

The effect of selected natural oils on the permeation of flurbiprofen through human skin

Amé Cowley

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Supervisor: Dr JM Viljoen

Co-supervisor: Prof J du Plessis

Assistant-supervisor: Dr M Gerber

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

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ABSTRACT

In pharmaceutical sciences, topical delivery is a transport process of an active pharmaceutical ingredient (API) from a formulated dosage form to the target site of action. For most topical delivery systems, the skin surface, or the specific skin layers, such as the outermost layer of the stratum corneum, the lipids amid the corneocytes within the stratum corneum, the corneocytes themselves, the epidermis, dermis, Langerhans cells, Merckle cells or the appendageal structures can be the target delivery location. When an API is delivered to the skin, it has to firstly diffuse from the formulation in which it is applied, to the skin surface. From there the API may partition into the stratum corneum, permeate across the stratum corneum and partition into the viable epidermis, from where it may partition further into the dermis and permeate across the dermis into the bloodstream (Wiechers, 2008:1-3, 7).

With respect to the barrier function of the skin, the intercellular spaces within the stratum corneum contain lipids and its main purpose is to operate as a barrier to water-loss and to provide an imperative diffusional barrier to the absorption of APIs. This resistance is comprised of a complex interaction of lipids that creates a hydrophilic and lipophilic penetration pathway. The fundamental aspect underlying the impermeability of the skin, therefore, is the lipophilic nature of the stratum corneum (Bouwstra *et al.*, 2003:4; Franz & Lehman, 2000:25; Walker & Smith, 1996:296).

A common approach for the promotion of poorly penetrating APIs in transdermal delivery is the incorporation of chemical penetration enhancers in delivery systems, in order to promote the partitioning of an API into the stratum corneum. These chemicals are also referred to as accelerants, promoters and absorption promoters. Penetration enhancers are added to topical formulations and usually also partition into the stratum corneum, where they temporarily and reversibly disrupt its fundamental diffusional barrier properties, hence facilitating the absorption of an API through the skin (Büyüktimkin *et al.*, 1997:358-359; Sinha & Kaur, 2000:1131; Walker & Smith, 1996:296). The mechanisms for the enhancement of diffusion of the API should therefore increase the solubility and partitioning of the drug from the formulation into the skin. It should further increase the solubility of the API within the skin and promote its permeability and diffusion coefficient (Rajadhyaksha *et al.*, 1997:489).

Fatty acids are recognised to effectively enhance the penetration of transdermally delivered hydrophilic and lipophilic APIs. Many penetration enhancers contain saturated and unsaturated hydrocarbon chains, and a popular fatty acid that has been used in this regard is oleic acid

(Williams & Barry, 2004:609-610). It is believed that fatty acids disrupt the lipid organisation of the intercellular lipids within the stratum corneum to cause fluidisation of these bilayers, making the stratum corneum more permeable to APIs. Excipients with polar (hydrophilic) head groups and long hydrophobic chains i.e. fatty acids, can penetrate into the intercellular lipids of the stratum corneum and disrupt these endogenous lipid components, thereby increasing diffusion of an API within the skin (Barry, 2006:9-10; Hadgraft & Finnin, 2006:367-368; Kanikkannan *et al.*, 2006:18; Williams & Barry, 2004:610).

Natural oils are widely used in topical formulations and were an obvious choice in this study. Oils are liquids at room temperature, whereas fats are in solid form. They are relatively easy to obtain from both plants and animals. The main constituents of fats and oils are triglycerides comprising of fatty acids and a glycerol. Oils control the evaporation of moisture from the skin, spread easily and evenly and are partly metabolised in the skin to release valuable fatty acids (Fang *et al.*, 2004:170,173; Lautenschläger, 2004:46; Mitsui, 1997:121-122).

The focus of this study was not formulation *per se*, but included the formulation of avocado-, grapeseed-, emu-, crocodile, olive and coconut oil into semisolid emulgel- and two foam formulations. This was done in order to investigate the penetration enhancing properties of their fatty acid content on flurbiprofen which was chosen as the marker API. The emulgels containing the natural oils were compared to the same emulgel formulation containing liquid paraffin, and a hydrogel without the inclusion of an oil.

Six natural oils were analysed by gas chromatography (GC) in order to quantify their fatty acid compositions, whilst also providing qualitative information by indicating the retention times of the materials with an alkyl chain composition (Mitsui, 1997:260). Data obtained with the GC indicated that olive- (76%), avocado- (68%), emu- (46%) and crocodile oil (40%) presented with high levels of oleic acid, also known as a mono-unsaturated fatty acid (MUFA). Lower levels of oleic acid were observed within grapeseed- (27%) and coconut oil (8%). The only oil demonstrating high levels of the poly-unsaturated fatty acid (PUFA), linoleic acid, was grapeseed oil (61%), whereas the remainder of the oils showed levels below 24%. Contrary, coconut oil seemed to have been the only oil high in saturated fatty acids (SFAs) and consisted of a lauric acid content of 52% and medium levels of myristic acid (21%). Average levels of palmitic acid (SFA) were found in crocodile- (21%) and in emu oil (21%), both of animal origin, whereas avocado-, grapeseed-, olive- and coconut oils from plants presented with levels below 15%. Stearic acid was also present in levels below 10% in all of these oils, with the oils of animal origin portraying the highest values.

A method was developed and validated to determine the concentration of the marker flurbiprofen after diffusion from the formulations into the skin, as well as concentrations of the

marker that diffused through the skin, by means of high performance liquid chromatography (HPLC). Franz cell membrane diffusion studies were conducted prior to the skin diffusion studies in order to verify the actual release of the marker from the semisolid formulations.

Skin diffusion experiments were performed using dermatomed excised, human skin to which the six emulgel formulations, containing the natural oils, were applied. A comparative study was performed utilising liquid paraffin and a hydrogel, in order to compare the diffusion of the marker, flurbiprofen, into and through the skin. The two oil emulgel formulations that had indicated the best flux values were subsequently formulated into foam preparations in order to compare the penetration enhancement properties on flurbiprofen of these two oils in a foam preparation, to those in the equivalent emulgels. The data generated for all ten the formulations were compared, and the formulations that yielded the best results with regards to median flux values and the flurbiprofen concentrations within the stratum corneum-epidermis and epidermis-dermis, were identified.

Application of the liquid paraffin emulgel (21.29 µg/ml) depicted the highest average concentration of the diffused lipophilic flurbiprofen within the stratum corneum-epidermis, followed by the olive oil foam (21.47 µg/ml), olive oil emulgel (17.82 µg/ml) and grapeseed oil emulgel (17.78 µg/ml). Very similar concentrations for the marker were demonstrated by the hydrogel (16.73 µg/ml) and crocodile oil emulgel (14.89 µg/ml), whereas a lower concentration was shown for coconut oil emulgel (7.18 µg/ml). The remainder of the formulations yielded concentrations below 3%, i.e. the avocado oil emulgel (2.72 µg/ml), the coconut oil foam (1.57 µg/ml) and finally the emu oil emulgel (1.25 µg/ml).

The penetration of the marker, flurbiprofen, being trapped within the skin seemed to have been enhanced more by the oleic acid (UFA) containing emulgels and foam, especially. This was followed by oils containing high linoleic acid values, which indicated that the more kinked shaped the fatty acids, the more difficult it became to insert themselves within the lipid structures of the stratum corneum, with a resulting accumulation of the marker (Fang *et al.*, 2003:318-319). It therefore seemed that those oils that predominantly consisted of unsaturated fatty acids (UFAs) (grapeseed-, crocodile- and olive oils) seemed to have increased the concentration of the diffused marker more significantly than those oils containing an almost even combination of MUFAs and PUFAs (avocado oil), or those mainly consisting of SFAs (coconut oil).

Average concentrations of the diffused flurbiprofen found in the epidermis-dermis region of the skin for all of the formulations demonstrated low concentrations, ranging between 0.97 - 5.39 µg/ml, with the exception of the emu oil emulgel that presented with a higher concentration of 16.15 µg/ml. The reason for the high accumulation of the marker might have

been as a result of epidermal proliferation, with subsequent accumulation of the marker within the epidermis-dermis due to high oleic- and linoleic acid values, as well as small amounts of palmitoleic acid present within this oil (Katsuta *et al.*, 2005:1011).

The resistance of the epidermis-dermis region to the general permeation of flurbiprofen might have been caused by its lipophilic nature, resulting in a reduced solubility within the hydrophilic environment of this region (Hadgraft, 1999:5).

Median results from the skin diffusion studies demonstrated that the hydrogel (23.79 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) had the highest flux, followed by the olive oil- (17.99 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), liquid paraffin- (15.70 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), coconut oil- (13.16 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), grapeseed oil- (11.85 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), avocado oil- (8.31 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), crocodile oil- (6.68 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) and emu oil emulgels (4.41 $\mu\text{g}/\text{cm}^2\cdot\text{h}$).

The fact that the hydrogel presented a higher flux value for the marker could have been as a result of its high water content that had caused hydration of the skin. Hydration opens up the dense lipid structures inside of the stratum corneum, due to swelling of the corneocytes, with a subsequent increase in the marker's flux (Benson, 2005:28; Ranade & Hollinger, 2004:213). The high flux value of flurbiprofen with the liquid paraffin emulgel might also have resulted from the fact that it occluded the skin, which increased the hydration of the stratum corneum, with a subsequent increase in the flux (Mitsui, 1997:124; Thomas & Finnin, 2004:699).

Results from the skin diffusion studies could be explained by the fact that the fatty acids differ in their hydrocarbon chain by (1) the length of the chain, and (2) the position- and number of the double bonds (Babu *et al.*, 2006:144). It is suggested that fatty acids with hydrocarbon (lipophilic) chains between C_{12} to C_{14} (also present within coconut oil) have an optimal balance of the partition coefficient and its affinity for the skin (Ogiso & Shintani, 1990:1067). It appears as though the branched UFAs, especially oleic acid, present in high quantities in olive oil, were more powerful enhancers of the diffusion of the marker, flurbiprofen (Chi *et al.*, 1995:270).

Foam formulations were manufactured with the olive- and coconut oil emulgels that had demonstrated the best median flux values of flurbiprofen from the natural oil emulgel formulations. These formulated foams, however, did not significantly increased flux values for flurbiprofen through the skin, but only achieved values of 5.56 $\mu\text{g}/\text{cm}^2\cdot\text{h}$ for the olive oil foam and 4.36 $\mu\text{g}/\text{cm}^2\cdot\text{h}$ for the coconut oil foam formulations. The low flux values could have been attributed to the nature of the formulation itself, which was filled with trapped air that could have resulted in the formulation not making optimal direct contact with the available skin surface.

Throughout this study, it became evident that olive oil, predominantly consisting of oleic acid (UFA), was most effective in enhancing the flux of the lipophilic marker, flurbiprofen, through the skin, closely followed by coconut oil consisting of SFAs, with lauric- and myristic acid as its main

constituents. Better enhancement effects were observed with those oils containing high amounts of oleic acid (MUFA), than oils consisting of almost equal amounts of both PUFAs and MUFAs (avocado-, emu- and crocodile oil), or oils mainly consisting of PUFAs (grapeseed oil) as its main components, but their effect was not more significant than the oil containing SFAs (coconut oil) as its key components.

Keywords: Transdermal, natural oils, fatty acids, penetration enhancers, Franz cell diffusion.

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UITTREKSEL

In die farmseutiese wetenskap, behels topikale aflewering die vervoerproses van 'n aktiewe farmaseutiese bestanddeel (AFB) vanaf 'n geformuleerde doseervorm na die beoogde plek van werking. Vir die meeste topikale doseervorme kan die oppervlak van die vel, of die spesifieke lae in die vel, soos die buitenste laag van die stratum korneum, die lipiede tussen-in, die korneosiete midde-in die stratum korneum, die korneosiete hulself, die epidermis, dermis, Langerhans- en Merckle-selle, of die appendageale strukture, die teikenareas vir aflewering wees. Wanneer 'n AFB in die vel afgelewer word, moet dit eerstens vanuit die formulering waarin dit aangewend word diffundeer tot op die oppervlak van die vel. Hiervandaan mag partisie van die aktief tot binne-in die stratum korneum plaasvind; dit mag deur die stratum korneum diffundeer en tot binne-in die lewensvatbare epidermis dring, vanwaar dit verder binne-in die dermis dring en deur die dermis tot binne-in die bloedstroom deursypel (Wiechers, 2008:1-3,7).

Wat die skansfunksie van die vel betref, bevat die intersellulêre spasies binne-in die stratum korneum lipiede waarvan hulle hoof funksie die voorkoming van ongewenste waterverlies deur die vel is en om as 'n noodsaaklike diffusie-skans teen die absorpsie van substansie vanuit die eksterne omgewing te dien. Hierdie komplekse interaksie van lipiede bestaan uit 'n hidrofiele- en lipofiele weerstandsoete. Die fundamentele aspek onderliggend aan die ondeurdringbaarheid van die vel is dus die lipofiele natuur van die stratum korneum (Bouwstra *et al.*, 2003:4; Franz & Lehman, 2000:25; Walker & Smith, 1996:296).

'n Algemene benadering tot die bevordering van swak deurdringbare AFBe in transdermale aflewering is die inkorporering van chemiese penetrasie-bevorderaars in afleweringsisteme, ten einde die partisie van AFBe tot binne-in die stratum korneum te verhoog. Hierdie chemiese middels staan ook as versnellers, bemiddelaars en absorpsie-bemiddelaars bekend. Chemiese penetrasie-versnellers word in topikale doseervorme geïnkorporeer en gewoonlik vind partisie daarvan tot binne-in die stratum korneum ook plaas waar hulle tydelik en omkeerbaar die fundamentele diffusie-skans ontwig om sodoende die absorpsie van die AFB tot binne-in die vel te fasiliteer (Büyüktimkin *et al.*, 1997:358-359; Sinha & Kaur, 2000:1131; Walker & Smith, 1996:296). Die meganisme vir hierdie verhoging in die diffusie van die AFB behoort dus die oplosbaarheid en partisie vanaf die formulering tot binne-in die vel te verbeter. Dit behoort voorts ook die oplosbaarheid van die AFB binne die vel te verhoog en sy deurdringbaarheid en diffusie-koëffisiënt te verbeter (Rajadhyaksha *et al.*, 1997:489).

Vetsure is daarvoor bekend dat hulle effektief daarin slaag om die penetrasie van transdermaal-afgelewerde hidrofiele en lipofiele AFB_e te verbeter. Baie penetrasie-versnellers bevat versadigde en onversadigde koolwaterstofkettings. Oleïensuur is 'n baie populêre vetsuur wat gereeld gebruik word (Williams & Barry, 2004:609-610) in transdermale formulerings. Vetsure versteur die lipied-organisasie van die intersellulêre lipiede binne die stratum korneum, wat hierdie lipied-dubbellae vloeibaar maak, ten einde die stratum korneum meer deurlaatbaar vir AFB_e te maak. Hulpmiddels met polêre (hidrofiele) groepe aan die bopunt en lang, hidrofobiese koolstofkettings kan tot tussen-in die intersellulêre lipiede van die stratum korneum penetreer, waar hulle dan hierdie endogene lipied-komponente versteur, om sodoende diffusie van 'n AFB binne-in die vel te verhoog (Barry, 2006:9-10; Hadgraft & Finnin, 2006:367-368; Kanikkannan *et al.*, 2006:18; Williams & Barry, 2004:610).

Natuurlike olies word algemeen in topikale doseervorme gebruik en was dus 'n voor-die-hand-liggende keuse in hierdie studie. Olies is in 'n vloeistofvorm by kamertemperatuur terwyl vette in 'n soliede vorm voorkom. Olies is relatief maklik bekombaar vanaf beide plante en diere. Die hoofbestanddele van vette en olies, is trigliseriedes wat bestaan uit vetsure en gliserol. Olies beheer die verdamping van vog vanaf die vel, smeer maklik en eenvormig aan en word gedeeltelik in die vel afgebreek om waardevolle vetsure vry te stel (Fang *et al.*, 2004:170,173; Lautenschläger, 2004:46; Mitsui, 1997:121-122).

Die fokus van hierdie studie was nie formulering as sodanig nie, maar het die formulering behels van avokado-, druiewepit-, emu-, krokodil- en klapperolie in semisoliede emulgelle asook twee skuimformulerings. Hierdie formulerings het ten doel gehad om vas te stel wat die penetrasie verhogende eienskappe van die ses natuurlike olies se vetsuur-inhoud op flurbiprofeen was, wat geskies was as die merker AFB. Die emulgelle is ook vergelyk met dieselfde emulgel formulering wat vloeibare paraffin bevat het sowel as 'n hidrogel sonder die insluiting van olie.

Die ses natuurlike olies is deur middel van gaskromatografie (GK) geanaliseer, ten einde die vetsuurinhoud daarvan te kwantifiseer, terwyl dit ook kwalitatiewe inligting verskaf het deur die retensie-tye van die bestanddele wat alkielkettings bevat het, aan te dui (Mitsui, 1997:260). Die data wat deur die GK gegenereer is, het aangedui dat olyf- (76%), advokado- (68%), emu- (46%) en krokodilolie (40%) 'n hoë oleïensuurwaarde getoon het (ook bekend as 'n mono-onversadigde vetsuur) (MOVS). Laer vlakke van oleïensuur is in druiewepit- (27%) en klapperolie (8%) waargeneem. Die enigste olie wat hoër vlakke van die poli-onversadigde vetsuur (POVS), linoleïensuur bevat het, was druiewepitolie (61%), terwyl die res van die olies vlakke laer as 24% aangetoon het. Daarteenoor wou dit voorkom asof klapperolie die enigste olie, ryk in versadigde versure (VVSe) was, wat lauriensuur (52%) en laer vlakke van miristiensuur (21%) bevat het. Gemiddelde vlakke van palmitiensuur (VVS) is waargeneem in

krokodil- (21%) en emu-olie (21%), beide van dierlike oorsprong, terwyl advokado-, druiwepit-, olyf- en klapperolie van plantaardige oorsprong, minder as 15% aangetoon het. Steariensuur was ook in vlakke onder 10% in al hierdie olies teenwoordig, terwyl die olies van dierlike oorsprong die hoogste waardes getoon het.

