is presented below.

A summary of the statistics is presented in the form of averages, medians and standard deviations. Graphical representation of the data and the bootstrap distributions are presented using box-plots. All statistical tests were performed at a 5% level of significance and all statistical analyses were done using SPSS (version 18) and the R statistical package (version 2.13).

C.2.9.2.1 The bootstrap statistical method

During the analysis of the data, the so-called ôbootstrapô statistical method was used. According to Felsenstein (1985:783) the bootstrap method involves the re-sampling of points from the obtained data (with replacement) of the same size as the original data. The bootstrap is thus a technique that can estimate population parameters and distributional properties of statistics by substituting the population mechanism used to obtain the parameter with an empirical equivalent. These estimates can be obtained analytically, but they are mostly obtained through the use of re-sampling and Monte-Carlo methods carried out on a computer. Monte-Carlo methods are employed in order to determine the approximate calculation of integrals and optimisation that employ a collection of random samples (Kastner, 2010:1590). Estimates of standard errors, as well as confidence intervals can be derived. Confidence intervals comprise two values, that are estimates of an interval between which the value of a specific parameter is likely to fall. One of the advantages of this method includes its simplicity (Efron & Tibshirani, 1986:54). In the application of this simulation method a specific test statistic was used. These test statistics are defined in Equations C.1 to C.4 where Equations C.1 and C.2 are the corrected average concentrations of the PheroidÊ and non-PheroidÊ formulations. The subscript *E* indicates an experimental group e.g., the 1% formulation.

$$\bar{Y}_{\text{Pheroid.E}} = \bar{X}_{\text{Pherid.E}} - \bar{X}_{\text{Pheroid Placebo}}$$ **Equation C.1**

$$\bar{Y}_{non-Pheroid\ E} = \bar{X}_{non-Pheroid\ E} - \bar{X}_{non-Pheroid\ Placebo}$$ **Equation C.2**

The test statistic that was used (in the bootstrap simulation) to measure the difference between the PheroidÊ and non-PheroidÊ formulations is defined in Equation C.3. Equation C.4, however, is an estimate of the standard error of the difference between the average corrected concentrations of the PheroidÊ and non-PheroidÊ formulations.

$$(\bar{Y}_{Pheroid,E} - \bar{Y}_{non-Pheroid,E}) / stderr(\bar{Y}_{Pheroid,E} - \bar{Y}_{non-Pheroid,E})$$ **Equation C.3**

$$stderr(\bar{Y}_{Pheroid,E} - \bar{Y}_{non-Pheroid,E})$$ **Equation C.4**

C.3 Results and discussion

C.3.1 Physicochemical properties

It is not possible to determine the diffusion of an API by making use of individual parameters alone, but a combination of these parameters can be used to predict the outcome of diffusion with a specific API. These parameters include aqueous solubility, the log P value, the molecular weight, melting point and the degree of ionisation of the API.

C.3.1.1 Aqueous solubility

According to Naik *et al.* (2000:319) for a drug to be able to penetrate the skin, it must have an aqueous solubility of 1 mg/ml or more. The aqueous solubility of sodium ascorbyl phosphate was determined to be 6.14 mg/ml in Milli-Q water at 32 °C, thus allowing it to permeate the skin sufficiently.

C.3.1.2 Octanol-water partition coefficient (log P)

Williams (2003:35) stated that in order for a drug to dissolve in both oil and water, ensuring the adequate penetration of the drug, through the skin, it should have a log P value of 1 to 3. The log P of sodium ascorbyl phosphate was found to be -0.005. This indicated that although the

drug is very soluble in water, the fact that it is less soluble in oil makes the penetration of the drug into the lipophilic part of the skin very difficult.

C.3.1.3 Degree of ionisation

After determining the appropriate pH value at which to perform the experiments, as well as the pKa or dissociation constant of the API, the percentage ionised and unionised API could be determined. The unionised species were determined to make up 99.37% of the API and the ionised species 0.63%. Mansoor (2003:302) stated that the unionised form of an API is mainly responsible for the penetration of the hydrophobic biological membranes, such as the skin. With an unionised species of 99.37%, it was expected that the API would be able to penetrate the skin effectively.

C.3.1.4 Molecular weight

A molecular mass of less than 600 Da is required in order for an API to penetrate the stratum corneum efficiently, because small molecules penetrate the skin faster than large ones (Barry 2002:512). The molecular mass of sodium ascorbyl phosphate is 322.05 Da which points to the ability of the API to penetrate the stratum corneum.

C.3.1.5 Melting point

The melting point of an API can be used to determine its solubility. Lower melting points (less than 40 °C) lead to better penetration according to Donnelly *et al.* (2012:214). Due to the linear correlation seen when the melting point of an API is plotted against its steady state flux, it is evident that lower melting points indicate higher solubility and thus increased penetration (Donnelly *et al.,* 2012:214). The melting point of sodium ascorbyl phosphate is approximately 260 °C, indicating that the solubility of the API should be low.

Having a combined look at the abovementioned properties of the API, it was expected that the drug would be less soluble when compared to other APIôs, because of its high melting point. The low log P value reinforced this as it indicated that although the drug was very soluble in water (6.14 mg/ml), its low lipid solubility would make the penetration of the drug into the lipophilic part of the skin very difficult. The unionised species of 99.37% was, however, expected to improve the lipid solubility of the API, as the unionised form of an API is usually more lipid soluble.

C.3.2 Membrane release studies

Membrane release studies were performed for each of the ten API formulations. The formulations included 1% PheroidÊ cream **(1)** and 1% cream **(2)**, 2% PheroidÊ cream **(3)** and 2% cream **(4)**, 3% PheroidÊ cream **(5)** and 3% cream **(6)**, 2% PheroidÊ cream with more paraffin liquid in the formula **(7)** and 2% cream with more paraffin liquid in the formula **(8)** as well as 2% PheroidÊ cream with less paraffin liquid in the formula **(9)** and 2% cream with less paraffin liquid in the formula **(10)**. Data acquired during the membrane studies are given in Table C.1.

Table C.1: Membrane release data of formulations

	Average %released after 6 h (%)	**Average cumulative concentration after 6 h (µg/cm^2)**
1% PheroidÊ cream **(1)**	1.940	360.696
1% Cream **(2)**	1.259	234.157
2% PheroidÊ cream **(3)**	0.278	103.250
2% Cream **(4)**	1.813	674.067
3% PheroidÊ cream **(5)**	1.384	772.185
3% Cream **(6)**	0.685	382.192
2% Pheroid cream more PL* **(7)**	0.683	253.922
2% Cream more PL* **(8)**	1.677	623.818
2% PheroidÊ cream less PL** **(9)**	0.594	220.814
2% Cream less PL** **(10)**	2.008	746.738

*More PL = more liquid paraffin

**Less PL = less liquid paraffin

Comparing the average %API released, the following was observed through the membranes after 6 h: **(10)** (2.008%) released the highest percentage API, followed by **(1)** (1.940%), **(4)** (1.813%), **(8)** (1.677%), **(5)** (1.384%), **(2)** (1.259%), **(6)** (0.685%), **(7)** (0.683%), **(9)** (0.594%) and lastly **(3)** (0.278%).

C.3.2.1 The effect of Pheroid™ on release of the API

When the PheroidÊ and non-PheroidÊ formulations were compared, the non-PheroidÊ formulations (**(2)**, **(4)**, **(6)**, **(8)**, **(10)**) were more likely to cross the membrane than the PheroidÊ formulations (**(1)**, **(3)**, **(5)**, **(7)**, **(9)**). This could be as a result of the artificial membrane not being

as intricate as the skin (Barry, 2002:518). It could also be that because of the hydrophilic nature of the creams (because of the lower oil concentrations) the oil soluble unionised species of the API were diffused from the formulation (Bickers, 2010:22).

C.3.2.2 The effect of the polarity of the carrier medium on the release of the API

No significant differences were found between the formulations with higher polarity versus the formulations with a lower polarity there was no significant difference. When comparing **(9)** and **(10)** both had a higher polarity, because of a part of the liquid paraffin total being substituted by water. When considering that water is polar and liquid paraffin non-polar, it is evident that with more water than liquid paraffin in a formulation, the polarity should increase. Formulation **(10)**ôs higher polarity proved to increase the API release from the cream. Once again because of the unionised species of the API being oil soluble, it would have a higher affinity for a less polarised environment (Wiechers *et al.,* 2004:175). In **(9)**, however, the higher polarity because of the liquid paraffin did not cause the API to release more. This is evident in the fact that **(6)**, **(7)** and **(9)** showed only a slight difference in the percentage API which penetrated the membrane. The aforementioned three formulations, as well as **(3)** showed a significantly lower percentage of the API crossing the membrane, when compared to the other formulations.