'n Hoë-druk vloeistofkromatografiese (HDVK) metode is ontwikkel en gevalideer om die konsentrasie van die merker, flurbiprofeen, na diffusie vanuit die formulerings in die vel in asook die konsentrasie wat deur die vel gediffundeer het te bepaal. Franz-sel membraandiffusie-studies is uitgevoer voor aanvang van die veldiffusie-studies, ten einde seker te maak dat die merker wel vanuit die semi-soliede formulerings vrygestel word.

Veldiffusie-studies is op dermatoom-uitgesnyde menslike vel, waarop die ses emulgelformulerings aangewend is, wat die natuurlike olies bevat het, uitgevoer. 'n Vergelykende studie is gedoen, deur van die emulgel met vloeibare paraffien en die hidrogel gebruik te maak, ten einde die diffusie van die merker, flurbiprofeen, in en deur die vel te vergelyk. Die twee olie-emulgelle wat die beste vloedwaardes gelever het, is daarna in skuimpreparate geformuleer, met die doel om die olies se penetrasie-versnellende vermoëns op flurbiprofeen in skuimvorm, met hulle penetrasie-versnellingseffekte in emulgelvorm te vergelyk. Die data van al tien formulerings is vergelyk en die formulerings wat die beste resultate met betrekking tot die mediaan vloedwaardes, asook die flurbiprofeen konsentrasies in die stratum korneum-epidermis en epidermis-dermis gelever het, is geïdentifiseer.

Aangewende vloeibare paraffien-emulgel (21.29 µg/ml) het veroorsaak dat die hoogste konsentrasie van die gediffundeerde flurbiprofeen binne die stratum korneum bereik is, gevolg deur olyfolie-skuim (21.47 µg/ml), olyfolie-emulgel (17.82 µg/ml) en die druiwepitolie-emulgel (17.78 µg/ml). Byna dieselfde konsentrasies van die merker, is deur die hidrogel (16.73 µg/ml) en die krokodilolie-emulgel (14.89 µg/ml) verkry, terwyl laer konsentrasies vir die klapperolie-emulgel (7.18 µg/ml) bereik is. Die res van die formulerings het konsentrasies onder 3% getoon, naamlik die advokado-olie-emulgel (2.72 µg/ml), klapperolie-skuim (1.57 µg/ml) en die emu-olie-emulgel (1.25 µg/ml).

Penetrasie van die merker, flurbiprofeen, is tot 'n hoër mate deur die oleïensuur- (onversadigde vetsuur) (OVS) bevattende formulerings en skuim verhoog. Dit is gevolg deur olies wat hoë linoleïensuur-konsentrasies getoon het, wat aangedui het dat hoe meer verbuig die koolstofketting binne die vetsure was, hoe moeiliker dit vir hulle geword het om hulself binne-in die stratum korneum in te dring, met 'n gevolglike akkumulاسie van die merker (Fang *et al.*, 2003:318-319). Dit het voorgekom asof die olies wat hoofsaaklik uit OVSe (druiwepit-, krokodil- en olyfolie) bestaan, die gediffundeerde merker se konsentrasie oënskynlik meer verhoog het

as daardie olies wat uit bykans gelyke hoeveelhede MOVSe en POVSe (advokado-olie), of hoofsaaklik uit VVSe (klapperolie) bestaan het.

Konsentrasies van die gediffundeerde flurbiprofeen wat in die epidermis-dermis-area van die vel vir al die formulerings gevind is, het redelike lae vlakke, wat tussen 0.97 - 5.39 $\mu\text{g/ml}$ gewissel het, getoon, met die uitsondering van emu-olie-, wat 'n hoër konsentrasie van 16.15 $\mu\text{g/ml}$ gelewer het. Die rede vir die hoër akkumulasie mag as gevolg van epidermale proliferasie met meegaande akkumulasie van die merker binne die epidermis-dermis wees, omrede hoë oleïen- en linoleïensuur vlakke en 'n lae palmitoleïensuur vlakke teenwoordig is in hierdie olie (Katsuta *et al.*, 2005:1011).

Weerstand van die epidermis-dermis-area teen die algemene deurdringbaarheid van die merker, flurbiprofeen mag as gevolg van flurbiprofeen se lipofiele natuur, wat tot 'n verlaagde oplosbaarheid midde die hidrofiele omgewing van hierdie area aanleiding gee, gewees het (Hadgraft, 1999:5).

Mediaan-resultate van die veldiffusie-studies het aangetoon dat die hidrogel (23.79 $\mu\text{g/cm}^2\cdot\text{h}$) die hoogste vloedwaarde het, gevolg deur olyfolie- (17.99 $\mu\text{g/cm}^2\cdot\text{h}$), vloeibare paraffien- (15.70 $\mu\text{g/cm}^2\cdot\text{h}$), klapperolie- (13.16 $\mu\text{g/cm}^2\cdot\text{h}$), druïewepitolie- (11.85 $\mu\text{g/cm}^2\cdot\text{h}$), advokado-olie- (8.31 $\mu\text{g/cm}^2\cdot\text{h}$), krokodilolie- (6.68 $\mu\text{g/cm}^2\cdot\text{h}$) en emu-olie-emulgels (4.41 $\mu\text{g/cm}^2\cdot\text{h}$).

Die feit dat die hidrogel 'n verhoogde vloedwaarde getoon het, kon as gevolg van sy hoë waterinhoud, wat hidrasie van die vel veroorsaak het, gewees het. Hidrasie maak die digte lipiedstrukture midde-in die stratum korneum oop, as gevolg van die swelling van die korneosiete, met 'n gevolglike verhoging in die vloed van die merker (Benson, 2005:28; Ranade & Hollinger, 2004:213). Die vloedwaarde van flurbiprofeen in die vloeibare paraffien-emulgel mag moontlik die gevolg gewees het van die feit dat dit die vel omsluit/afseël, wat hidrasie in die stratum korneum verhoog, met 'n gevolglike verhoging in die van die vloed van die merker in die vel in (Mitsui, 1997:124; Thomas & Finnin, 2004:699).

Die resultate van die veldiffusie-studies kan verklaar word deur die feit dat die vetsure verskil in hul koolwatersof-ketting wat betref (1) die lengte van die ketting, en (2) die posissie- en die getal dubbel-bindings (Babu *et al.*, 2006:144). Daar word aanvaar dat vetsure met koolwaterstof- (lipofiele) kettings tussen C_{12} en C_{14} (ook teenwoordig in klapperolie) 'n optimale balans tussen die partisie-koëffisiënt en hul affiniteit vir die vel het (Ogiso & Shintani, 1990:1067). Daarteenoor blyk dit dat die vertakte OVSe, veral oleïensuur, teenwoordig in hoë hoeveelhede in olyfolie, meer kragtige versnellers van die diffusie van die merker, flurbiprofeen is (Chi *et al.*, 1995:270).

Skuimformulerings is met die olyf- en klapperolie-emulgelle, wat die beste mediaan vloedwaardes vir flurbiprofeen vanuit die formulerings van die natuurlike olies getoon het, vervaardig. Hierdie geformuleerde skuime het egter nie goeie vloedwaardes vir flurbiprofeen deur die vel getoon nie, maar het waardes van slegs $5.56 \mu\text{g}/\text{cm}^2\cdot\text{h}$ vir die olyfolie-skuim en $4.36 \mu\text{g}/\text{cm}^2\cdot\text{h}$ vir die klapperolie-skuim gelever. Die lae vloedwaardes is moontlik aan die natuur van die formulerings self toeteskryf, aangesien dit vol vasgevangde lugborrels was, wat daartoe aanleiding kon gee dat die skuimformulering nie optimale direkte kontak met die beskikbare veloppervlak kon maak nie.

Regdeur die studie het dit duideliker geword dat olyfolie, wat hoofsaaklik uit oleïensuur (OVS) bestaan, die beste daarin geslaag het om die vloedwaarde van die lipofiele flurbiprofeen deur die vel te verhoog, gevolg deur klapperolie, bestaande uit laurien- en miristiensuur (VVS) as sy hoofbestanddele. Beter penetrasie-verhogende effekte is vir daardie olies, met hoë oleïensuur-inhoud (MOVSe) waargeneem, as vir olies wat uit bykans gelyke hoeveelhede van beide MOVSe en POVSe (advokado-, emu- en krokodilolie) bestaan het, of olies wat hoofsaaklik POVSe (druiwepitolie) as hul hoofbestanddeel bevat het, maar hul effek was nie meer as die olie wat hoofsaaklik uit VVSe (klapperolie) bestaan het nie.

Sleutelwoorde: Transdermaal, natuurlike olies, vetsure, penetrasie- bevorderaars, Franz-sel diffusie.

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

Transdermal drug delivery offers certain advantages to other routes of administration, in that it is non-invasive and a patient-friendly option for therapeutic treatment. Transdermal delivery may (1) improve bioavailability by avoiding the hepatic first-pass effect, (2) avoid gastrointestinal irritation, (3) have a longer duration of action, (4) reduce the dosing frequency and therefore have the potential of better patient compliance, (5) reach the deeper underlying tissue without the risk of systemic side effects, and (6) control the plasma levels of certain potent active pharmaceutical ingredients (APIs), as their input may be promptly interrupted, should toxicity occur (Pfister, 1997:38-39; Ranade & Hollinger, 2004:208; Singh, 1999:598).

The site of action for most topical formulations is the surface of the skin, or the specific layers within it. Various target sites on or within the skin can be distinguished (see Figure 2.1), i.e. the outermost layer of the stratum corneum; the lipids between the corneocytes (filled with keratin fibres) within the intercellular, lipid rich matrix of the stratum corneum; the epidermis; dermis; hair follicles; sebaceous- or eccrine glands; melanocytes, and the Langerhans or Mercle cells (Ghosh & Pfister, 1997:5; Moghimi *et al.*, 1996:117; Wiechers, 2008:3). The corneocytes are attached to each other by a protein-rivet structure, called desmosomes that contributes to the overall cohesion of the stratum corneum. The stratum corneum thus constitutes the main barrier to cross in the transdermal delivery of APIs (Asbill & Michniak, 2000:36; Charoo *et al.*, 2005:346). Human skin therefore acts as a very protective shield to substances from the environment, including pharmaceutical and cosmetic commodities (Boelsma *et al.*, 1996:729). To diminish the barrier properties of the skin temporarily, penetration enhancers are employed (Nanayakkara *et al.*, 2005:130). Fatty acids are frequently used penetration enhancers in formulations for transdermal and topical delivery of substances. These fatty acids comprise an individual group with very prominent properties (Boelsma *et al.*, 1996:729) and are a familiar component of the human skin (Lampe *et al.*, 1983:120).

Therapeutic APIs are applied to the surface of the skin for (1) dermatological delivery within the skin, (2) localised-regional delivery, and (3) transdermal, systemic delivery (Walters & Brain, 2002:320). APIs with low molecular weight and high lipid solubility are especially suitable for transdermal drug delivery (Schulmeister, 2005:48). Given that diffusion of an API is a passive process, APIs with low solubility and low affinity for hydrophilic and lipophilic elements within the

stratum corneum would in theory partition slowly. Therefore, a common method used for the promotion of certain inadequately structured APIs into the skin, is the incorporation of an enhancer into a formulation that serves as an adequate vehicle for delivery (Walker & Smith, 1996:296). These types of penetration enhancers commonly partition into the skin and interact with the different skin constituents (Büyüktimkin *et al.*, 1997:358-359), whilst facilitating the absorption of APIs through the skin (Sinha & Kaur, 2000:1131).

Natural oils are multifaceted compounds that contain saturated and unsaturated fatty acids and have been tested as penetration enhancers. These oils are often used in the cosmetic industry and are thus considered a safe option (Büyüktimkin *et al.*, 1997:433). It is believed that fatty acids increase the skin's permeation by disrupting the packed structure of the intercellular lipid domain within the stratum corneum, and increase the diffusion and partitioning of APIs across this lipid barrier. Fatty acids have mainly been examined as penetration enhancers for lipophilic drugs (Babu *et al.*, 2006:138,154), since in transdermal permeation, it has become known that lipophilic APIs tend to absorb faster than hydrophilic APIs. The lipophilic nature of an API is therefore a desired feature for a chosen transdermal API (Williams, 2003:37).

The aim of the present study was not formulation as such, but to investigate the potential penetration enhancing effects of a series of natural oils from plant and animal origins, using flurbiprofen, a lipophilic API, as a marker to be able to distinguish between the enhancing effects of these oils. The saturated and unsaturated fatty acids present within these oils were assessed, as well as their potential activity on the delivery of flurbiprofen into and across the skin. In order to determine the effects of a series of fatty acids on the skin, *in vitro* diffusion studies were conducted. Membrane diffusion studies were performed beforehand, in order to determine the release of the marker from all of the formulations tested. Thereafter, dermatomed excised human skin was used to determine the impact(s) of these fatty acids on the transdermal permeation of the marker. It was believed that the applied emulgel formulations, containing the specific natural oils with their selection of fatty acids, would react with the intercellular lipids within the stratum corneum by temporarily altering the fluidity thereof and to allow an enhanced partitioning of the marker into and through the skin.

During this study, six oils were investigated, four from plant -and two from animal origin. They were avocado-, grapeseed-, olive-, coconut-, emu- and crocodile oils. These oils were each incorporated into a standard emulgel formulation and compared to an equivalent emulgel formulation containing liquid paraffin, as well as to a hydrogel without the inclusion of oil. The aim of these experiments was to determine the extent to which these oils would affect the permeation of the lipophilic marker, flurbiprofen. Two oil emulgels that showed the best flux values were then formulated into foam applications and subsequently tested *in vitro* on the skin in order to determine their penetration effects on the marker.

The objectives of this study were to:

- develop and validate a high performance liquid chromatography (HPLC) method to quantify the flurbiprofen extracted receptor phase sampled during the membrane and *in vitro* diffusion studies; as well as the concentration of the diffused flurbiprofen in the different skin layers;
- determine the fatty acids' percentage composition of each of the oils used in this study by gas chromatography (GC);
- formulate six emulgel delivery systems, consisting of the selected natural oils, as well as two emulgels, one containing liquid paraffin and another without the inclusion of oil (hydrogel), for testing during comparative studies;
- formulate foams consisting of the natural oil emulgels that depicted the highest flux values;
- determine the release rate of the marker flurbiprofen from the formulated emulgels and hydrogel through membrane diffusion studies, prior to conducting the skin diffusion studies;
- determine whether the fatty acids have improved delivery into (topical) the skin by conducting tape stripping studies, using dermatomed excised, human skin; and to
- determine whether the fatty acids have improved delivery through (transdermal) the skin by conducting *in vitro* skin diffusion studies, using dermatomed excised, human skin.

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CHAPTER 2

THE EFFECTS OF NATURAL OILS ON TRANSDERMAL DELIVERY WITH FLURBIPROFEN AS MARKER

2.1 INTRODUCTION

In recent years, there has been a renewed interest in the transport of substances through human skin, although it has been one of the world's oldest researched methods for delivering APIs (Stanos, 2007:343). The skin's popularity stems from the fact that it represents the largest and most easily accessible organ of the human body. Despite being an excellent biological barrier that prevents the flux of foreign chemicals to permeate through the skin, whilst keeping water and other essential substances in and unfamiliar materials out (Cevc & Vierl, 2010:279; Franz & Lehman, 2000:15), more healthcare providers and the pharmaceutical industry are reassessing the probable advantages associated with topical applications (Stanos, 2007:343).

Permeation of compounds across the skin is normally controlled by the stratum corneum (Walters & Brain, 2009:475-476), the uppermost layer of the skin and classified as the rate limiting step in transdermal absorption of chemicals (Asbill & Michniak, 2000:36; Barry, 2007:569). Due to the lipophilic nature of the stratum corneum, the physicochemical properties of an API are of utmost importance when considered for transdermal delivery (Bonner & Barry, 2006:257). These properties determine the rate at which an API will permeate across this layer *via* the intercellular lipids, as the main penetration route of the skin (Walters & Brain, 2009:478). Transdermal delivery is often limited, due to the reduced permeability of an API that does not possess of ideal physicochemical properties (Boelsma *et al.*, 1996:729; Fang *et al.*, 2003b:153; Fang *et al.*, 2004:164).

Research has been focusing on methods to increase the permeability characteristics of the intact human stratum corneum. Amongst these methods is the employment of penetration enhancers in transdermal formulations (Benson, 2005:23), aimed at increasing the diffusion coefficient of the applied formulation into the stratum corneum (Williams & Barry, 2004:605). Warner *et al.* (2001:1151-1152) conclude that the barrier properties of the stratum corneum were reduced when permeation enhancers were included in transdermal formulations.

Penetration enhancers act on the intercellular lipid layers of the stratum corneum by disrupting the packing of these lipids. Furthermore, in several cases it was found that penetration enhancers promoted a localised effect by increasing the drug concentration of an API within the skin (Foldvari, 2000:419). However, according to Charoo *et al.* (2008:301), numerous chemicals that have shown successful penetration enhancing abilities, unfortunately possess localised and systemic side effects. Therefore, investigation of possible penetration enhancers that are easy to obtain, economical, safe and effective, is therefore imperative.

Natural products, e.g. oils consisting of a mixture of glycerol and fatty acids, were used during this study as enhancers, due to their “safety profiles”. Worldwide, it has become extremely popular to employ natural products to benefit people’s health. Vegetable oils, for example, are being used as emollients, whereas fatty acids are used as moisturisers and antioxidants. Fatty acids are also frequently used as penetration enhancers to promote skin permeation, of which oleic acid is an excellent example. An important penetration enhancer, which has been successfully tested to increase the permeability of a variety of APIs, is the highly lipophilic Azone®. Azone®, having a similar structural activity as fatty acids, consists of a polar head and lipophilic alkyl chain with saturated and unsaturated hydrocarbon bonds. It interferes with the lipid spheres within the stratum corneum, thus causing the bilayers to become disrupted and in doing so, enhances permeability of APIs. Fatty acids may possess a similar mechanism of action, due to its similar structural activity (Aungst, 1989:246; Charoo *et al.*, 2008:301; Fang *et al.*, 2004:163; O’Brien, 2009:265; Schneider, 2009:466; Williams & Barry, 2004:608-609). Fang *et al.* (2004:170) studied the fatty acid-containing, *Botryococcus braunii*, a type of green algae (high in poly-unsaturated fatty acids), using lipophilic flurbiprofen as a marker API. This study showed encouraging results regarding the extraction of the intercellular lipid bilayers of the stratum corneum and the effective promotion of the absorption of flurbiprofen into and across the skin.