C.3.2.3 The effect of concentration of API on release

Lower API concentrations also proved to further the diffusion of the release of the API from the formulation, as is evident in formulas **(1)**; **(3)** and **(4)**. Each of these formulations showed a higher release rate, from which it can be deduced that the lower the API concentration in the formulations, the higher the release. This could be the result of higher stability at lower API concentrations in the formulations. According to Zussman *et al.,* (2010:515) concentrations of the API lower than 20% (m/m) proved to be the most stable in topical formulations, whilst still being able to penetrate the skin sufficiently.

C.3.3 Tape stripping

Table C.2. Tape stripping data of formulations

	n	Average [ ] stratum corneum-epidermis (µg/ml)	n	Average [ ] epidermis-dermis (µg/ml)
1% PheroidÊ **(1)**	3	0.217	10	0.656
1% non-PheroidÊ **(2)**	7	0.407	10	0.226
2% PheroidÊ **(3)**	12	0.127	12	0.530
2% non-PheroidÊ **(4)**	11	0.217	11	0
3% PheroidÊ **(5)**	11	0.115	6	0.220
3% non-PheroidÊ **(6)**	11	0.247	11	0
2% PheroidÊ more LP* **(7)**	8	0.247	4	0.297
2% non-PheroidÊ more LP* **(8)**	12	0.130	12	0
2% PheroidÊ less LP** **(9)**	10	0.457	6	0.231
2% non-PheroidÊ less LP** **(10)**	12	0.259	12	0
PheroidÊ placebo **(11)**	12	0	12	0
Non-PheroidÊ **(12)**	12	0	6	0.554

*More LP = More liquid paraffin

**Less LP = Less paraffin liquid

n= Number of cells used for determination of average

C.3.3.1 Stratum corneum-epidermis

The tape stripping data of the formulations are given in Table C.2. Only small concentrations of the API were found in the epidermis-dermis or on the tape strips (stratum corneum-epidermis), after stripping.

C.3.3.1.1 The effect of the polarity of the carrier medium on diffusion of the API

The formulation with the highest API concentration in the stratum corneum-epidermis was **(9)**, which showed a concentration of 0.457 µg/ml. Formulation **(10)** also proved to penetrate the stratum corneum-epidermis relatively well, as this formulation was the formula with the third highest penetration rate, with a concentration of 0.259 µg/ml. The higher polarity of these two formulas (higher amount of water in the formulations) could be the reason for their observed penetration. Due to the higher polarity in the formulations, the oil soluble unionised species of the API could possibly have a higher affinity for a less polar environment such as the stratum

corneum-epidermis (Bickers, 2010:22). Compared to the two formulations with lower polarities (**(7)** and **(8)**) they performed significantly better. Formulation **(7)** had a concentration of 0.246 µg/ml and **(8)** a much lower concentration of 0.130 µg/ml, it was thus evident that the formulations with the higher polarities performed better than formulations containing less water.

C.3.3.1.2 The effect of concentration of API on transdermal delivery

Comparing the formulations with higher API concentrations to the formulations with lower concentrations of the drug, the lower the concentrations of API in the formulation the better the penetration was. In the case of the 1% formulations (**(1)** and **(2)**) they penetrated the stratum corneum-epidermis better when compared to **(3)** and **(4)**, with the 2% API in the formula. Formulation **(1)** and **(2)** had concentrations of 0.217 µg/ml and 0.407 µg/ml, respectively; and **(3)** and **(4)** had concentrations of 0.127 µg/ml and 0.217 µg/ml, respectively. The addition of a phosphate group in the second position on the cyclic ring could be a contributing factor to the improved stability of the API (Ġpiclin *et al.,* 2002:68). Increased stability could be due to the API (when formulated in formulations with concentrations lower than 20% (m/m) of the API) penetrating the skin better (Zussman *et al.,* 2010:516).

C.3.3.1.3 The effect of Pheroid™ on transdermal delivery

The non-PheroidÊ formulations proved to penetrate the stratum corneum-epidermis better on average, than the PheroidÊ formulations. Once again it could be because of the higher affinity of the oil soluble unionised species for less polar surroundings (Wiechers *et al.,* 2004:175).

C.3.3.2 Epidermis-dermis

C.3.3.2.1 The effect of Pheroid™ on delivery

Only one of the non-PheroidÊ formulations penetrated the epidermis-dermis. Formulation **(2)** with an average concentration of 0.226 µg/ml penetrated the epidermis-dermis, whilst each of the PheroidÊ formulations penetrated this layer of the skin. The formula with the highest penetration rate was **(1)** with an average concentration of 0.656 µg/ml, followed by **(3)** (0.530 µg/ml). It is thus clear that the API encapsulated in the PheroidÊ penetrated the epidermis-dermis to a higher extent and that the PheroidÊ enhanced the absorption of the API (Grobler *et al.,* 2008:297).