Natural oils are internationally available and their extraction methods from plants and animals can be reproduced effortlessly. If found to have penetration enhancing properties, they could serve as more acceptable natural excipients that may offer economical alternatives to the usually expensive, commercially available, pure fatty acids (Fang *et al.*, 2004:170,173). This study therefore focused on examining the penetration enhancing abilities of the fatty acids found in selected natural oils, using flurbiprofen, as a marker, with the purpose of comparing the enhancing abilities, if any, of these natural oils.

As discussed next, it is imperative to understand the structure and characteristics of human skin in order to explain differences found in the permeation effects of the various oils.

2.2 THE STRUCTURE AND FUNCTIONS OF HUMAN SKIN

The surface area of the skin of an average adult person is approximately 1.6 m², whilst its thickness varies with age, sex and site. Male skin is commonly thicker than that of women, although the average woman has a thicker subcutaneous layer of fat (Mitsui, 1997:13). On average, a square centimetre of skin contains 10 hair follicles, 15 sebaceous glands, 12 nerves and 100 sweat glands, 360 cm of nerves and three blood vessels (Asbill & Michniak, 2000:36).

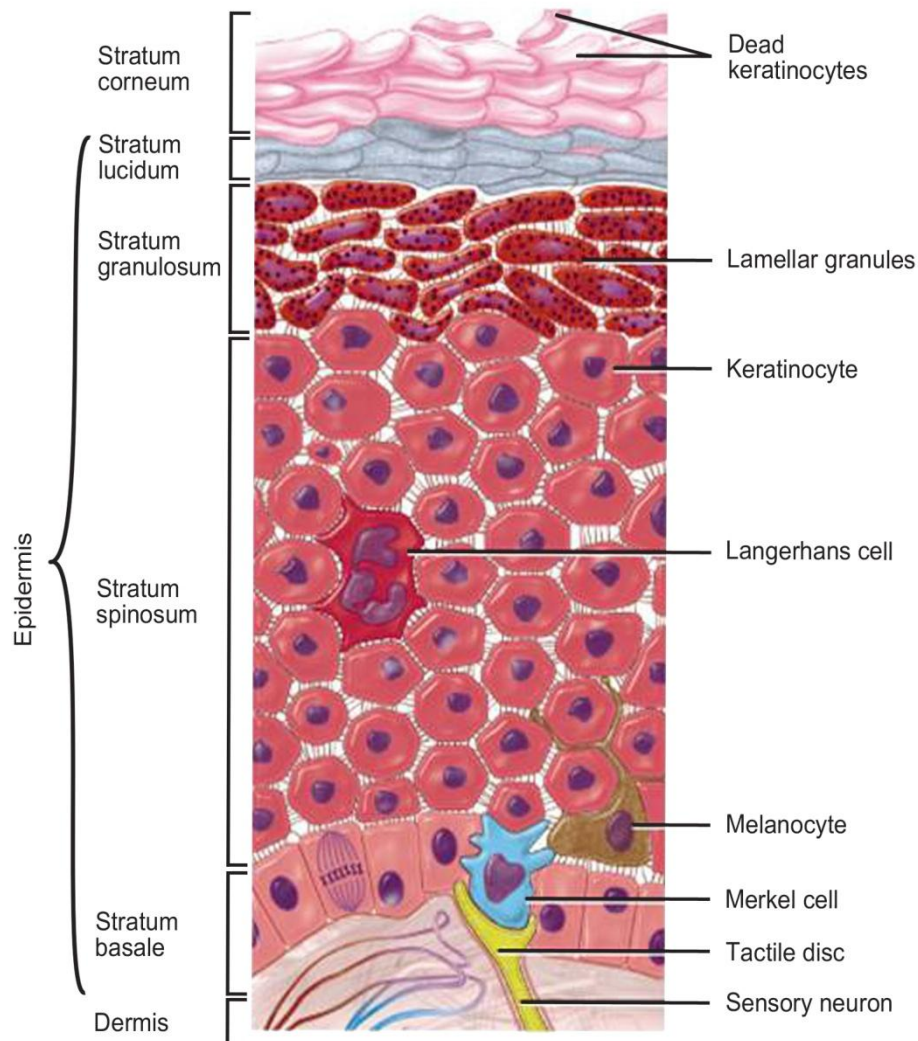


Figure 2.1: Anatomical view of the three main structures of the human skin (Adapted from: Morrell, 2009).

The main functions of the skin are to prevent the body from losing water to the environment and to block the entry of exogenous agents into the skin (Barry, 1983:17). The functions of the skin can be summarised as follows (Flynn, 2002:188):

- protection barrier from harmful external stimuli and mechanical shock, as well as a microbiological-, chemical-, electrical- and radiation barrier;

- mechanical function (confining underlying tissue);
- intra-species identification (also through apocrine secretions);
- synthesis and metabolism;
- containment of body fluids and tissues;
- sensory perception;
- absorption of fat soluble materials (water soluble materials are not readily absorbed);
- blood pressure regulation through circulation and evaporation methods; and
- thermoregulation (primarily responsible for maintaining body temperature at 37 °C) (Barry, 1983:1, 16-20; Mitsui, 1997:13, 19-21).

Human skin comprises of several layers, i.e. the stratum corneum (outermost layer), the viable epidermis, the dermis and the lower layers of adipose tissue. The skin forms an appealing and accessible route for the systemic delivery of APIs. However, as a result of its effective barrier properties that prevent the permeation of any agent, only a few APIs are able to penetrate the stratum corneum (Asbill & Michniak, 2000:36-37). Figure 2.1 is an anatomical illustration of the three main structures of the skin, i.e. the stratum corneum, the viable epidermis and the dermis. These layers, as well as the skin appendages (not shown in the figure) are discussed next.

2.2.1 Skin surface

Table 2.1: The composition of human skin surface lipids (Adapted from: Downing *et al.*, 1969:326)

Lipids	Mean percentage (%)
Cholesterol	1.4
Cholesterol esters	2.1
Diglycerides	2.2
Fatty acids	16.4
Squalene	12.0
Triglycerides	41.0
Wax esters	25.0

The principal structural characteristic of this skin layer is to operate as a protective acid mantle. It has a pH value of 4.2 - 5.6 (Barry, 1983:32). The skin surface is the first zone any API must cross in order to be absorbed into the skin and is regarded as a surface film of “emulsifying lipids” (sebum) (Mezei, 1994:172). Skin surface lipids are derived from and excreted to the skin surface by way of the hair follicle (Downing & Strauss, 1974:228; Mitsui, 1997:17). They consist of a mixture of sebum from the sebaceous glands and lipids formed by the keratinising, viable

epidermis (Greene *et al.*, 1970:240). These skin surface lipids moisturise and keep the stratum corneum supple, as well as prevent transepidermal water loss (TEWL) from the skin. These lipids play an important role in the barrier functioning of the stratum corneum and avert entry of harmful substances and bacteria (Bouwstra *et al.*, 2003:5; Mitsui, 1997:17). The composition of human skin lipids is shown in Table 2.1, and approximately 57% of the total amount is made up of triglycerides and fatty acids. Free fatty acids are released from the triglycerides by bacteria inside the hair canal and on the skin surface (Downing & Strauss, 1974:231; Katsuta *et al.*, 2005:1008).

2.2.2 The stratum corneum

The lipophilic stratum corneum consists of 10 - 15 layers of corneocytes, varying in thickness, depending on its level of hydration. It is approximately 15 µm thick in the dry state and 40 µm when hydrated (Benson, 2005:24). When the stratum corneum is exposed to moisture, the corneocytes swell out in a vertical way to open up this compact structure (Barry, 2001b:969). Corneocytes are mainly composed of insoluble, bundled keratins that are stabilised through covalently bound lipids and cross-linked proteins (Asbill & Michniak, 2000:36). Anatomically, the stratum corneum is a multifaceted membrane that comprises of lipids and proteins. These components are arranged in a complex, interlocking structure that is highly impermeable (Shah, 1994:20). Hence, it is considered to be the rate limiting barrier in selective chemical transdermal permeation of most APIs, because of its diffusional resistance (Barry, 1983:7, 17).

The most important barrier for an API to cross is the “brick” (keratin rich corneocytes) and “mortar” (intercellular lipid rich matrix) arrangement of the stratum corneum (Benson, 2005:24; El Maghraby *et al.*, 2008:204). The mortar of lipid layers consists of ceramides, fatty acids, cholesterol and cholesterol esters (Barry, 2006:4-5). Stratum corneum lipids average about 5 - 15% of the dry tissue weight, whilst the principal lipid class comprises the ceramides (Moghimini *et al.*, 1999:518). Permeation enhancement methods aim at disrupting or circumventing this brick and mortar domain (Barry, 2006:5). Neutral, interstitial lipids further contribute to the barrier function of the stratum corneum. The main lipid composition of the stratum corneum comprises of neutral lipids, whilst both saturated and unsaturated fatty chains exist within these lipids, with the unsaturated chains dominating. Unlike other biological membranes, the stratum corneum contains no phospholipids. The transdermal permeation of APIs is classified as an interfacial division into the lipid skin surface and the aqueous phase that facilitates the molecular diffusion of APIs through this barrier. Hydrolysis of triglycerides (lipids), derived from subcutaneous fat through lipase action, produces fatty acids on the skin surface. The sebaceous fatty acids generally comprise of C₁₆ - C₁₈-carbon chains and high proportions of mono-saturated and branched fatty acids. The stratum corneum contains more

C₂₀-carbon chains, saturated and straight chained fatty acids (Barry, 2006:4-5; Ghosh & Pfister, 1997:6; Hadgraft & Finnin, 2006:362; Norlén, 2008:64; Roy, 1997:141).

2.2.3 The viable epidermis

Underneath the hydrophobic stratum corneum is the hydrophilic viable epidermis that is less than 200 µm thick (Robinson *et al.*, 1997:60). As illustrated in Figure 2.1, it consists of four distinct layers, i.e. the stratum germinativum (basal layer), stratum spinosum (spinous layer), stratum granulosum (granular layer) and the stratum lucidum (Barry, 1983:6; El Maghraby *et al.*, 2008:204).

The epidermis contains keratinocytes (each at varying stages of differentiation), melanocytes (produces the skin pigment, melanin), Langerhans cells (for antigen presentation and immune response with a protective mechanism against the invasion of foreign materials), and Merkel cells (involved in sensory perception) (Asbill & Michnaik, 2000:36; Mitsui, 1997:13). The nerves present in the epidermis and dermis are called the cutaneous nociceptors (Stanos, 2007:344). Epidermal cells (keratinocytes) divide rapidly, mature and finally desquamate (Moghimi *et al.*, 1999:516).

Since the epidermis does not contain any blood vessels, the nutrients and waste products have to diffuse across the dermal-epidermal junction (El Maghraby *et al.*, 2008:204).

2.2.4 The dermal-epidermal junction

Below the basal cell layers of the epidermis resides this complicated junction that is not flat, and when observed using light microscopy, individual papillae's can be seen. The dermal-epidermal junction decreases in surface area with age and may be involved in the permeation of large molecular weight proteins and peptides (Asbill & Michnaik, 2000:36; Barry, 1983:4).

2.2.5 The dermis

Underneath the layered epidermis resides the dermis with a thickness of about 2 000 - 5 000 µm (Robinson *et al.*, 1997:60). The dermis contains endothelial cells, mast cells and fibroblasts. The dermis is the primary site in which inflammation of the skin occurs in reaction to any form of injury. In cases of inflammation, psoriasis and wound healing macrophages, lymphocytes and leukocytes may also be observed (Asbill & Michniak, 2000:36; Singh, 1999:597).

Mast cells produce histamine and serotonin, responsible for immediate allergic responses, while fibroblasts synthesise and secrete the extracellular matrix of which collagen is the principle protein. Elastic fibres (elastin) within the extracellular matrix are connected to each other to

form cross-links that maintain tissue elasticity, whereas collagen maintains the form of the tissue to act as a mechanical scaffold of strength. Collagen accounts for about 70% of the dry weight of the skin (Asbill & Michnaik, 2000:36; Mitsui, 1997:14).

Embedded within the dermis are blood and lymphatic vessels, nerve endings, hair follicles, and sebaceous- and sweat glands. Sweat glands and hair follicles open directly on the skin surface and are called the appendageal route in skin permeation (Barry, 1983:7-8; El Maghraby *et al.*, 2008:204). Blood supply in the dermis is imperative to the systemic absorption of substances applied to skin (Singh, 1999:597). The dermal papillary layer is so rich in capillaries that most APIs that cross the stratum corneum clear within minutes (Barry, 2001a:102). The dermis needs a rich supply of blood to control temperature and pressure, whilst it also absorbs and systemically dilutes most APIs that penetrate through the stratum corneum and epidermis. This ensures that an API's concentration gradient across the epidermis is at a maximum, as the concentration gradient provides the driving force for diffusion. The generous supply of blood volume within the dermis also functions as a "sink" with regards to the diffused APIs reaching it during transdermal absorption (Barry, 1983:9).

2.2.6 The hypodermis

The hypodermis consists of a fatty, subcutaneous layer, with its main function being the regulation of the body's temperature (Mitsui, 1997:15; Moghimi *et al.*, 1999:515). Serving as a protective cushion between the external skin layers and internal structures, i.e. bone and muscle, it also insulates the body against the outside environment. This interior layer of adipose tissue functions as support to both the epidermis and dermis and allows for skin mobility, whilst moulding the body and adding contours, whereas it also serves as an energy source to the body (Barry, 1983:10; Singh, 1999:597).

2.2.7 The skin appendages

The stratum corneum is breached by appendages, consisting of the following:

- hair follicles;
- eccrine and apocrine sweat glands; and
- sebaceous glands.

These appendages provide parallel diffusion pathways through the stratum corneum (Shah, 1994:20) that provide an alternative diffusional route for hydrophilic and high molecular weight APIs (Ghosh & Pfister, 1997:6). These appendages are discussed further in Section 2.3.2.

2.3 ROUTES OF API TRANSPORT THROUGH HUMAN SKIN

The first step in transdermal delivery is the partitioning of the API into the stratum corneum. Only molecules bordering the skin can partition from the applied topical formulation into the tissue, with molecular diffusion within the formulation depending on the nature of the formulation (i.e. viscosity). As illustrated in Figure 2.2, once the API has partitioned through the outermost layer of the stratum corneum, it diffuses through parallel pathways. After reaching the stratum corneum, the molecule partitions into the viable epidermis and diffuses through it. At the dermal-epidermal junction, it partitions into the dermis and into the capillaries. The molecule then partitions through the capillaries and enters the blood stream (Williams, 2003:29-30).

Any API will penetrate the skin through that route that offers the least resistance to passive diffusion. The physicochemical properties of the API, the formulated dosage form and the state of the skin determine which pathway will dominate (Robinson *et al.*, 1997:61).

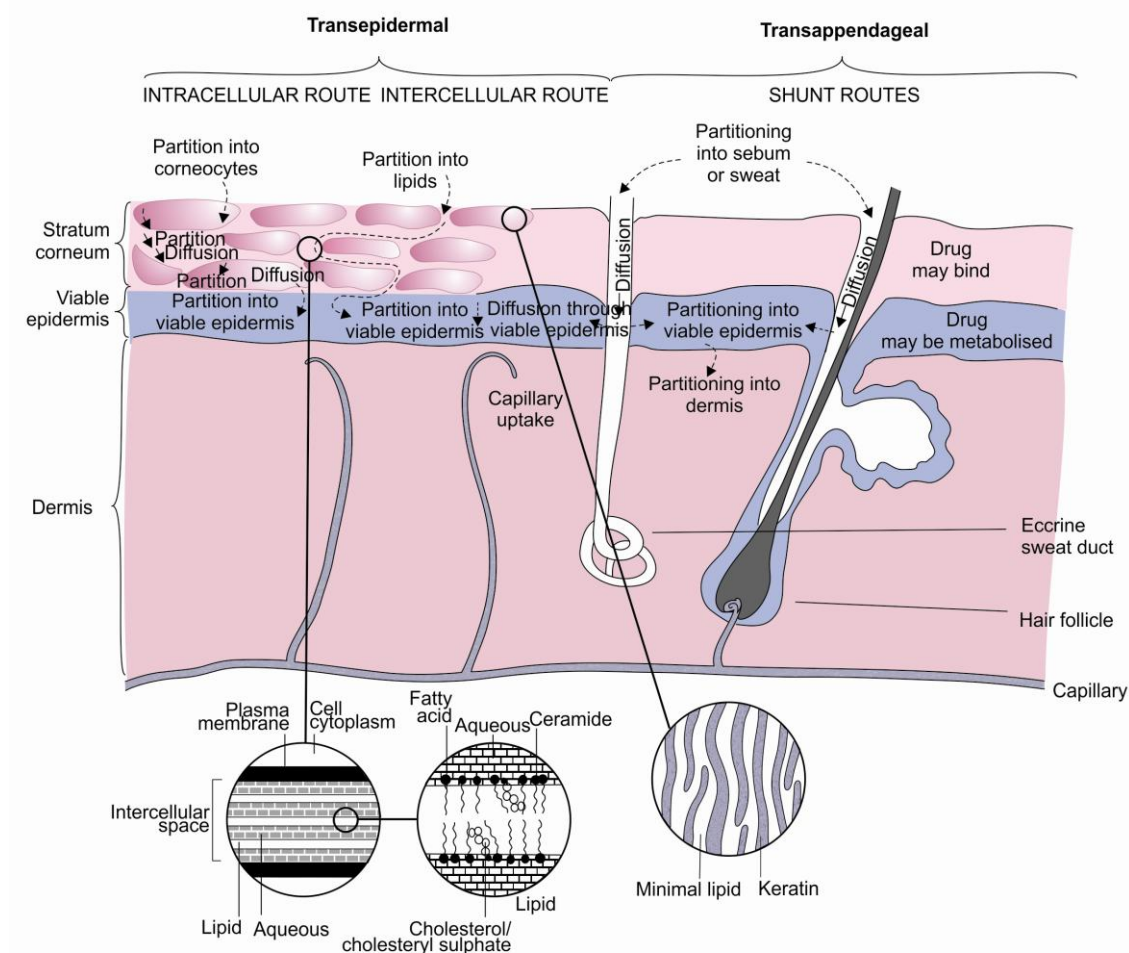


Figure 2.2: API transport through the skin (Adapted from: Barry, 2006:5; Williams, 2003:29).

Sections 2.3.1 and 2.3.2 describe (as illustrated in Figure 2.2) the possible transport mechanisms by which any molecule that is applied to the skin may cross the intact stratum corneum. The two macro routes through the stratum corneum that may be followed include:

- transepidermal (partitioning of non-electrolyte APIs that possess hydrophilic and hydrophobic properties); and
- transappendageal (partitioning of electrolytes, polar- and large molecules) (Robinson *et al.*, 1997:61).

2.3.1 Transepidermal pathway

Despite the relatively small skin surface ratio, the transepidermal pathway dominates permeation through the skin. An API has to permeate across the intact stratum corneum and can follow any of the following two potential routes (El Maghraby *et al.*, 2008:205):

- paracellular (intercellular) route: The API permeates between the cells through the intercellular lipid domains (El Maghraby *et al.*, 2008:205). It is essentially a lipophilic route (Williams, 2003:38). Hence, the lipophilic marker flurbiprofen may prefer to partition through the intercellular route (Fang *et al.*, 2003b:319).
- transcellular (intracellular) route: The API permeates through the protein filled keratinocyte cells and across the lipid rich region (Ghosh & Pfister, 1997:6).

In the intracellular region, the API has to diffuse and partition through the keratin bricks and across the intercellular lipids. The intercellular lipids thus play a key role in the barrier nature of the stratum corneum (El Maghraby *et al.*, 2008:205).

The intercellular route is considered the major pathway for permeation of most API across the lipid stratum corneum. Therefore, most of the penetration enhancing techniques used to enhance permeation across the skin are aimed at manipulating the solubility in the lipid domain, or modification of the ordered structure in this region of the stratum corneum (El Maghraby *et al.*, 2008:205; Benson, 2005:24).

The rate at which permeation of an API takes place is largely dependent on its physicochemical characteristics, as is described in Section 2.5, with the most important being the ability to partition into the intercellular lipids (Walters & Brain, 2009:478). An API with a partition coefficient, $\log P_{(\text{octanol/water})}$ of 1 - 3, would probably dominate the intercellular route, while more hydrophilic molecules ($\log P < 1$) would tend to cross the stratum corneum through the transcellular route. Highly lipophilic APIs ($\log P > 3$) would solely cross the stratum corneum through the intercellular route (Williams, 2003:36). Any API requires further diffusion through the rest of the epidermis and dermis (Ranade & Hollinger, 2004:214).

2.3.2 Transappendageal route

For ions and highly polar molecules, skin appendages provide the main portals of entry into the sub-epidermal layers of the skin (Moghimi *et al.*, 1999:516). These routes, *via* the hair follicles and the aqueous passageways of the sweat glands, allow for diffusion into the epidermis and direct permeation into the dermis (Ranade & Hollinger, 2004:214). Sweat glands and hair follicles act as diffusional shunts in the transport of certain APIs through the skin (Barry, 2006:4; Robinson *et al.*, 1997:61).

2.3.2.1 Hair follicles

It is understood that a lipophilic API penetrates the skin more easily through the transfollicular (along the hair shaft) route than a hydrophilic API, thus leaving only small amounts of lipophilic APIs within the stratum corneum (Wiechers, 2008a:13). Polymers, colloidal particles and small crystals will also target the follicular route (Barry, 2006:4). Follicular delivery bypasses the intact stratum corneum and presents a target for API delivery. Particles larger than 10 μm remain on the skin surface, whereas particles of approximately 3 - 10 μm converge inside the hair follicle. Other smaller particles of less than 3 μm penetrate follicles and the stratum corneum (Barry, 2006:11-13).

2.3.2.2 Sweat glands

Sweat glands secrete perspiration and are classified into two types. The eccrine glands open on the skin's surface *via* a duct running through the dermis and epidermis. Eccrine secretion is somewhat acidic and the principle element is sodium chloride that suppresses bacterial activity. Other components include urea, lactic acid, creatinine and amino acids (Mitsui, 1997:18-19).

Apocrine glands are significantly larger than the eccrine sweat glands and extend into the subcutaneous tissue. Most ducts open into the neck of the hair follicle and only a few exit on the skin surface. Apocrine gland secretion contains lipids, protein, lipoproteins and saccharides (Barry, 1983:11). These secretions are a combination of perspiration and viscous material with a distinct odour. Although the perspiration is weakly alkaline, bacterial infections may occur easily (Mitsui, 1997:19).

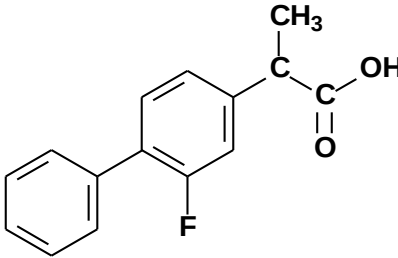
2.3.2.3 Sebaceous glands

The lipids being produced by these glands are excreted through the sebaceous gland ducts onto the skin surface through the hair follicle. These excreted lipids are mixed with other lipids that are derived from the epidermis (Table 2.1) and called skin surface lipids (Mitsui, 1997:19-20).

2.4 PHYSICOCHEMICAL CHARACTERISTICS OF FLURBIPROFEN

Flurbiprofen or 2-(2-fluoro-4-biphenyl)-propionic acid was found as being one of the most efficient members of a series of phenylalkanoic acids that act as anti-inflammatory, analgesic and antipyretic agents. It is used for the effective treatment of rheumatoid arthritis and musculoskeletal pain in humans. These types of disorders are widespread and exert a huge toll on a person's quality of life and the cost to society. Upon oral administration, the most common side effect is abdominal discomfort (Anderson & Conradi, 1985:815; Charoo *et al.*, 2008:300; Stanos, 2007:342, 346). Topically applied analgesics may provide a safe and effective therapeutic alternative for certain musculoskeletal pains (Fang *et al.*, 2003b:153).

Table 2.2: Physicochemical characteristics of flurbiprofen

Chemical formula¹	C ₁₅ H ₁₃ FO ₂
Molecular weight¹	244.3 g/mol
Chemical structure¹	
Melting point¹	114 - 117 °C
Appearance¹	White or almost white crystalline powder.
Aqueous solubility²	2.94 mg/ml (phosphate buffer saline at pH 7.4 at 32° C) 1.306 mg/ml (water at 32° C). Practically insoluble in water.
Log P³ (octanol-water partition coefficient)	4.16
Detection on spectrophotometer¹	247 nm
Elimination half-life³	± 3 h
Pk_a value⁴	4.27
Solubility¹	Freely soluble in ethanol and methylene chloride. Dissolves in aqueous solutions of alkali hydroxides and carbonates.

1.(British Pharmacopoeia, 2012)

2 (Ambade, 2008:37)

3 (Martindale, 2012a)

4 (Craig, 1990:541)

Table 2.2 summarises the physicochemical characteristics of flurbiprofen. It has a short half-life of ± 3 h and hence requires frequent dosing. Flurbiprofen has a rather high log P value of 4.16 and is therefore highly lipophilic. These properties are indicative of a possibly low permeation

rate through the skin, as the ideal log P value for topically applied APIs is between 1 and 3 (González-Mira *et al.*, 2011:243; Walters & Brain, 2009:479).

Several studies have confirmed the poor skin permeability of flurbiprofen and have suggested the inclusion of penetration enhancers in topical formulations in order to maintain effective blood levels. Penetration enhancers may enhance the flux of flurbiprofen through the skin (Charoo *et al.*, 2005b:493; Charoo *et al.*, 2008:300-301).

2.5 PHYSICOCHEMICAL PROPERTIES INFLUENCING TRANSDERMAL ABSORPTION

An API is typically applied to the skin in a vehicle (formulation). An API, neighbouring the stratum corneum surface, will partition into the membrane, depending on its physicochemical properties. Generally, a lipophilic API (for example, flurbiprofen), released from a saturated aqueous formulation, will show enhanced partitioning into the stratum corneum lipids compared to when released from an oily formulation. Therefore, lipophilic APIs will present with a lower propensity to leave an oily formulation. Lipophilic APIs with log P values above 2 depict high oil/water partition coefficients and bind to the hydrocarbon part of the lamellar structure of the stratum corneum, whereas polar APIs (log P values < 0) often have negative oil/water partition coefficients and concentrate in the aqueous region of the stratum corneum (Barry, 1991:240; Williams, 2003:28).

During the permeation process, various chemical characteristics (e.g. solubility, lipophilicity, ionisation and stability) influence the transport and distribution of the API across the skin. These properties control the concentration differences within the skin, but have little or no effect on diffusivity. Diffusion of an API is rather associated with its physical properties (e.g. molecular weight and size) (Smith, 1990:25; Barry, 1991:240). Certain aspects of an API's physicochemical properties can be used as parameters to predict permeability and attainable delivery through the skin (Peterson *et al.*, 1997:260). These properties are discussed in the following sections.

2.5.1 API concentration and solubility

Smith (1990:25) states that the rate at which an API is carried across the skin is dependent upon the concentration and solubility of the API in the formulation. More specific, it is rather the API concentration within the skin that controls the rate of transport across the layers, which in effect, is dependent upon the thermodynamic activity of the API. If an API is dissolved in a formulation at a concentration less than saturation, it results in a lower flux through the skin. A rapid rate of transfer will only occur when the API is available on the skin surface at a concentration nearing saturation (Smith, 1990:25).

For an API to be effectively transported across the skin, its solubility parameter should be similar, or close to that of the skin. The solubility parameter of the skin is approximately $10 \text{ (cal/cm}^3)^{1/2}$ (Asbill & Michnaik, 2000:37; Hadgraft & Finnin, 2006:367). According to Naik *et al.* (2000:319), the aqueous solubility of an API considered for skin permeation should be higher than 1 mg/ml. The marker flurbiprofen possess aqueous solubility values of 2.94 mg/ml in phosphate buffer solution (pH 7.4 at 32 °C) and 1.306 mg/ml in water at 32 °C (Ambade, 2008:37). Consequently, flurbiprofen should be able to permeate through the skin.

2.5.2 Melting point

It is generally known that the lower the melting point of a substance, the higher its solubility (Barry, 2007:579; Benson, 2005:26). Ideally, a melting point lower than 200 °C is preferred to ensure adequate solubility (Barry, 2001a:102; Barry, 2006:6; Naik *et al.*, 2000:319). The marker, flurbiprofen has a melting point of between 114 - 117 °C, which therefore depicts adequate solubility (British Pharmacopoeia, 2012).

2.5.3 Molecular weight and size

The permeability of an API into the skin is directly related to its molecular size, i.e. the smaller the molecules, the more rapidly it would penetrate into the skin. The larger the molecules in a non-homogenous medium (i.e. skin), the harder it is to move and therefore its diffusivity is reduced. Both diffusion and the partition coefficient of an API are thus reliant on molecular weight (Barry, 2007:579; Benson, 2005:26; Smith, 1990:28). Furthermore, molecules smaller than 200 Da (Dalton) will be absorbed by the skin through the intercellular route, whereas larger molecules smaller than 500 Da will transverse through intracellular passive diffusion. The smaller the molecular weight of the API, therefore, the more rapidly it will cross the skin (Ashford, 2007:295; Williams, 2003:36). The marker flurbiprofen, having a molecular weight of 244.3 Da (British Pharmacopoeia, 2012), could be expected to adequately penetrate the skin. It was suggested by Fang *et al.* (2003a:319) that the marker, flurbiprofen would possibly diffuse through the intercellular route.

2.5.4 pH, pKa and state of ionisation

Most APIs are weak acids and bases whilst only the unionised form possesses considerable lipid solubility. The scale of ionisation of an API is a function of its pK_a value and the pH at the surface of the skin. Membranes are usually more permeable to the unionised form of an API, whereas charged (ionised) molecules do not readily penetrate the stratum corneum. However, ionised molecules do cross the stratum corneum to a limited extent, due to their increased aqueous solubility. Whilst ionised APIs cross the stratum corneum *via* the transappenageal (shunt) route, unionised APIs cross *via* the intercellular (lipid) routes. The stratum corneum is

also very resistant to pH alterations, tolerating a pH range of 3 - 9 (Barry, 2006:7; Barry, 2007:576; Malan *et al.*, 2002:388; Williams, 2003:38).

Since flurbiprofen has a pK_a value of 4.27, in a solution having a pH of 7.4, only 7% of the flurbiprofen would be in the unionised form, resulting in inadequate diffusion. However, permeability would significantly increase as the pH decreases (at pH 4, 65% of the flurbiprofen would be in the unionised form).

2.5.5 Permeability coefficient (Kp)

The permeability coefficient describes the rate by which the API is transported per unit time in distance/time (cm/h) (Williams, 2003:27). The permeation tendency of an API can be predicted from its log P value, its molecular mass and its melting point (Hadgraft & Finnin, 2006:365). It is therefore possible that flurbiprofen, with a melting point of 114 - 117 °C and a molecular mass of less than 500 Da, should permeate the skin well.

2.5.6 Diffusion coefficient (D)

Absorption through the skin involves the passage of an API from the surface of the skin into the stratum corneum under the influence of a concentration gradient, followed by diffusion into the underlying epidermis and dermis, after which the API finally transverses into the blood circulation. The diffusion coefficient describes this process of absorption and is measured in area/time (cm²/h or cm²/s). Most APIs are transdermally absorbed through a passive diffusion process that is described by Fick's law of diffusion (Equation 2.1). Passive diffusion is energy independent and the flow of molecules in a certain direction occurs due to a chemical potential variation, or because of a concentration difference between two membranes. Diffusion through the skin is controlled by the stratum corneum and can be classified as the diffusion of a molecule through a passive membrane from a high to a low concentration. According to Fick's law, two physicochemical properties that influence the API flux across the skin are (1) the diffusivity and (2) the concentration gradient of the applied API within the skin (Benson, 2005:24; Hsieh, 1994:11; Panwar *et al.*, 2011:334; Malan *et al.*, 2002:386; Sinha & Kaur, 2000:1131; Thomas & Finnin, 2004:699; Williams, 2003:27).

$$\frac{dm}{dt} = J = \frac{DCoP}{h}$$

Equation 2.1

Where:

- J = steady state flux per unit area
- D = diffusion coefficient
- h = membrane thickness or diffusional path length
- P = partition coefficient (between the stratum corneum and the applied formulation)
- C_o = constant donor concentration
- m = cumulative mass of the diffused
- t = time (Barry, 2007:571-572; Benson, 2005:25; Hadgraft, 2004:292).

2.5.7 Partition coefficient (log P) and lipophilicity

The partition coefficient is the primary factor that determines permeation of an API across the skin and plays an important role in establishing flux rates after equilibrium has been established. It is generally concentration independent and is an indication of the lipid solubility of an API. The partition coefficient shows the ratio of API distribution in a lipid/oily phase (usually represented by octanol) and an aqueous phase (water). It is also called the *n*-octanol-water partition coefficient, or log P, and establishes the tissue distribution (stratum corneum lipids) of an API in transdermal delivery studies. Compounds with log P values above 3 are lipophilic (possess low water solubility) and are thus highly permeable, whereas Log P values below 1 indicate poor permeability and poor partitioning into the skin lipids (hydrophilic compounds). Optimal permeability is accomplished when an API's log P value is between 1 and 3. Such an API possesses an acceptable solubility in both oils and water and therefore has adequate solubility within the stratum corneum to allow for diffusion through this lipid domain, whilst also allowing for partitioning through the hydrophilic viable epidermis. Since the partition coefficient is occasionally indicated as (K), it should not be confused with the permeability coefficient (K_p) (Ashford, 2007:286; Barry, 1983:71; Behl, 1990:7; Benson, 2005:25; Hadgraft, 2004:292; Hadgraft & Finnin, 2006:365; Malan *et al.*, 2002:386; Shargel *et al.*, 2005:255; Smith, 1999:27; Malan *et al.*, 2002:386; Wiechers, 2008b:23-24; Williams, 2003:27, 36).

The marker, flurbiprofen, has a lipophilic character, having a log P value of 4.16 (Charoo *et al.*, 2008:300; Martindale, 2012a). It should therefore be highly permeable into the stratum corneum, whilst partitioning through the epidermis-dermis would probably be restricted. Manipulation of transdermal formulations hence becomes necessary (Benson, 2005:25) and may be accomplished through the incorporation of a chemical permeation enhancer into the formulation.

2.6 TRANSDERMAL PENETRATION ENHANCERS

Penetration enhancers provide infinite opportunities to deliver APIs through the skin, the largest and most easily accessible portal of entry to the human body and into the systemic circulation (Büyüktimkin *et al.*, 1997:446).

By definition, a penetration enhancer is anything that is used to improve delivery of a chemical substance across a chemical barrier (Sloan *et al.*, 2006:52). These substances facilitate absorption of a permeant through the skin by temporarily and reversibly diminishing the barrier function of the stratum corneum. Partitioning and diffusion of an API through the skin are thereby increased (Barry, 1983:29-30; Rajadhyaksha *et al.*, 1997:478; Sinha & Kaur, 2000:1131).

The stratum corneum forms this rate limiting, lipophilic barrier (Barry, 2006:11) and therefore penetration enhancers aim at modifying its barrier properties (Shah, 1994:20). Most molecules penetrate the skin through the stratum corneum's inert-cellular, micro route and therefore many enhancing techniques would try to either disrupt or bypass this elegant molecular architecture of the stratum corneum (Barry, 2001a:102). Penetration enhancers can promote API absorption by way of either the intra- or intercellular routes (Shen & Lin, 1994:37).