C.3.3.2.2 The effect of API concentration on transdermal delivery

As with the penetration through the skin and into the stratum corneum-epidermis, the lower the concentration of API in the formula, the better the penetration was (Zussman *et al.,* 2010:516). According to Ġpiclin *et al.* (2002:68) the protection of the enediol system from oxidation, by the addition of a phosphate group in the second position of the cyclic ring, is also one of the attributing factors to the increased stability of the API.

C.3.3.2.3 The effect of the polarity of the carrier medium on transdermal delivery

Formula **(7),** with the higher amount of liquid paraffin, penetrated the skin more effectively than the formulation with the higher polarity **(9)**. These formulationsô respective concentrations were 0.297 µg/ml and 0.231 µg/ml. The higher polarity of the formulations could once again possibly be the reason for the API penetrating the skin as well as it did. The lipid soluble unionised species of the drug preferred the less polar environment of the skin and were therefore released from the formulation into the skin (Wiechers *et al.,* 2004:175).

C.3.4 Diffusion studies

Table C.3: Data obtained from diffusion studies

	Average %diffused after 12 h	**Average concentration after 12 h [(µg/cm^2)]**	**Corrected average concentration after 12 h (µg/cm^2)**
1% PheroidÊ **(1)**	0.026	4.772	3.057
1% non-PheroidÊ **(2)**	0.029	5.422	3.555
2% PheroidÊ **(3)**	0.012	4.486	2.771
2% non-PheroidÊ **(4)**	0.015	5.628	3.761
3% PheroidÊ **(5)**	0.007	2.511	0.796
3% non-PheroidÊ **(6)**	0.014	5.211	3.344
2% PheroidÊ more LP* **(7)**	0.005	1.895	0.180
2% non-PheroidÊ more LP* **(8)**	0.008	2.920	1.053
2% PheroidÊ less LP** **(9)**	0.009	3.208	1.493
2% non-PheroidÊ less LP** **(10)**	0.012	4.432	2.565

*More LP = More liquid paraffin

**Less LP = Less liquid paraffin

The data as obtained from the skin diffusion studies are given in Table C.3. Two placebo formulations were also used in separate skin diffusion studies to determine the effect of the natural vitamin C found in the skin (Shindo *et al.,* 1994:123) on the diffusion data. Both of the placebo creams (**(11)** and **(12)**) succeeded in extracting a certain amount of the vitamin C in the skin. This was evident in the average concentration after 12 h for each of the placebo formulations. The average placebo effect was deducted from each of the formulations in order to obtain actual percentage diffused values. The average concentrations for the PheroidÊ and non-PheroidÊ placebos were 1.715 and 1.867 µg/cm^2, respectively.

C.3.4.1 The effect of Pheroid™ on delivery

From the data, it is evident that **(2)**, **(4)** and **(6)** proved to penetrate the skin most effectively, with the average concentration after 12 h for (**4**) being only slightly higher than that of the other two formulations. The same slight difference (as noticed between formulas **(2)**, **(4)** and **(6)**) was observed between **(1)**, **(3)** and **(10)**. Once again, comparison of the PheroidÊ (**(1)** and **(3)**) and non-PheroidÊ formulations (**(2)**, **(4)**, **(6)**, **(10)**) showed, contrary to what was expected, that the non-PheroidÊ formulations penetrated the skin better than the PheroidÊ formulations. This can be attributed to the fact that the relatively oil soluble unionised species of the API had a higher affinity for the less polar surrounding of the skin (Bickers, 2010:22). The remaining formulations, including **(5)**, **(7)**, **(8)** and **(9)** all showed a significantly lower average percentage concentration after 12 h, as well as average percentage diffused after 12 h.

C.3.4.2 The effect of the polarity of the carrier medium on transdermal delivery

A comparison between the formulations with the higher amount of liquid paraffin (**(7)** and **(8)**) and formulations with a lower amount of liquid paraffin in formulations **(9)** and **(10)**, showed that the more polar formulations (**(9)** and **(10)**) penetrated the skin better. This could be because of the higher polarity of these two formulations, because of the increased amount of water in the formula and the APIôs affinity for a less polar environment, namely the stratum corneum. Small non-polar and relatively lipophilic APIôs penetrate the skin best and highly polar compounds less (Schaefer *et al.*, 2011:224). During the literature study of this project the unionised (oil soluble) species of the API was found to be 99.37%. This does indicate that the drug should thus be able to cross the skin. Furthermore, the molecular size of the API (322.05 Da) is also perfect for

penetration of the skin, as smaller molecules tend to cross the membrane easier than larger ones (Barry, 2002:512).