A penetration enhancer should enhance the flux of an API safely, temporarily and sufficiently in order to supply therapeutic benefits (Foldvari, 2000:421). It does, however, require a careful balancing of enhancers' toxicity and the permeation enhancement benefit (Aungst, 1989:245; Boelsma *et al.*, 1996:729). As many potent enhancers can irritate the skin by interfering with viable cell membranes, the choice of suitable enhancers is limited (Barry, 2006:10).

Ideal characteristics of penetration enhancers can be summarised as:

- pharmacologically inactive;
- non-toxic, non-irritating, non-allergenic;
- odourless, colourless, tasteless;
- high degree of permeation enhancement;
- onexpensive;
- release the API from the formulation;
- acceptable solvent properties;
- the effects should be reversible (the skin should regain its barrier properties upon the removal of the enhancer);
- may not lead to a loss of bodily fluids, electrolytes or any endogenous material;

- should be compatible with other molecules;
- hydrate the stratum corneum; and
- have a cosmetically acceptable 'feel' (Barry, 2006:9; Büyüktimkin *et al.*, 1997:358-360; Charoo *et al.*, 2008:301; Sinha & Kaur, 2000:1131; Williams & Barry, 2004:604).

Many penetration enhancers display some of these characteristics of which Azone[®] and fatty acids are examples (Charoo *et al.*, 2008:301).

It is generally accepted that the appendages comprise a small portion that contributes to a steady state flux of most APIs. This has resulted in the majority of penetration enhancement techniques focusing on increasing the transport across the stratum corneum, rather than *via* the appendages. Exceptions are iontophoretic delivery, which uses an electrical charge to drive a molecule into the skin *via* the appendageal routes (Benson, 2005:23). As mentioned before, since the stratum corneum only allows a limited number of APIs in minimal amounts through the skin, chemical enhancers have been developed. These chemical enhancers, when co-applied with an API, increase the flux of skin reversibly (Naik *et al.*, 1995:300).

Many research efforts have focused on finding methods for enhancing API penetration and absorption through human skin by employing chemical (modification of the stratum corneum) and physical (bypassing or removing the stratum corneum) methods (Shah, 1994:19-20).

2.6.1 Stratum corneum bypass or removal

Bypassing or removal of the stratum corneum is known as a physical method of penetration enhancement, which can be accomplished by the following three methods:

1. Bypassing the stratum corneum by means of microneedle array, follicular delivery and ablation.
2. Removing the stratum corneum through chemical peels (operating at different tissue layers), dermabrasion, microdermabrasion and high powered, laser pulses (leaving permeable regions), tape stripping, micro-infusion, or with the aid of an epidermatome (Barry, 2006:12).
3. Electrically assisted penetration enhancement through iontophoresis, electroporation and ultrasound (Barry, 2006:13; Shah, 1994:21).

2.6.2 Stratum corneum modification

The barrier properties of the stratum corneum can be modified or reduced by hydration or through chemical enhancers (Barry, 2001b:968; Benson, 2005:28). Marjukka Suhonen *et al.* (1999:151-152) state that to enhance the penetration of an API across the skin requires

alteration of the properties of the principle pathways linked to penetration within the stratum corneum, namely the intercellular lipid- and polar pathways. Therefore, interaction with the stratum corneum's intercellular lipids is important for the effectiveness of these types of penetration enhancers (i.e. chemical and hydration).

2.6.2.1 Enhancement through hydration

Skin is normally partially hydrated and it maintains consistent water content of approximately 5 - 15%. It remains unaffected by the humidity of the environment, as the stratum corneum plays a significant part in maintaining this concentration. Hydration, however, occurs when water diffuses from underlying epidermal layers or perspiration accumulation, after the application of an occlusive. Due to the polar nature of water, it interacts with the polar heads of the lipid bilayers within the intercellular domain and disrupts the packing of this layer. Increased water content within the skin results in the swelling of the hydrophilic domains of the stratum corneum and causes an enhanced diffusion of polar APIs. Hydration can be achieved by applying an occlusive to the skin, with lipid rich formulations being the most effective (Barry, 2007:577; Marjukka Suhonen *et al.*, 1999:153; Zhang *et al.*, 2006:67). Lipophilic materials (i.e. paraffins, oils, fats, waxes, fatty acids and esters) thus prevent water loss from the skin and increase the effect of an API on the skin (Barry, 2007:577).

By occluding the surface of the stratum corneum with formulations containing fatty acids and fatty acid esters, perspiration is blocked and remoisturisation is induced. Occlusives act by filling microscopic cracks in the stratum corneum, sealing them off and stabilising this bilayer structure to maintain moisture. The keratin layer of the skin contains natural moisturising factors (NMFs), with hydrophilic, moisture absorbing compounds, known to play an important role in moisturising the skin. Other emollients, containing glycols, replenish the NMF and also assist in retaining moisture of the stratum corneum. Cosmetics should aim at imitating this natural moisture maintenance method (Flynn, 2002:200; Mitsui, 1997:134).

The safest and most cost effective penetration enhancer is water. Most substances depict enhanced penetration through a hydrated stratum corneum (Barry, 2006:8-9). Increased hydration of the stratum corneum enhances the flux of a variety of API molecules, whereas the degree to which the skin is hydrated is very important to the API's release potential in a transdermal formulation (Büyüktimkin *et al.*, 1997:372). In general, tissue hydration seems to increase the transdermal delivery of hydrophilic and lipophilic APIs (Williams & Barry, 2004:606).

The physicochemical properties of a selected enhancer should be compared with those of the API. In other words, the solubility parameter of the enhancer should be similar to that of the skin, which has been noted in literature as being $\pm 10 \text{ (cal/cm}^3)^{1/2}$ (Asbill & Michnaik, 2000:37).

When hydration occurs, even the most harmless formulations tend to change the character of the stratum corneum (Ranade & Hollinger, 2004:211).

2.6.2.2 Chemical enhancement

The most utilised approach in penetration enhancement is the use of chemical penetration enhancers, which disrupt the normally ordered arrangement of the lipid layers within the stratum corneum (Walker & Smith, 1996:297). Some of these chemical enhancers' structures typify polar head groups and long alkyl chains which cause fluidity within the stratum corneum lipids (Pellet *et al.*, 1997:92). A chemical penetration enhancer enters the skin and alters the barrier properties of the stratum corneum reversibly, to enhance the penetration of an API into the skin and to increase the flux of the API across the skin. It therefore increases the partitioning of an API from the formulation into the stratum corneum (Büyüktimkin *et al.*, 1997:358-360; Tojo, 1997:114; Barry, 2001b:969; Nanayakkara *et al.*, 2005:130; Ranade & Hollinger, 2004:212).

A chemical penetration enhancer shifts the solubility structure of the skin in the direction of the API, by reversibly altering the physicochemical resistance of the stratum corneum (Benson, 2005:30; Charoo *et al.*, 2008:300-301). The skin tissue should be able to lapse back to its normal integrity and normal barrier properties upon removal of these chemical enhancers (Shah, 1994:20).

As discussed in Section 2.5.7, the altering aqueous and hydrophobic phases within the bilayers of the stratum corneum allow for diffusion of certain polar and non-polar APIs, provided an API has suitable physicochemical characteristics. Most APIs lack these characteristics and need compounds that enhance their permeation and diffusion through the skin. Chemical penetration enhancers achieve this by either increasing the partition coefficient of the penetrating API from the formulation, or by interacting with the skin components (Nanayakkara, 2005:130).

A simplified classification of chemical penetration enhancers is done through the lipid-protein partitioning (LPP) concept (Barry, 2006:9-10), which explains the penetration enhancer activity in human skin. Of the three macro routes for API penetration through human skin, the penetration across the stratum corneum (intracellular and intercellular) is of particular interest in this theory (Barry, 1991:237-238). This hypothesis proposes that many chemical enhancers operate in one or more of three main mechanisms, hence providing an easy means of categorising both chemical enhancers and of rationalising their different modes of action (Barry, 2006:9). The three main mechanisms of action of chemical enhancers as per the LPP theory include (Table 2.3):

➤lipid action;

- protein alteration (not a preferred method as it can split the stratum corneum, which is clinically unacceptable); and
- partitioning promotion (Barry, 2006:10).

Table 2.3: Classification of chemical penetration enhancers by using the LPP theory (Barry, 2001a:106; Barry, 2006:9-10; Benson, 2005:28)

Lipid-protein partitioning (LPP) theory		
Lipid action	Protein alteration (Not preferred)	Partitioning promotion
Process		
•Disrupts the lipid organisation (fluidisation) within the intercellular bilayer of the stratum corneum. •Increases the permeability (P) of the stratum corneum by making it more permeable to the API. •Raises the diffusion coefficient (D) of the API.	•Opens up the dense keratin protein structures inside the intercellular corneocytes of the stratum corneum. •Modifies the protein bilayer of the stratum corneum. •Increases the permeability (P) of the stratum corneum. •Raises the diffusion coefficient (D) of the API.	•Alters the chemical environment of the stratum corneum. •Increases the partition coefficient (P) of a companion molecule (i.e. co-enhancer or co-solvent, water) into the stratum corneum. •In theory this route also increases the partition coefficient for lipid APIs with the help of non-solvent enhancers.
Examples		
•Azone® •Terpenes •Fatty acids •Alcohols •Dimethylsulphoxide (DMSO)	•Ionic surfactants •DMSO	•Ethanol •Propylene glycol

From Table 2.3 it is evident that chemical enhancers increase API diffusion into the stratum corneum by either dissolving the skin lipids or by denaturising the skin proteins (Shah, 1994:20). Enhancer action on the intercellular lipid bilayers of the stratum corneum is thus achieved by disordering the packing of the lipids (Foldvari, 2000:419), which enhances delivery of an API (Barry, 2001a:107).

The fatty acids in natural oils that are believed to play a role in promoting the enhancement of the marker, flurbiprofen, are discussed next under natural oils.

2.7 NATURAL OILS (NON-VOLATILE)

Seeds and fruits generally bear oils, whereas animal products produce fats (O'Brien, 2004:1). Vegetable fats and oils occur primarily in seeds and in fruits. In animals, the fat depots are derived from the subcutaneous tissue, intramuscular connective tissue, the liver and the abdominal cavity. It is also believed that fat in the animal body may originate from ingested fat, carbohydrates, as well as from protein, but interestingly enough; the differences in fatty acid composition of herbivores and carnivores do not vary significantly, as would be expected (Kirschenbauer, 1960:1, 25-26).

Fats and oils and all substances associated with them are known as lipids (Kirschenbauer, 1960:5; Strayer *et al.*, 2006:1). Lipophilic substances all show characteristic features of being more or less insoluble in water and are used to support the lipid layer of the skin. These substances make the skin water resistant by reducing transepidermal water loss, and thus protect the skin against dehydration. Lipophilic substances can also cause noticeable smoothening of the skin (Lautenschläger, 2004:46).

The term, lipid, is given to the portion of animal or vegetable tissues that dissolve when extracted with a non-polar, fat solvent, for example, ether, benzene, petroleum, chloroform, or carbon disulfide. Natural oils mainly consist of building blocks, called triglycerides, and minor components include mono- and diglycerides, free fatty acids, phosphatides, sterols, fat soluble vitamins, tocopherols, pigments, waxes and fatty alcohols. Fatty acids and triglycerides are the prominent lipids found in natural oils (O'Brien, 2009:7-8; White *et al.*, 1964:61). The physical properties of fats and oils vary significantly, due to the extent of variation in the fatty acid components and triglyceride structures for each fat and oil. The chemical and physical properties of fats and oils are largely determined by the fatty acid constituents, as well as their positions within the triglyceride molecules (O'Brien, 2009:7). The solubilities of fats and oils decrease with an increase in molecular weight of the fatty acids present (Mitchell, 1918:11).

Natural fats and oils are widespread in the plant and animal kingdom. Natural oils, from plant or animal origin, are complex mixtures of a variety of ingredients containing saturated and unsaturated fatty acids. Various oils have been tested as penetration enhancers, but it is the unsaponifiable fractions of oils that are thought to contribute to their penetration enhancing activities. Penetration enhancement activity seems to be related to an increase in the diffusion coefficient of an API across the skin and not to an increase in API solubility, seeing that the polarity of oils appear to be an important factor. Non-polar oils have been used in previous studies as vehicles in pharmaceutical and cosmetic preparations. When natural oils (lipophilic substances) are metabolised in the skin, they release valuable free fatty acids. Natural oils are widely used as components in cosmetics, in which case they are processed to remove smell

and colour. Depending on the type, fats and oils may be used as hardened oils, when processed by partial or complete hydrogenation. These types may even be used after the solid fats have been removed through a cooling process. A study done by Takahashi *et al.* (1991:156-157) established that olive oil had shown penetration enhancing abilities. The importance of selecting a formulation vehicle that has a low affinity for an API, as well as for the oil enhancer, and the significance of increasing the API concentration in the specific oil, were also emphasised (Büyüktimkin *et al.*, 1997:433-434; Lautenschläger, 2004:46; Mitsui, 1997:122; Takahashi *et al.*, 1991:154, 157).

Natural oils are referred to as fixed oils (liquids) and fats (solids). The state of such a compound depends on temperature, i.e. if solid at room temperature ($\pm 25^{\circ}\text{C}$), they are referred to as fats and if in a liquid state at $\pm 25^{\circ}\text{C}$, they are called oils (Heinrich *et al.*, 2004:65; Strayer *et al.*, 2006:1; Zamora, 2005). These terms are normally used interchangeably, based on the physical state in which the material appears at ambient temperature, or based on tradition (O'Brien, 2009:1).

Frequently, vegetable oils contain fat soluble vitamins and/or provitamins. Avocado oil, for example, contains beta-carotene and vitamin A, whereas, vitamin E is present in almost every vegetable oil. Other components included in vegetable oils are phytosterols, which are similar in structure to cholesterol and are therefore able to substitute cholesterol molecules in the skin. This can be noticed by a specific barrier strengthening effect. Avocado oil contains particularly high percentages of phytosterols and in older skin they have specific skin caring effects (Lautenschläger, 2004:46).

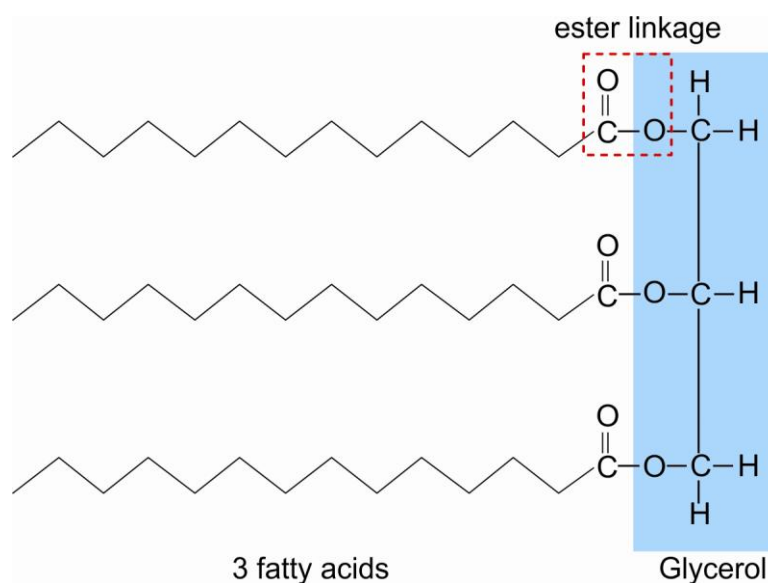


Figure 2.3: The chemical structure of the triglyceride molecule (Adapted from: Goebel, 2008; Ophardt, 2003a).

Chemically speaking, natural fats and oils are combinations of various triglycerides (triacylglycerol) that combine one unit of glycerol with three units of fatty acids through ester links (Figure 2.3) (O'Brien, 2004:237; O'Brien, 2009:7-8).

Glycerol (glycerine) has three alcohol-containing hydroxyl (-OH) groups that can bind up to three fatty acids to form either a mono-, di- or triglyceride (classified as esters). The three possible locations where a fatty acid may attach itself to a glycerol molecule are designated alpha, beta and gamma. Inside the triglyceride structures, the fatty acids may vary in combination (i.e. all three may have the same structure or their structures may differ). Since the glycerol unit does not vary in structure, the types and combinations of fatty acids determine the properties of the triglyceride molecule. Naming of the triglycerides is based upon the constituent fatty acid within the molecule. Triglycerides with more than one identical fatty acid are called mono-acid triglycerides. The more common forms are the mixed triglycerides where more than one type of fatty acid is present (Kirschenbauer, 1960:24; O'Brien, 2004:237; O'Brien, 2009:7-8; Strayer *et al.*, 2006:1-2; White *et al.*, 1964:62, 71; Zamora, 2005).

2.7.1 Fatty acids

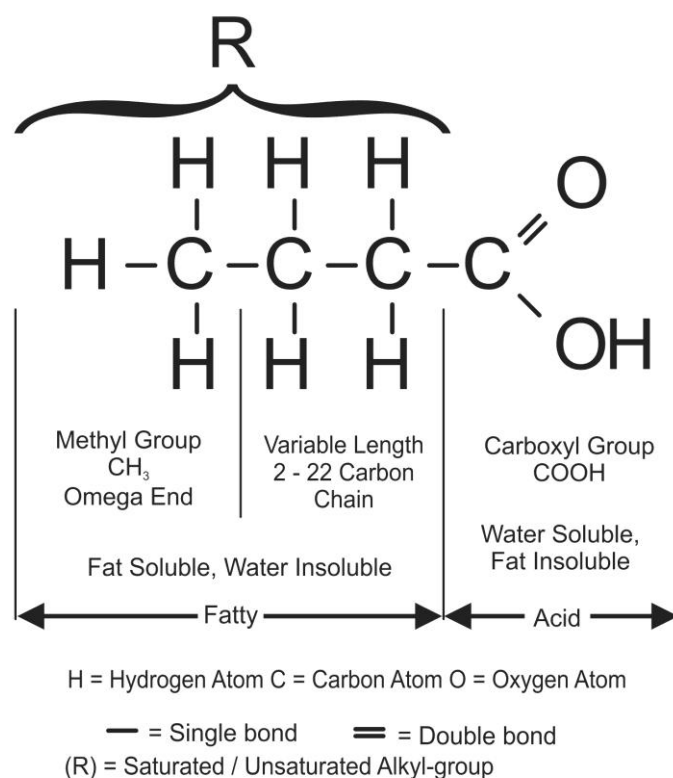


Figure 2.4: The chemical structure of the fatty acid (Adapted from: Healthknot, 2009; Hidden-diabetes-cures, 2005).