The percentage diffused after 12 h from the formulations ranged between 0.002% **(5)** and 0.01% **(4)**. This significant difference in values can be due to the large number of outliers in the data. The same reason can be given for the significant difference in the average concentration values, after 12 h. The average concentration values ranged from 0.021 µg/cm^2 for **(7)** to 3.601 µg/cm^2 for **(4)**.

C.3.4.3 The effect of API concentration on transdermal delivery

It was once again evident that the formulations with a lower concentration of the API in the formula penetrated the skin better than those with higher amounts of API. Smaller amounts of the weak acidic API in the formulations tend to improve the stability of the formulations, when compared to the formulations with higher API concentrations. Zussman *et al.* (2010:516) stated that formulations with a concentration lower than 20% (m/m) API penetrated the skin best. The higher stability of the API can also be attributed to its chemical structure. The enediol system is protected from oxidation by the introduction of a phosphate group in the second position of the cyclic ring (Ġpiclin *et al.*, 2002:68).

C.3.5 Statistical results

A box-plot representation of the data is presented in Figure C.1. From this graph it is clear that there was no visible difference between the concentrations of the placebo group. However, a clear difference was observed for the 3% formulations (**(5)** and **(6)**). These findings were subsequently investigated, using statistical methods as described in Section C.2.9.2.

Table C.4 Comparison of placebo formulations

	Non-PheroidÊ		**PheroidÊ**			
	Average concentration (µg/cm^2)	Median of placebos	Average concentration (µg/cm^2)	Median of placebos	t-test	Mann-Whitney
Placebo ((11) & 12))	1.87 ± 1.05	1.74	1.72 ± 0.82	1.39	0.703	0.806

However, the average placebo concentrations were corrected in order to present corrected summary statistics of the experimental groups (a comparison of the placebo formulations is given in Table C.4). This statistical analysis is presented next according to the two correction approaches described in Section C.2.9.2.

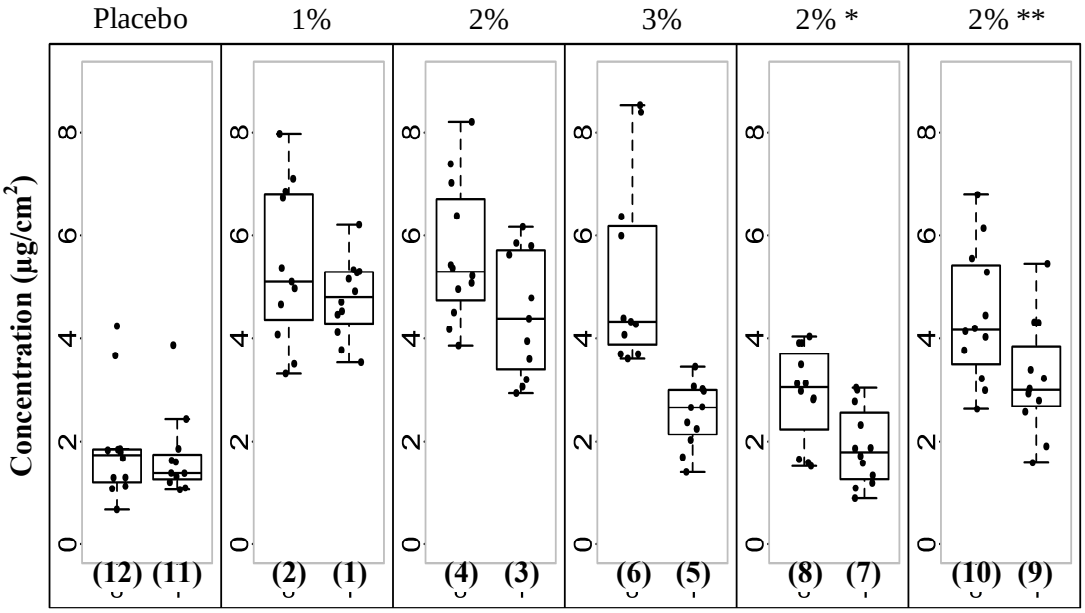

Figure C.1: Box-plot representations of the concentration values (εg/cm^2) after skin diffusion: before the correction of endogenous vitamin C (data are superimposed on the graph as filled black dots; *more liquid paraffin; **less liquid paraffin in the formulations)

C.3.5.1 Conventional method

Using the conventional correction method (see Section C.2.9.2), summary statistics as well as the p-values for the PheroidÊ versus non-PheroidÊ comparisons were obtained. These values are presented in Table C.5.