A fatty acid compound consists of a general chemical formula (RCOOH), where R is either a saturated or an unsaturated alkyl group (Mitsui, 1997:125). It contains an aliphatic hydrocarbon

chain and a carboxyl (polar) group at one end, as illustrated in Figure 2.4 (Babu *et al.*, 2006:144).

Numbering of carbons in fatty acids starts with the carbon of the carboxyl group (parent carbon). Structures of fatty acids are usually denoted by a systematic name after the organisation of the parent hydrocarbon, with shorthand designation conveniently presenting the number of carbon atoms and the number of the double bonds, i.e. linoleic acid (18:2) indicates that this fatty acid consists of an 18-carbon chain and 2 double bonds. The double bonds, together with the length of the carbon chain, are indicated after the name of the fatty acid. The hydrocarbon chain of each fatty acid can differ in (1) the length of the carbon chain, (2) the number of double bonds, (3) the position of the double bonds, (4) configuration of the double bonds i.e. *cis* or *trans*, and (5) the position where the fatty acids are attached to the glycerol. Certain properties of fatty acids may be due to the contribution of the sum of the aforementioned factors (Babu *et al.*, 2006:144; Heinrich *et al.*, 2004:67; O'Brien, 2009:7, 265; White *et al.*, 1964:62; Zamora, 2005).

pH describes the hydrogen-ion concentration in an aqueous solution. Since oils are water-insoluble, their acidity cannot be measured in terms of pH. Important to note is that at normal physiological pH (4.0 - 7.4), the carboxyl group is readily ionised, which would render a negative charge onto fatty acids in bodily fluids. APIs that are delivered dermally and transdermally would also be ionised over the normal physiological pH range. One should therefore ensure that no incompatibilities, which could possibly interfere with the formulation that contains the API being used, exist (Hadgraft & Valenta, 2000:243; Olive oil source, 2012). At pH above the pK_a , the fatty acid present on the skin surface will ionise (Green *et al.*, 1988:103).

Fatty acids are divided into two groups, namely:

- saturated fatty acids (SFAs), consisting of only single bonds; and
- unsaturated fatty acids (UFAs), consisting of one or more double bonds (Heinrich *et al.*, 2004:65; O'Brien, 2009:7, 269).

The most widespread SFAs are lauric-, myristic-, palmitic-, stearic- and arachidic acids, whereas the most important mono-unsaturated fatty acids (MUFAs) are oleic and eucic fatty acids. Linoleic and linolenic acids are poly-unsaturated fatty acids (PUFAs). These two acids are also known as essential fatty acids (EFAs), due to the fact that the human body lacks the enzymes necessary to manufacture them. EFAs must therefore be obtained from the diet and are known as the omega-3 (ω 3) and omega-6 (ω 6) fatty acids. Omega-9 (ω 9) fatty acids are technically not classified as EFAs, due to the fact that the body is able to produce a limited amount only, provided, omega-3 and -6 are present in sufficient amounts. The Greek symbol, omega (ω), is used to designate the terminal carbon atom the farthest away from the carboxyl group. The number attached to the (ω) denotes the position of the double bond for the specific

fatty acid closest to the methyl end of the molecule. For example, oleic acid (C18:1) has its double bond, nine carbons away from the methyl end of the molecule and is thus known as a (C18:1 ω 9) fatty acid (O'Brien, 2009:7,271-272; Omega-9 fatty acids, 2008; Strayer *et al.*, 2006:4, Zamora, 2005).

2.7.1.1 Saturated fatty acids

Saturated fatty acids (SFAs) can generally be found in high concentrations in animal and tropical oils. Coconut oil, for example, contains high concentrations of lauric (C12:0) and myristic (C14:0) acids, whereas the most common SFA naturally present in animals is palmitic (C16:0) and stearic (C18:0) acid. Myristic (C14:0)- and lauric (C12:0) acid, as well as some longer chain acids are also present, but in smaller concentrations (Kris-Etherton, 2007:1600; White *et al.*, 1964:63-65).

SFAs consist of straight, evenly numbered, single carbon-to-carbon chains (Figure 2.5). They are the least reactive of all the fatty acids and have higher melting points than their unsaturated counterparts with the same chain lengths, due to their unbranched structures (O'Brien, 2004:93).

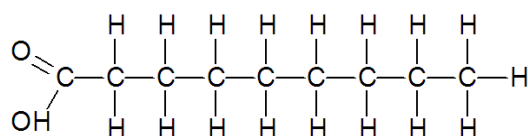


Figure 2.5: The chemical structure of the saturated fatty acid (Adapted from: Carter, 1996).

The SFAs of vegetable oils are predominantly, evenly numbered carbon atoms that can vary from 4 to 24 carbon atoms. Contrary, animal fats also contain evenly numbered carbon chains with the addition of uneven chains, ranging between 15 and 17 carbon atoms. SFAs of less than 10 carbon atoms are liquids at physiological temperature ($\pm 25^{\circ}\text{C}$), whereas those containing more than 10 carbons are in solid form. Due to their solubility properties, acetic acid (C2:0) and propionic acid (C3:0) will therefore form a homogeneous mixture with water (O'Brien, 2009:267; White *et al.*, 1964:62).

Chain lengths of SFAs vary from short, to medium to long. Short chain SFAs comprise of 2 to 6 carbon chains (i.e. acetic-, butyric- and caproic acid) and can be found in coconut oil (O'Brien, 2009:267). Butyric acid (C4:0), which is a liquid at room temperature, is predominantly found in cows and coconut milk (O'Brien, 2009:267). It, however, shows limited solubility in water (White *et al.*, 1964:62). Butyric acid usually associates with caproic-, caprylic- and longer chained SFAs (Ralston, 1948:19), and is responsible for the characteristic flavour of butter (Zamora, 2005). Caproic acid (C6:0), instead, is a minor component of many animal fats and of coconut oil. It is

a colourless, oily liquid, with an unpleasant smell and is never present in large amounts in any fat or oil. It usually associates with caprylic- and longer chained SFAs (Ralston, 1948:21).

Medium chain SFAs consist of 8 to 12 carbon chains (i.e. caprylic-, capric- and lauric acid) (O'Brien, 2009:267). Caprylic acid (C8:0), also known as octanoic acid, has an unpleasant, persistent odour. One of the best sources for caprylic (C8:0) acid seems to be coconut oil and to a lesser extent grapeseed oil. In animal and vegetable fats and oils it generally occurs in smaller concentrations. Capric acid (C10:0) is a common component in naturally occurring fats and oils and has a less distinctive penetrating odour. Coconut oil is the best source of capric acid (Ralston, 1948:25, 29). Lauric acid (C12:0) is a longer, medium chained SFA of coconut and palm nut oils (Mitsui, 1997:125). It is the most widely distributed SFA in nature, with a low melting point (O'Brien, 2009:267-268).

Long chain SFAs consist of 14 to 24 carbon chains (i.e. myristic-, palmitic- and stearic acid) (O'Brien, 2009:268). Myristic acid (C14:0), which is odourless, occurs in small concentrations in most fats and oils. However, it is present in higher concentrations in nut oils, but occurs in almost insignificant concentrations in animal fats. Palmitic acid (C16:0), however, appears to be more prevalent in animal fats and also occurs in most vegetable oils. It is used in cosmetics as an oily base in creams and milky lotions. Stearic acid (C18:0) is also one of the most widely used SFAs in nature (Healthknot, 2009; Mitsui, 1997:125; O'Brien, 2009:267-268; Ralston, 1948:33-34).

2.7.1.2 Unsaturated fatty acids

Generally, the melting points of unsaturated acids are low, whilst their solubilities are enhanced in non-polar solvents, due to the presence of one or more carbon-to-carbon double bond(s) (Figure 2.6) (White *et al.*, 1964:63).

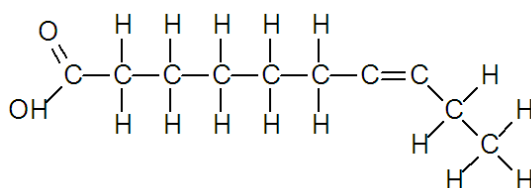


Figure 2.6: The chemical structure of the unsaturated fatty acid (Adapted from: Carter, 1996).

The presence of double bonds makes UFAs more reactive than SFAs and their reactivity increases as the double bonds increase. Double bonds are usually sited on the 9th and 10th positions on the carbon chain and give rise to isomeric fatty acids, known as *cis*- and *trans*-isomers. Naturally occurring fatty acids generally contain the *cis* configuration. Identical UFAs, except for the position of the double bond, are called isomers, and this feature is the reason for

UFAs being more chemically reactive than SFAs. They furthermore exist in a liquid state at room temperature (O'Brien, 2009:269; Strayer *et al.*, 2006:4-5; White *et al.*, 1964:63-65; Zamora, 2005).

UFAs are subdivided into mono-unsaturated fatty acids (MUFAs) and poly-unsaturated fatty acids (PUFAs). MUFAs (i.e. palmitoleic-, oleic- and erucic acid) have only one double bond and are the least reactive of all the UFAs. Palmitoleic acid (C16:1) is to a lesser extent present in the natural occurring oils, as it is unusual for a vegetable oil to contain more than 1% of palmitoleic acid. However, palmitoleic acid is commonly found in olive oil (O'Brien, 2009:269; Ralston, 1948:97-98). Contrary, oleic acid (C18:1) is one of the most widely occurring, natural fatty acids. It is found in most vegetable oils and animal fats, as well as in nuts and avocados. Oleic acid possesses high oxidative stability and has minimal levels of *trans*-isomers. Due to olive oil's high oleic acid content ($\pm 80\%$), it is considered a flavour stable oil (Kris-Etherton, 2007:1600; O'Brien, 2009:269-270). Studies have shown that oleic acid primarily modulates the stratum corneum's extra-cellular lipid layer (Naik *et al.*, 1995:300). Another relevant MUFA is erucic acid (C22:1), which is a component of canola oil. It has a possible physio-pathological harming effect, when its content in oil is too high (O'Brien, 2004:242).

PUFAs have two or more double bonds and their chemical reactivity increases as the number of double bonds increases. Examples of PUFAs include (ω -6) linoleic acid (C18:2) di-unsaturated, and (ω -3) linolenic acid (C18:3) tri-unsaturated, predominantly found in vegetable oils and of nutritional interest in the human diet. These examples of EFAs must all be ingested from food sources, as humans and several animals are unable to produce these PUFAs (Kris-Etherton, 2007:1559; O'Brien, 2004 237; O'Brien, 2009:270; Zamora, 2005).

Longer chained unsaturated oils release linoleic acid, responsible for preventing scaly skin, as it is integrated into the barrier substances. Gamma-linoleic acid (omega-6 fatty acid) has anti-inflammatory and anti-pruritic effects and is therefore also used in topical preparations for the treatment of dehydrated and atopic skin conditions (Lautenschläger, 2004:46). Lipids are essential components of the human skin and of these; linoleic acid is recognised as a significantly important component. It plays an essential role in skin functioning and when applied topically, has shown to regenerate a defective skin barrier (Muggli, 2005:247).

2.7.1.3 Fatty acids as penetration enhancers

Fatty acids have been established as effective penetration enhancers for transdermal delivery of (Kanikkannan *et al.*, 2006:18) both hydrophilic and lipophilic APIs (Williams & Barry, 2004:610). Shah (1994:27) avers that a possible mechanism of action for their promotional effects stems from their "surface- and chelating" activities. Fatty acids increase the skin's permeation by disordering the structure of the ordered lipids inside the intercellular spaces of

the skin barrier. Due to disruption of the alkyl chains, the lipid fluidisation of the stratum corneum is increased, thus enhancing the diffusivity and partitioning of APIs across the stratum corneum (Aungst, 1989:244; Babu *et al.*, 2006:138; Benson, 2005:28; Kanikkannan, 2006:19; Shah, 1994:20). Fatty acids have the advantage of being endogenous constituents of human skin and their structural variations influence their effects as penetration enhancers (Santoyo & Ygartua, 2000:245).

Saturated and unsaturated fatty acids have been established as skin penetration enhancers, especially medium (C_6 - C_{10}) to long (C_{12} - C_{24}) chain fatty acids (Babu *et al.*, 2006:138). Transdermal absorption has been increased by a variety of long chain fatty acids, with oleic acid being an especially popular fatty acid. Oleic acid is commonly used as penetration enhancer and primarily operates by modulating the intercellular lipids of the stratum corneum that causes the permeability of a co-administered API to increase. It therefore reduces the stratum corneum's diffusivity through fluidity of the lipids in the skin. It is believed that saturated alkyl chain lengths of around C_{10} - C_{12} , attached to a polar head group; seem to have great enhancing abilities, while alkyl chains of C_{18} seem optimal (Naik *et al.*, 1995:300; Williams & Barry, 2004:609). Oleic acid has been investigated extensively as a skin penetration enhancer, and it was found that the extent of its enhancement ability is affected by several factors, i.e. fatty acid chain length, the presence of double bonds and the formulation (vehicle) in which the fatty acid is dissolved (Foldvari, 2000:421). Disappointing though, is the fact that unsaturated fatty acids seem to irritate the skin more than saturated fatty acids, while also inducing inflammatory mediators in the skin (Babu *et al.*, 2006:154).

According to Babu *et al.* (2006:150), fatty acids may essentially distribute in the stratum corneum, due to their lipophilicity. They then possibly interact with the lipids inside of the stratum corneum and cause a "pulling" effect of the API from the formulation. Those fatty acids that remain within the formulation create a "pushing" effect that increases the thermodynamic activity of the API within the formulation (Babu *et al.*, 2006:150). As mentioned before, a specific, established penetration enhancer, called Azone[®], also contains saturated and unsaturated hydrocarbon chains and some structural relationships with fatty acids have been investigated (Williams & Barry, 2004:609). Fatty acids are important both as formulation agents and as vehicles in the pharmaceutical industry, whilst they are also used as components in the cosmetic industry and in soaps (Heinrich *et al.*, 2004:65).

2.7.2 Natural oils used in this study

As discussed above, various fatty acids can and have been used as penetration enhancers in pharmaceutical formulations. However, little research has been conducted on the penetration enhancing effects of natural oils consisting of different fatty acids. The natural oils that were

investigated during this study included oils of both plant- and animal origins, and are briefly discussed in the following sections.

2.7.2.1 Avocado oil

Avocado (*Persea americana*), also called alligator pear, is used topically as an emollient (Martindale, 2012b). Avocado oil contains high amounts of phytosterols and provitamins. As the structure of phytosterols is similar to that of cholesterol, phytosterols can be substituted for cholesterol in the skin (Kirschenbauer, 1960:190; Lautenschläger, 2004:46). Avocado oil predominately consists of UFAs, with the highest percentage being oleic acid (Kris-Etherton, 2007:1611e).

2.7.2.2 Grapeseed oil

Grapeseed (*Vitis vinifera*) is a well-known oilseed crop, which produces 8 - 15% of oil. It is becoming increasingly popular in the pharmaceutical and cosmetic industries, due to its high, unsaturated, fatty acid content (oleic- and linoleic acid). The main fatty acids present in grapeseed oil are palmitic-, oleic- and linoleic fatty acids (Kirschenbauer, 1960:26; Passos *et al.*, 2009:48).

2.7.2.3 Emu oil

Ratites (also called emus) are flightless birds and opportunistic feeders that prefer fruits, seeds, flowers and insects, which can have a significant impact on their fatty acid content. Emu oil's pharmaceutical and cosmetic potential have only recently been investigated and research still continues. The main fatty acids present in emu oil seem to be oleic- and palmitic acid (Angel, 1996:241; Shimizu, 2003:57-58, 60). It is also interesting to note that emu oil is not a clear oil, which may be attributed to the myristic- and stearic acids present, which seems to be semisolid at room temperature (Aungst, 1989:245).

2.7.2.4 Crocodile oil

The Nile crocodile (*Crocodylus niloticus*) is native to Africa and can grow up to 7 m in length. Meat fat of crocodiles contains concentrations of palmitic-, palmitoleic-, stearic-, oleic- and linoleic acids. However, due to their monogastric animal diet, the fatty acid composition of crocodile fat may vary (Osthoff *et al.*, 2010:64).

2.7.2.5 Olive oil

Olive oil originates from the fruit of the olive tree (*Olea europaea*). Its major fatty acid constituents are oleic acid (65 - 86%), palmitic acid (7 - 20%) and linoleic acid (5 - 15%). Olive oil is used in cosmetics to control moisture evaporation from the skin and to enhance the

product's feel upon use. The unsaturated fatty acid, oleic acid, causes the oil to spread more easily and therefore olive oil is readily used in skin care products (Kirschenbauer, 1960:26,188; Lautenschläger, 2004:46; Mitsui, 1997:122).

Olive oil usually has a greenish-yellow colour, with a characteristic olive flavour and odour. Virgin olive oil's colour is mainly related to the presence of chlorophyll and pheophytin. The olive plant must be harvested as soon as it reaches maturity and the olives have to be processed immediately, as they are fruits, not seeds, hence they cannot be dried and preserved (O'Brien, 2004:40-43, 62).

2.7.2.6 Coconut oil

Coconut palms (*Cocos nucifera*) are traditionally found near the coastal regions. The oil is obtained from kernels of the nuts of palm trees and the endocarp consists of coconut meat and milk. Fresh coconut meat contains an oil content of 60 - 80% (Kirschenbauer, 1960:167). The colour of the oil varies from a light yellow to a brownish yellow colour. The oil is also referred to as copra oil and contains average amounts of caproic- (0.2 - 0.8%), caprylic- (5 - 9%), palmitic- (7 - 11%), stearic (1 - 3%) and oleic- (5 - 8%) acids. More than 90% of the fatty acids in this oil are of low molecular weight and are saturated, which makes coconut oil the richest source of medium chain, fatty acids (C_6 -, C_8 - and C_{10}). Only small concentrations of UFAs (i.e. oleic acid) are present in coconut oil, hence a good indicator of its excellent oxidative stability. This also explains why this oil is less prone to turning rancid. Due to significant concentrations of lauric acid (SFA), this oil will pass abruptly from a brittle solid to a liquid, within a narrow temperature range. It, furthermore, melts rapidly and completely below body temperature, due to the low molecular weight of the SFA (Kirschenbauer, 1960:188; Lautenschläger, 2004:46; Martindale, 2012c; O'Brien, 2004:50; Strayer *et al.*, 2006:6; Zamora, 2005).

Table 2.4 summarises the free fatty acid concentrations (obtained from the literature) present in the natural oils described above. It is important to remember that this study dealt with fatty acids that form part of the triglyceride molecule. These concentrations are presented as grams of the fatty acid per 100 grams of oil and are generally known as the free fatty acidity of the oil in percentages. Some of the fatty acid values that were negligibly small were not incorporated, while very long chain fatty acids ($> C_{20}$) that tend to be more solid at room temperature (e.g. waxes), did not constitute appreciable amounts in these oils. The melting and boiling points (BP) of these oils in Table 2.4 show that as the chain lengths of SFAs increase, the melting points increase. UFAs further depict lower melting points (MP), as the number of double bonds increase. The BPs and MPs of these fatty acids raise with an increase in chain length, hence the evenly numbered, carbon chain, SFAs shorter than ten carbons are liquids at room temperature, while the longer chained SFAs are solids. Due to animal (species, health, feeding

habit, diet and maturity, etc.) and natural environmental variations (climate, type of soil, season, health and maturity of the plant, genetic variations of species, etc.), the chemical and physical characteristics of fatty acids in different oils may vary slightly, and therefore, cannot be guaranteed (Hilditch, 1956:12; Kirschenbauer, 1960:194; O'Brien, 2004:7, 237; O'Brien, 2009:7; Olive oil source, 2012; White *et al.*, 1964:62, 71).

Table 2.4: Approximate fatty acid compositions of several natural oils (in percentage by weight of total fatty acids per 100 g) and their melting- and boiling points (in °C)

	Capric	Lauric	Myristic	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	Linolenic	Arachidonic
	SFA					MUFA		PUFA		
Oil or fat	10:0	12:0	14:0	16:0	18:0	16:1	18:1	18:2	18:3	20:4
Avocado ¹				10.9	0.7		67.9	13.5	12.6	
Grapeseed ¹				6.7	2.7		15.8	69.6	0.1	
Emu ²				25	9	5	50	10	1	
Crocodile ³			0.4	21	8	4	34	29	2	1
Olive ¹				11	2.2		72.5	7.9	0.6	
Coconut ¹	6	44.6	16.8	8.2	2.8		5.8	1.8		
HB ⁴	2	5	8	25	8		35	9	1	
MP (°C) ⁵	31.6	44.2	54.4	62.9	69.6	-0.5	13.4	-6.5	-13.0	-49
BP (°C) ⁶	269	130	114	167	184	134	153	202	157	-50 ⁷

¹ (Kris-Etherton, 2007:1611e); ² (Shimizu & Nakano, 2003:58); ³ (Osthoft *et al.*, 2010:66); ⁴ HB is human butterfat (Strayer *et al.*, 2006:4); ⁵ (O'Brien, 2009:266); ⁶ (Kirschenbauer, 1960:8); ⁷ (Ophardt, 2003b).

The natural oils used in this study were also compared with human (*Homo sapiens*) butterfat in Table 2.4. Older and recent literature similarly indicated human butterfat as having an average chemical constitution of saturated palmitic acid (24 - 25%), unsaturated palmitoleic acid (5 - 7%) and unsaturated oleic acid (53 - 57%). Human fat shows an average melting point of 12.5 - 17.5 °C (Hilditch, 1956:12; Kirschenbauer, 1960:194; White *et al.*, 1964:71) and has a very similar composition to lard fat that also consists of the highest percentage of oleic acid (Zamora, 2005).

It is important to understand the differences between the origin of the fatty acids in human butterfat and human stratum corneum lipids. Stratum corneum lipids consist of a mixture of sebum from the sebaceous glands, as well as lipids from the epidermis. The first detailed quantitative analyses of human stratum corneum lipids determined fatty acid composition, regardless of the site of origin (abdomen, leg, face and sole) on the skin. Fatty acid chain lengths present in these lipids ranged from C₁₂-C₂₄. However, fatty acids consisting of longer

carbon chains (C₁₆ and C₁₈) were found in higher percentages. SFAs and UFAs were encountered in all fractions, with the UFA concentrations being significantly higher (Greene *et al.*, 1970:240; Lampe *et al.*, 1983:120, 125).

2.8 SUMMARY

Because the stratum corneum seems relatively impermeable, it provides the main, rate limiting barrier against transdermal absorption of chemicals. Although the stratum corneum allows no molecules to pass readily, most materials penetrate it to some extent. Permeation also does not only occur through the appendageal route or the intercellular route, but also across the bulk of the stratum corneum. Intercellular keratin presents with polar and non-polar regions in which substances dissolve and diffuse according to their chemical affinities. Because the stratum corneum consists of dead cells, diffusion is a passive process. With regards to electrolytes and larger molecules with low diffusion coefficients (most topical APIs), diffusion through appendages may be significant. Once the molecules pass the stratum corneum, they permeate rapidly through the living tissue of the epidermis and dermis and into the systemic circulation (Barry, 1983:119). One method being employed to decrease the diffusional resistance of the stratum corneum is the use of penetration enhancers. These materials interact with some components within the stratum corneum and thus increase the permeation of an API ingredient. Transdermal, pharmaceutical formulations also provide advantages of a non-invasive nature for therapy of systemic and local disease states (Charoo *et al.*, 2005a:346).

Because any given enhancer would not necessarily increase the permeation of all types of APIs (Asbill & Michniak, 2000:37; Tojo, 1997:363), the use of natural oils were employed for the purpose of this study, in order to represent a non-invasive method of permeation enhancement, by investigating their effects as chemical enhancers through modification of the stratum corneum. Since natural oils contain fatty acids, which are endogenous to human skin, it was assumed that no allergic reactions would occur during the permeation process. This study hence focused on assessing the potential penetration enhancement activity of a series of saturated and unsaturated fatty acids, present in natural oils.

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CHAPTER 3

ARTICLE FOR PUBLISHING IN THE JOURNAL OF NATURAL MEDICINE

Chapter 3 is written in article format for the purpose of publication in the Journal of natural medicine. A complete guide for authors of this journal is given in Appendix E and from there it is advised that the authors will be briefed in US English. For publishing purposes Chapter 3 contains no formatting, except for double-line spacing and font specifications written in Times New Roman 12 as described by the instructions for authors.

Effects of selected natural oils on the delivery of flurbiprofen as marker active pharmaceutical ingredient in topical dosage forms

Amé Cowley, Joe M Viljoen*, Minja Gerber, Jan du Preez and Jeanetta du Plessis.

Unit for Drug Research and Development, North-West University, Private Bag X6001,
Potchefstroom 2520, South Africa.

*Corresponding author: Tel: +2718 299 2273; Fax: +2718 299 2248. E-mail address:
11320036@nwu.ac.za (JM Viljoen).

Abstract

Context: Natural oils are frequently used as components in topical dosage forms. Their main constituents are triglycerides comprising of fatty acids and glycerol. These oils are metabolized in the skin and release fatty acids. It has been reported to effectively enhance transdermal delivery of hydrophilic and lipophilic active pharmaceutical ingredients (APIs). Fatty acids are used as penetration enhancers to overcome the lipophilic barrier of the stratum corneum in the skin. **Objective:** This study focused on possible penetration enhancing properties of fatty acids generally found in avocado-, grapeseed-, emu-, crocodile-, olive- and coconut oils, utilizing flurbiprofen as marker. Emulgel formulations consisting of these oils were compared to a liquid paraffin emulgel and a hydrogel without the inclusion of an oil. Two oil emulgels depicting the highest flux values were manufactured as foam formulations and also compared.

Materials/Methods: Franz cell diffusion studies and tape stripping methods were used to investigate the delivery of the marker, flurbiprofen, within the formulations through and into the skin. **Results and discussion:** The hydrogel depicted the highest flux, followed by the olive oil emulgel. Liquid paraffin portrayed a slight higher flux than the coconut oil emulgel. **Conclusion:** Oils containing predominantly mono-unsaturated oleic acid on average increased the flux of the marker to a larger extent than oils containing an almost even mixture of both mono- and poly-unsaturated fatty acids. Oils containing predominantly fatty acids with alkyl chains between C₁₀ to C₁₂, generally showed good enhancing properties, but not significantly more than oils containing mainly oleic acid.

Keywords: Natural oils, penetration enhancers, fatty acids, transdermal, Franz cell diffusion studies, tape stripping.

1 Introduction

The permeation of a compound that crosses the skin is normally controlled by the stratum corneum [1], which is the uppermost layer of the skin and is classified as the rate limiting step in transdermal delivery of chemicals [2], [3]. Due to the lipophilic nature of the stratum corneum, the physicochemical properties of an active pharmaceutical ingredient (API) are of utmost importance when considered for transdermal delivery [4]. These properties determine the rate of permeation of the API across the stratum corneum *via* the intercellular lipids, which is considered the main penetration route into the skin [1].

Research focusing on methods to increase the permeability characteristics of the intact stratum corneum, have employed penetration enhancers in transdermal formulations [5]. Penetration enhancers reduce the barrier properties of the stratum corneum temporarily and reversibly [6], [7] by acting on the intercellular lipid layers within the stratum corneum to disrupt the packing of these lipids [8]. Natural products, e.g. natural oils, have been used as penetration enhancers, due to their “safety profiles” [9]. Natural oils consist of a mixture of glycerol and fatty acids [10] and it is their unsaponifiable fractions that are thought to contribute to their penetration enhancing activities [11]. Fats and oils, therefore, chemically speaking, are triglycerides consisting of a glycerol molecule and three fatty acids linked through an ester bond [12]. The fatty acids present within natural oils, of which oleic acid is an excellent example, are often used as penetration enhancers to promote the permeability of the skin [13]. Fatty acids and triglycerides are some of the most prominent lipids found within natural oils [14]. The state of natural fats and oils largely depends on temperature, and if in a liquid form at room temperature ($\pm 25^{\circ}\text{C}$), they are referred to as oils, whereas if solid at room

temperature, they are called fats [15], [16]. Valuable free fatty acids are released when natural oils (lipophilic substances) are metabolized within the skin [17]. A fatty acid consists of an aliphatic hydrocarbon chain (non-polar) and a carboxyl (polar) group at one end [14], [18]. The hydrocarbon chain of a fatty acid can differ in (i) the length of the hydrocarbon chain, (ii) the number of double bonds present, (iii) the positions of the double bonds in the hydrocarbon chain, (iv) the configuration of the double bonds, e.g. *cis* or *trans*, and (v) the location of the fatty acid attached to the glycerol molecule [18], [19]. Structures of fatty acids are generally denoted by a systemic name, after the organization of the parent hydrocarbon with shorthand designation, presenting the number of carbon atoms, as well as the number of double bonds within the chain. These double bonds, together with the length of the carbon chain, therefore, are indicated after the name of the fatty acid, e.g. linoleic acid (C18:2). This name, for example indicates that the linoleic acid consists of an 18-carbon chain with two double bonds.

Fatty acids are divided into two groups, namely saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs). SFAs consist of single bonds only, whereas UFAs consist of one or more double bonds within the hydrocarbon chain [15], [19]. Lauric- (C12:0), myristic- (C14:0), palmitic- (C16:0) and stearic acids (C18:0) are some of the most widespread SFAs, whereas the most important mono unsaturated fatty acids (MUFAs), having one double bond, is oleic acid (C18:1). Linoleic- (C18:2) and linolenic acids (C18:3) are the most important poly unsaturated fatty acids (PUFAs) and are also called essential fatty acids (EFAs), due to the fact that the human body lacks the enzymes to manufacture them [15],[19].

SFAs and UFAs have been recognized to effectively enhance transdermal delivery [18], [20] of hydrophilic and lipophilic APIs [21]. Fatty acids increase the skin's

permeability by disordering the ordered lipids (alkyl chains) inside the intercellular spaces resulting in lipid fluidization within the stratum corneum. This enhances the diffusivity and partitioning of an API across the skin barrier [18], [20], [5], [22]. This modulation of the intercellular lipids of the stratum corneum thus increases the permeability of the co-administered API [23]. The degree of penetration enhancement within the skin is affected by (i) the fatty acid chain length, (ii) the presence of double bonds, and (iii) the formulation in which the fatty acid is dissolved [8]. It is believed that SFAs with alkyl chain lengths of approximately C_{10} to C_{12} , and attached to a polar head group, may have enhancing abilities, whereas alkyl chains of C_{18} show optimal enhancing properties [21]. Fatty acids have the benefit of being endogenous constituents of the human skin, whilst their structural variations affect their abilities as penetration enhancers [24].

The aim of this study was not to formulate topical dosage forms as such, but to investigate the penetration enhancing properties of a series of SFAs and UFAs present within a range of natural oils. These oils were from both plant and animal origins; and the lipophilic flurbiprofen ($\log P$ 4.16) [25] was used as a marker API to determine its permeation through the stratum corneum and into the underlying structures of the skin. The six oils used in this study were avocado-, grapeseed-, emu-, crocodile-, olive- and coconut oil. Each of these natural oils was formulated into an emulgel and their affects were compared to an emulgel containing liquid paraffin, as well as a hydrogel without the inclusion of an oil. The formulated emulgels were a hydrogel **(1)**, liquid paraffin emulgel **(2)**, avocado oil emulgel **(3)**, grapeseed oil emulgel **(4)**, emu oil emulgel **(5)**, crocodile oil emulgel **(6)**, olive oil emulgel **(7)** and coconut oil emulgel **(8)**. Skin diffusion studies showed the highest flux values for emulgels **(7)** and **(8)**, respectively;

and therefore two foam formulations namely: olive oil foam **(9)** and coconut oil foam **(10)** were manufactured. These foams were also compared to the other formulations, and the effect(s) these oils had on the permeation of the marker were established. *In vitro* diffusion studies were performed on human dermatomed skin, utilizing vertical Franz diffusion cells.

2 Materials and Methods

2.1 Materials

Flurbiprofen was obtained from DB Fine Chemicals (Johannesburg, South Africa). Liquid paraffin, Span[®] 60, Tween[®] 80, propyl- and methyl parabens, HPLC grade methanol, HPLC grade ethanol, potassium dihydrogen orthophosphate (KH₂PO₄), orthophosphoric acid (H₃PO₄) and sodium hydroxide (NaOH) pearls (used to prepare the phosphate buffer solution) were all obtained from Merck Chemicals (Wadeville, South Africa) and Merck Laboratory Supplies (Midrand, South Africa). Polyethylene glycol (PEG) 400 was obtained from Saarchem (Krugersdorp, South Africa) and xanthan gum from Warren Chem Specialities (Johannesburg, South Africa). The deionized HPCL grade water used in this study was prepared with a Milli-Q[®] (Millipore, Milford, USA) water purification system. Whatman[®] filter paper, Parafilm[®] and Dow Corning[®] vacuum grease were acquired from Separations (Randburg, South Africa). The avocado-, grapeseed- and olive oils were purchased from Nautica Organic Trading CC (Umhlanga, South Africa) and the emu oil from Emuphoria (Potchefstroom, South Africa). Crocodile oil was kindly donated by Croc City Crocodile Farm (Sandton, South Africa); coconut oil by ENCO Fuels CC (Potchefstroom, South Africa); whilst the pharmaceutical grade aerosol gas, Solkane[®] 134a pharma, used as propellant in the manufacturing of the foams, was

donated by Solvay Chemicals (Rheinberg, Germany). All other chemicals and solvents used were of analytical grade.

2.2 HPLC method

2.2.1 Determination of the flurbiprofen concentration for diffusion studies

An HPLC method for the analysis of flurbiprofen was developed and validated at the North-West University, Potchefstroom Campus, South Africa. Samples collected from the donor phase of the Franz diffusion cells for all the diffusion studies and tape-stripping experiments were analyzed with HPLC, to accurately determine the concentrations of the diffused flurbiprofen within the samples. An Agilent® 1100 series HPLC system, equipped with an Agilent® 1100 pump, diode array detector, UV-detector, autosampler injection mechanism and Chemstation Rev. A.10.03 software for data acquisition and analysis (Agilent Technologies, Palo Alto, CA), were used. A high performance, silica based, reversed phase C₁₈-2, 5 µm particle size, endcapped, 150 x 4.60 mm column (Venusil XBP Agela Technologies, Newark, DE) was used. The mobile phase consisted of a mixture of acetonitrile: Milli-Q® water: acetic acid (70:30:1), and degassed using an ultrasonic bath prior to use. The flow rate was set to 1.0 ml/min and the injection volume was 25 µl. UV-detection of flurbiprofen was set at 247 nm with a run time of 8 min; and the retention time of flurbiprofen was approximately 3.9 min. A phosphate buffered solution was used as solvent in the diffusion studies.

2.2.2 Standard preparation

A standard solution was prepared prior to each analysis on the HPLC to serve as a control for the samples obtained from the receptor phases of the Franz cells used in the diffusion studies. It was prepared by accurately weighing approximately 10 mg

flurbiprofen and transferring it into a 100 ml volumetric flask and made up to volume with phosphate buffered solution (pH 7.4). From this solution 5 ml was diluted to 50 ml with the phosphate buffer solution. Approximately 2 ml of this standard solution was transferred into an HPLC autosampler vial and injected in duplicate at different volumes in order to establish a concentration range. The values of the obtained peak areas were plotted against time to calculate the concentration of the values of the marker after a linear regression line was drawn.