From Table C.5 it was clear that a highly significant statistical difference was observed between **(5)** and **(6)**. This is determined by the p-value of 0.001 between these two formulations. The smaller the p-value is, the more significant the data (Mendelhall *et al.,* 2009:352). From a conservative point of view, neither **(3)** and **(4)**, nor **(9)** and **(10)** differed significantly at a 5% significance level, when comparing their p-values. The p-value for formulations **(3)** and **(4)** was determined to be 0.079 and the value for formulations **(9)** and **(10)** was determined to be 0.036.

However, on a 10% significance level a significant difference could be concluded. In contrast, **(7)** and **(8)** (p-value of 0.017) indicated a significant difference between these two formulations. Concerning the results of **(3)**, **(4)**, **(7)**, **(8)**, **(9)** and **(10)** it is suggested that these experiments should be repeated, with a larger number of cells to validate the above findings. All of the above statistical findings were an indication of the conventional method.

Table C.5: Comparison of formulations, using corrected data

	Non-PheroidÊ		**PheroidÊ**			
	Average concentration ($\mu g/cm^2$)	Median	Average concentration ($\mu g/cm^2$)	Median	t-test	Mann-Whitney
1% (1 & 2)	3.55 ± 1.55	3.23	3.06 ± 0.75	3.09	0.349	0.580
2% (3 & 4)	3.76 ± 1.34	3.42	2.77 ± 1.22	2.67	0.079	0.097
3% (5 & 6)	3.34 ± 1.84	2.46	0.80 ± 0.62	0.95	0.001	0.000
2% more LP* (7 & 8)	1.05 ± 0.90	1.19	0.18 ± 0.75	0.08	0.017	0.038
2% less LP (9 & 10)**	2.56 ± 1.28	2.30	1.49 ± 1.07	1.29	0.036	0.065

*More LP = More liquid paraffin

**Less LP = Less liquid paraffin

C.3.5.2. Bootstrap method

The distributions of the corrected bootstrap averages, for each of the experiments, i.e., 1%, 2% etc. are presented in Figure C.2. From this graph a large difference is again observed in the case of the 3% formulation.

In order to determine the statistical significance of the PheroidÊ compared to non-PheroidÊ differences, 95% bootstrap percentile confidence intervals were calculated for the discrepancy test statistic defined in Section C.2.9.2. These confidence intervals are presented in Table C.6. If the value zero (0) is included in the confidence interval, it indicates an insignificant statistical difference between the relating formulations. If zero is however excluded from the confidence interval data, as in the case of formulations **(5)** and **(6)** a significant difference exists between the two compared formulations. The confidence intervals are given to state the existence of a significant statistical difference, when using the bootstrap method, as p-values are given when using the more conventional statistical method.

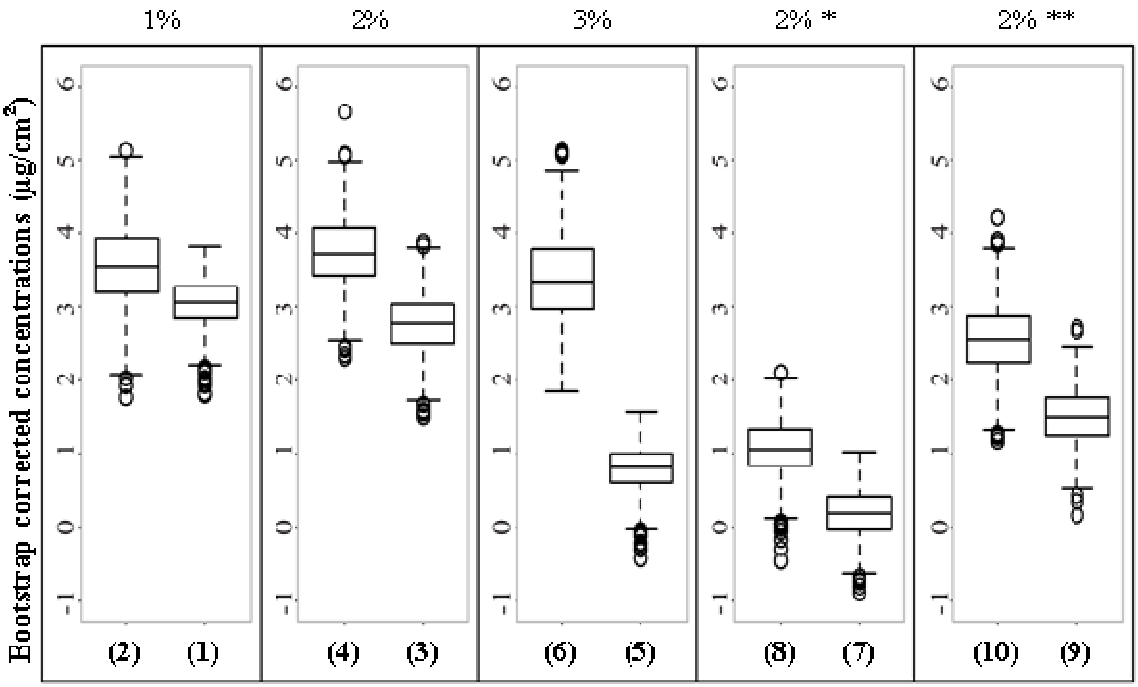

Figure C.2: Bootstrap corrected concentrations (*more liquid paraffin; **less liquid paraffin in the formulations)

Formulations **(3)**, **(4)**, **(7)**, **(8)**, **(9)** and **(10)** produced confidence intervals for which the upper bounds are close to zero. This suggested, as in the case of the conventional analysis, that a repetition of the study was required with larger numbers.

Table C.6 Bootstrap confidence intervals for each experiment

Formulations	**95% Bootstrap percentile Confidence Interval**
1% **(1)** and **(2)**	[-2.89:1.12]
2% **(3)** and **(4)**	[-3.61:0.49]
3% **(5)** and **(6)**	[-5.71:-2.16]
> 2% **(7)** and **(8)**	[-4.10:0.18]
< 2% **(9)** and **(10)**	[-4.06:0.22]

In conclusion, two different vitamin C concentration correction methods have been employed to investigate differences amongst the PheroidÊ and non-PheroidÊ formulations. The results and conclusions of these two methods mostly correspond except for formulations **(7)** and **(8)**. For

this experiment the conventional method showed a statistically significant difference (t-test: 0.017; Mann Whitney: 0.038) whilst the bootstrap confidence interval was [-4.10:0.18] which included zero. This contradictory result should be viewed in a positive light, as the bootstrap methodology does not have any distributional assumptions and discloses the need for further investigation, with larger sample numbers.

C.4 Conclusion

The aqueous solubility of the API was found to be 6.14 mg/ml. This indicated that the API would be able to penetrate the skin sufficiently (Naik *et al.,* 2000:319), in contrast to the log P value of -0.005, which suggested that penetration might not be optimal (Williams, 2003:35). The high melting point (260 °C) also suggested that the solubility of the API would not be ideal to facilitate its penetration through the skin (Donnelly *et al.,* 2012:214), but the high percentage of lipid soluble unionised species (99.37%) of the API was however a clear indication that the API would be able to penetrate the skin (Mansoor, 2003:302). Furthermore, the molecular size of 322.02 Da made it possible for the API to cross the stratum corneum (Barry, 2002:512), as the molecules were smaller than the required 600 Da.

In the membrane release study **(10)** was found to be the most efficient cream in crossing the membrane. The average percentage diffused after 6 h for this formulation was 2.008%. Formulation **(1)** was the cream with the second highest average percentage diffused after the 6 h membrane release study. The average percentage diffused for this formulation was 1.940%. The higher polarity of formulation **(10)** could have been the cause of its effective release of the water soluble API. The concentrations of API found in the samples overall after the membrane release studies were drastically higher than the concentrations found after the diffusion studies. This can be because of the fact that the membrane does not comprise the intricate layer which the skin does (Barry, 2002:518). Hhigh concentrations of API found after the membrane studies do, however, point to the fact that the 99.37% unionised oil soluble species of the API was successfully released from the relatively hydrophilic surroundings of the cream (Barry, 2002:511).

Formulations **(2)**, **(4)** and **(6)** were the three formulations, which penetrated the skin most effectively during the skin diffusion study. These three formulations all had average concentrations of between 3.761 and 3.344 µg/cm^2 with **(4)** being the formulation with the

highest average concentration after 12 h. When the diffusion data were compared with the tape stripping data, the non-PheroidÊ formulations proved to be the better formulations in the diffusion studies. The PheroidÊ formulations, however, were definitely the formulations which penetrated the epidermis-dermis to a higher extent. This could be attributed to the PheroidÊ which encapsulated the API and enhanced its penetration of the lipid bilayer of the skin (Grobler e*t al.,* 2008:297). Another reason for the diffusion of the API could be that the unionised species of a drug is usually lipid soluble and can pass readily across the stratum corneum (Barry, 2002:511). During the literature study of this project the unionised species of the API was found to be 99.37%.

Non-PheroidÊ formulations, however, proved to penetrate the stratum corneum-epidermis better on average. The formulations with the higher polarities proved to be in the top three formulations which penetrated the skin. Due to the hydrophilic nature of the non-PheroidÊ creams, the unionised oil soluble species of the API could have had a higher affinity for the oil lipid rich membrane of the skin (Bickers, 2010:22). The formula with the highest API concentration in the stratum corneum-epidermis was **(9)** which had a concentration of 0.457 µg/ml. Formulation **(10)**, a non-PheroidÊ formulation, with less liquid paraffin also proved to penetrate the stratum corneum-epidermis relatively well as this formulation depicted the third highest penetration rate, with a concentration of 0.259 µg/ml. The PheroidÊ formulations, however, proved to penetrate the epidermis-dermis significantly better than their non-PheroidÊ counterparts.

Only one of the non-PheroidÊ formulations delivered API in small concentrations in the epidermis-dermis. Concentrations of 0.226 µg/ml were found in the skin for **(2)**. Formulation **(1)** was the formulation with the highest concentration API in the epidermis-dermis, with a concentration of 0.656 µg/ml compared to the closest competitor **(3)**, with a concentration of 0.530 µg/ml. The formula with the higher amount of liquid paraffin i.e., **(7)** (0.297 µg/ml) penetrated the skin more effectively than the formulation with the higher polarity i.e., **(9)** (0.231 µg/ml). The formulations with the lower concentrations of API penetrated both the stratum corneum-epidermis and epidermis-dermis more effectively. This could be a result of the improved stability of the formulations when the API was used in lower concentrations (Zussman *et al.,* 2010:516).

Formulation which proved to be the best overall, was the 1% PheroidÊ cream **(1)**. In both the membrane release and diffusion studies it was one of the top four formulations when considering the API concentrations determined during the experimental work. It was also the formulation which best penetrated the skin, with a concentration of 0.656 µg/ml in the epidermis-dermis. It had a low concentration of API and the PheroidÊ encapsulated the API (Grobler *et al.,* 2008:297) improving its penetration of the skin. It is thus clear that the PheroidÊ in the formulations was effective in enhancing the penetration of the API into the skin and more specifically the epidermis-dermis (target site). The lower concentrations of API was effectively released from the formulations, as well as diffused through the skin, as was expected (Zussman *et al.,* 2010:516). Improved stability of the API could also be because of the addition of a phosphate group in the second position in the cyclic ring, which protects it from oxidation (Ġpiclin *et al.,* 2002:68). Regarding the polarity of the formulation, it was relatively neutral when compared to the formulations with more or less liquid paraffin in the formula, but still released the API effectively. This could have been, as previously stated, because the lipid soluble unionised species of the API preferred a less polar surrounding such as the skin. The physicochemical properties of the API were thus a good indication of the results that were obtained during the experiments.

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APPENDIX D

SKIN PHARMACOLOGY AND PHYSIOLOGY: AUTHORS GUIDE

D.1 Scope of the journal

Within the past decade research into skin pharmacology has developed dramatically with new and promising drugs and therapeutic concepts being introduced regularly. Recent examples include immunosuppressive topicals and new strategies in the therapy of malignant melanoma and wound healing. New concepts have also been developed in traditional areas of skin therapy and skin care which have led to discussions of issues such as ócosmeceuticalsô in the United States, óquasi-drugsô in Japan, and the 6th Amendment of the Cosmetic Regulation in the European Union. There are many open questions which reflect the importance of the subject and the need for a new international scientific forum where they can be discussed in extenso. Dermatologists, pharmaceutical chemists, pharmacologists, toxicologists, and others interested in the subject are invited to contribute to this exciting project and to submit their best research work for publication.

D.2 Submission

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1. Editorials
2. Reviews
3. Original Papers
4. Short Communications
5. Letter to the Editor

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Chatel J-M, Bernard H, Orson FM: Isolation and characterization of two complete Ara h 2 isoforms cDNA. Int Arch Allergy Immunol 2003;131:14-18.

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(c) Monographs:

Matthews DE, Farewell VT: Using and Understanding Medical Statistics, ed 3, revised. Basel, Karger, 1996.

(d) Edited books:

Parren PWHI, Burton DR, DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, Dubois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124ï 137.

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