2.3 1% flurbiprofen in semisolid formulations for the Franz cell donor phase

The donor phase for the skin and membrane studies used during this study contained 1% flurbiprofen in each of the emulgel, hydrogel and foam formulations. The six natural oils and liquid paraffin emulgels consisted of 20% oil, whilst the hydrogel contained Milli-Q[®] water. Subsequently, two of the emulgels that indicated good flux values, were placed into new foam containers and sealed. A propellant was forced, under pressure, into the containers to produce the final foam product.

2.3.1 Ingredients

Ingredients used in these semisolid formulations and their functions were as follow: flurbiprofen (API/model drug), natural oil/liquid paraffin (oil phase), Span[®] 60 and Tween[®] 80 (emulsifiers and non-ionic surfactants), methyl- and propyl parabens (preservatives), ethanol and polyethylene glycol (PEG) 400 (polar solvents for flurbiprofen), xanthan gum (polymeric thickener/gelling agent) and Milli-Q[®] water (solvent).

2.3.1.1 Emulgel formulations containing natural oils and liquid paraffin

The emulgel formulations containing the natural oils and liquid paraffin were prepared by weighing the ingredients into two separate glass containers (container 1: Phase A

and C; container 2: Phase **B**). Phase **C** (oil phase) contained the natural oil/liquid paraffin (20%), Span[®] 60 (3.55%), Tween[®] 80 (1.35%), methyl paraben (0.40%) and propyl paraben (0.08%), and was heated to 80 °C. Phase **A** (active phase) contained a mixture of ethanol/PEG 400 (6%), which was used to dissolve flurbiprofen (1%). Phase **A** was added to Phase **C** directly after it reached 80 °C. Phase **B** (water phase), consisting of xanthan gum (1%) and approximately 66.62 g Milli-Q[®] water, was prepared in the second glass container. The xanthan gum was slowly added to the water in small quantities and dissolved by stirring with a glass rod after the water reached 80 °C. The mixture containing phases **A** and **C**, was added to Phase **B**, after reaching the required temperature (80 °C) and homogenized at 13500 rpm, until cooled to 40 °C. Each formulation was allowed to cool to room temperature, and was stored at 2 - 8 °C until the following morning in order to conduct the diffusion studies.

2.3.1.2 Hydrogel formulation

In preparing the hydrogel formulation, the same method used to prepare the emulgels was used. The only exception was that the oil phase was omitted and replaced with Milli-Q[®] water (20%). In order to increase the viscosity, more xanthan gum (1.5%) was added to the formulation.

2.3.1.3 Foam formulation

In order to produce the two foams, previously prepared emulgels were combined with a pharmaceutical grade propellant, norflurane (HFA 134a), also known as Solkane[®] 134a pharma. The emulgels were measured into new open foam containers and sealed by crimping an aluminum valve seat to these containers. Solkane[®] 134a pharma was then forced under pressure into the sealed containers to produce the final product. The emulgel and gas were weighed before and after filling the containers, to ensure that

equal amounts of the propellant were used for each of the foam formulations. Foam formulations were prepared in duplicate.

2.4 Fatty acid methyl ester (FAME) analysis

GC was performed to determine the fatty acid composition of the natural oils.

Trimethylsulphonium hydroxide (TMSH) was used as the transesterification catalyst for the triglycerides in the oils, thus converting the fatty acids into their corresponding methyl esters [26], [27]. Quantitative analysis of the FAME determined percentage values of lauric- (C12:0), myristic- (C14:0), palmitic- (C16:0), stearic- (C18:0), palmitoleic- (C16:1), oleic- (C18:1), linoleic- (C18:2) and linolenic acids (C18:3) in each of the natural oils tested.

2.5 Franz cell diffusion experiments

2.5.1 Skin preparation for diffusion studies

Caucasian, female, abdominal skin was obtained after abdominoplastic surgery, following written consent from anonymous patients. Prior approval for the use of biological material obtained from human subjects for the Franz cell skin diffusion experiments was provided by the Research Ethics Committee of the North-West University (NWU) (reference no. NWU-00114-11-A5). In order to minimize biological variability among the specimens, each of the ten diffusion studies were performed on a single source of harvested skin. Twelve skin samples, 5.0 x 2.4cm, with a thickness of approximately 200 - 400 µm were harvested per study, using an electric dermatome (Zimmer® LTD, Swindon, Wiltshire, UK). The removed skin samples comprised of the stratum corneum, epidermis and upper dermis. Excised skin samples, with the stratum corneum facing up, were prepared by wiping the samples with paper tissue, soaked in Milli-Q® water and by then wiping once with paper tissue soaked in ethanol, in order to

remove any remaining residual subcutaneous fats and lipids. With the stratum corneum facing up, the harvested skin samples were tapped dry with tissue paper and placed on Whatman[®] filter paper. These samples were covered with aluminum foil, placed in Ziploc[®] plastic bags and kept frozen at -20 °C until used during the experiments. Preceding skin diffusion studies, the skin samples were defrosted at room temperature, punched into approximately 15 mm diameter circles with a punch and hammer; and mounted onto the Franz cells.

2.5.2 Receptor phase solution for diffusion studies

A phosphate buffer solution (pH 7.4) was used as the receptor phase solution in the Franz diffusion cells and prepared by accurately weighing 13.62 g potassium dihydrogen ortho-phosphate into a 500 ml volumetric flask and filling it to volume with Milli-Q[®] water. In another flask, 3.14 g sodium hydroxide pearls was weighed and dissolved in 768.80 ml Milli-Q[®] water. The two solutions were mixed and stirred with a magnetic stirrer. The pH was adjusted to 7.4 by adding either 2.0 M orthophosphoric acid (20% m/v) or 2.0 M sodium hydroxide (8% m/v) solution.

2.5.3 Franz cell skin diffusion experiments

The twelve-hour, *in vitro* skin diffusion studies were conducted using vertical Franz cells. These cells consisted of a receptor capacity of approximately 2ml and a diffusion area for the skin samples of approximately 1.075 cm² in diameter. The donor area received the semisolid emulgel (1g) formulation or the foam, whereas the receptor compartment contained the phosphate buffer solution (pH 7.4). To maintain an invariant concentration within the donor compartment, it was filled up periodically with the semisolid formulations, covered with Parafilm[®] and secured with a plastic cap to prevent evaporation or leakage. Harvested human skin samples were placed between

the donor and receptor compartments, with the stratum corneum facing up. Dow Corning[®] vacuum grease was used as sealant to prevent leakage in or out of the cells. Each cell was securely held together with a horseshoe clamp. The receptor compartments were filled with 2 ml of the pre-heated (32 °C) phosphate buffer solution, taking care to remove any trapped air by tilting the cell. Subsequently, the prepared Franz cells were placed in a tray on a Variomag[®] stirrer plate at 750rpm, within a Grant[®] water bath and maintained at approximately 37 °C. A total of twelve Franz cells were used during each of the skin and membrane diffusion studies, of which ten cells received either an emulgel or a foam formulation containing the marker flurbiprofen. Two cells received placebo formulations with no marker. The entire content of the receptor compartment was extracted and immediately replaced with pre-heated phosphate buffer solution at 20, 40, 60, 80, 100 and 120 min, and thereafter at 2, 4, 6, 8, 10 and 12 h. The recovered contents from the receptor compartments were injected into HPLC autosampler vials and directly analyzed on HPLC without any further processing.

2.5.4 Membrane diffusion experiments

Six-hour membrane diffusion studies were carried out prior to the skin diffusion experiments in order to determine whether the six formulated emulgels would release the marker, flurbiprofen. The exact same procedure was used as described in Section 2.5.3, except that polytetrafluoroethylene (PTFE) membranes were used instead of excised human skin. Phosphate buffer solution from the receptor compartments were extracted every hour for six hours.

2.5.5 Tape stripping procedure

Tape stripping of the skin was performed in order to determine the concentration of the marker within the stratum corneum-epidermis and epidermis-dermis. Following the

Franz cell skin diffusion experiments, the skin samples were removed after the final receptor withdrawal at 12 h. All of the skin circles were placed on Whatman[®] filter paper, the stratum corneum facing up, and tapped dry with tissue paper to ensure the removal of any residual formulation from the skin surface. The diffused area of skin was then partially removed using 3M Scotch[®] Magic[™] tape strips. Every first strip per skin sample was discarded and regarded as part of the cleaning process. The next fifteen strip samples taken from the specific skin sample were all placed in a single politop, containing 5 ml phosphate buffer solution (pH 7.4). A glistening epidermal layer was an indication that the entire stratum corneum was removed [28]. The remaining epidermis-dermis diffusion area of each skin sample was cut into small pieces and placed into a politop containing 2 ml phosphate buffer solution (pH 7.4). The politops with the stratum corneum-epidermis and epidermis-dermis samples were kept overnight for 12 h at 4°C. Samples were extracted with 0.45µm syringe filters from the politops and placed in HPLC autosampler vials and analyzed on HPLC without any further processing.

2.5.6 Data analysis

A standard solution was prepared as described in Section 2.2.2 in order to ensure linearity. The calibration curve was then constructed to determine the concentrations of each individual Franz cell within the skin diffusion experiments. The cumulative concentration ($\mu\text{g}/\text{cm}^2$) of the marker flurbiprofen that had permeated the skin was plotted against time and the slope of the straight line was used to determine the flux. All ten Franz cells containing the marker were initially used, but some outlier cells were omitted from the data due to either leakage of the cells, abnormal flow or depletion prior to the completion of the experiments.

2.5.7 Statistical data analysis

Data was statistically analyzed using inferential and descriptive statistics. For the descriptive statistics the mean and median values were calculated. Median value measures central location and is the statistical centre point. For data that is normally distributed, the mean is useful, whereas the median is unaffected by outliers in the data [29]. It was therefore suggested that the median would be a more accurate method for comparing the obtained flux values [30]. Data from skin diffusion experiments were graphically represented by means of scatter- and box-plots. Inferential statistics used in these experiments involved the analysis of variances (ANOVA), as well as a non-parametric, hypothesis testing. For the investigation of significant differences between the flux values of the different formulations, one-way ANOVA was used. This was followed by a post-hoc Tukey test that identified those formulations that had differed from each other. The Kruskal-Wallis test was performed to generate group medians, actual p values and test statistics and found that the median flux values would be a more accurate measurement of the true flux values [29], [31]. This test was followed by Dunn's multiple group comparison. In order to investigate the accumulation concentration of the marker for tape stripping data within the stratum corneum-epidermis and epidermis-dermis, a two-way ANOVA for significant interactions was performed. This was followed by a one-way ANOVA and non-parametric, Kruskal-Wallis test for the stratum corneum-epidermis and epidermis-dermis, respectively, followed by a Dunn's multiple group comparison. With regards to significance level $p < 0.05$ was indicative of a statistically significant difference. For the purpose of this study, interpretation of the more conservative Dunn's test for the pharmaceutical

interpretations was preferred, due to less differences having been reported without contradicting the Tukey post-hoc results.

3 Results and discussion

3.1 Fatty acid methyl ester (FAME) analysis

Collected data (Table 1) for the analysis of the natural oils showed high oleic acid (MUFA) values in olive- (76%), avocado- (68%), emu- (46%) and in crocodile oil (40%). Lower levels of oleic acid were found within grapeseed- (27%) and coconut oil (8%). Linoleic acid (PUFA) was present in high concentrations within grapeseed oil (61%), whereas the rest of the oils presented with levels less than 25%. Lauric acid (SFA) yielded high levels within coconut oil (52%), with average levels of myristic acid (21%) (SFA). Palmitic acid (SFA) was present in medium levels within crocodile- (21%) and emu oil (21%), whereas avocado-, grapeseed, olive- and coconut oil depicted levels below 15%. Stearic acid portrayed levels below 10% in both crocodile- and emu oil.

3.2 Franz cell diffusion experiments

3.2.1 Membrane diffusion experiments

Upon comparison of the median flux values (Figure 1) measured after 6 h of permeation through the membrane, the highest flux was observed with **(5)** ($144.82\mu\text{g}/\text{cm}^2$). This was followed by **(2)** ($137.42\mu\text{g}/\text{cm}^2$), **(7)** ($110.39\mu\text{g}/\text{cm}^2$), **(6)** ($104.28\mu\text{g}/\text{cm}^2$), **(8)** ($101.18\mu\text{g}/\text{cm}^2$), **(1)** ($93.95\mu\text{g}/\text{cm}^2$), **(3)** ($65.42\mu\text{g}/\text{cm}^2$) and lastly **(4)** ($48.06\mu\text{g}/\text{cm}^2$). These results clearly indicated that the marker flurbiprofen had been released from all of the formulations. It was also evident that formulations **(5)**, **(7)** and **(6)**, containing high amounts of oleic acid (MUFA), released the marker to a better extent than formulation **(4)**, which presented with high amounts of the branched linoleic acid (PUFA).

Formulation **(8)**, high in lauric acid (SFA), also released the marker better than **(3)** and **(4)**, which consisted of an average mixture of MUFAs and PUFAs.

Figure 1 shows that minor differences existed between the average and median flux values measured. This indicated that not too many outliers in the collected data existed and for that reason the average and median values could be used in the determination of the flux values. However, small differences were noted for formulation **(2)**, whereas a slightly larger difference was noted for formulation **(1)**. Therefore, the use of the median would be a more accurate representation of the true flux, due to it not being affected by outliers in the data [30].

3.2.2 Tape stripping experiments

3.2.2.1 Concentrations of flurbiprofen in the stratum corneum-epidermis

Average concentrations (Figure 2) of the marker flurbiprofen in the stratum corneum-epidermis following the skin diffusion experiments, could be placed in the following rank order: **(9)** ($21.47\mu\text{g}/\text{cm}^2$) $\geq$ **(2)** ($21.29\mu\text{g}/\text{cm}^2$) $>$ **(7)** ($17.82\mu\text{g}/\text{cm}^2$) $\geq$ **(4)** ($17.78\mu\text{g}/\text{cm}^2$) $>$ **(1)** ($16.73\mu\text{g}/\text{cm}^2$) $>$ **(6)** ($14.89\mu\text{g}/\text{cm}^2$) $>>$ **(8)** ($7.18\mu\text{g}/\text{cm}^2$) $>>>$ **(3)** ($2.72\mu\text{g}/\text{cm}^2$) $>$ **(10)** ($1.57\mu\text{g}/\text{cm}^2$) $>$ **(5)** ($1.25\mu\text{g}/\text{cm}^2$).

Small differences were noted (Figure 2) between the average and median concentrations of formulations **((1) to (10))**. However, large differences among the average and median values for formulations **(1)**, **(2)** and **(8)** indicated that use of the median flux values would give a more precise indication of the true concentrations due to the fact that it does not get affected by a distortion in the spread of the data [30].

3.2.2.1.1 Effects of hydration on the concentration of the marker

Natural oils present within formulations **(3)** to **(10)**, as well as the liquid paraffin in formulation **(2)**, may have acted as occlusives. These lipophilic materials [32] could

have added to the occlusive effect by increasing the hydration of the lipid structures within the stratum corneum [3]. It was therefore believed that occlusion of the skin increased the diffusion of the marker through a hydration process and therefore enhanced the concentration of the marker within the skin [33], [34]. Hydration that could have occurred through formulation **(1)** as a result of its high water content, may have also promoted the diffusion of the marker by opening up the dense, lipid structures within the stratum corneum, with subsequent accumulation of the marker [3], [35].

3.2.2.1.2 Effects of MUFAs and PUFAs on the concentration of the marker

The predominant UFA present within formulations **(9)**, **(7)** and **(6)** were oleic acid (C18:0). This fatty acid has been shown to have good enhancing properties [36]. The acid found in high concentrations within formulation **(4)** was linoleic acid (C18:2), whilst lower levels of oleic acid (C18:1) were observed. These MUFAs and PUFAs cause conformational kinks within their carbon chains due to the double bonds present. The more kink shaped, *cis*-double bonds found within these acids, the more difficult it becomes for these fatty acids to insert themselves within the stratum corneum. This may cause subsequent accumulation of the marker within the stratum corneum [36], [37]. In a study by Fang *et al.*, they concluded that the disruption and fragmentation of the stratum corneum lipids due to oleic acid (C18:1), may have enhanced the permeation of the marker within the skin. Their study indicated an accumulation of the API within the stratum corneum, which caused a reservoir effect of the API due to these UFAs present [38].

3.2.2.1.3 Effects of longer chain SFAs on the concentration of the marker

Relatively low concentrations of diffused flurbiprofen from formulations **(3)**, **(5)**, **(8)** and **(10)** may have been due to the palmitic acid (C16:0) present within these

formulations. It is believed that an increase in the hydrocarbon chain length of these fatty acids above C₁₄ decreases their enhancement effects. The longer chain fatty acids have a higher affinity toward the lipids within the stratum corneum and may have stalled the permeation of the marker within the skin. It is understood that due to the hydrophobic interactions within the stratum corneum, these longer chain SFAs show a high affinity toward the lipids within the stratum corneum as a result of their lipophilic nature, which might therefore have caused diffusional retardation of the marker within the skin [39].

3.2.2.1.4 Effects of SFAs and MUFAs on the concentration in foam formulations

Characteristics of formulation (10) as well as the shorter chain fatty acids present may have been responsible for the poor permeability of the marker. Short chain fatty acids have inadequate lipophilic properties to actively permeate the lipophilic stratum corneum [39]. Contrary, the high concentration of flurbiprofen within the stratum corneum released from formulation (9), could have been due to the fact that it predominantly contain oleic acid (C18:1) (MUFA) that has been established as a good enhancer, as previously described [36].

3.2.2.1.5 Concentration of the marker in the stratum corneum

Due to the lipophilic nature of the marker it automatically partitioned into the lipophilic stratum corneum, which probably resulted in the high concentrations found within this region for most of the formulations. The challenge would therefore be to keep a hydrophilic API inside of the lipophilic stratum corneum [40], [41]. The total concentration of the marker that succeeded in partitioning into the stratum corneum might have had a small propensity to be taken up into the viable epidermis, which may have been the reason for some of the formulations showing lower flurbiprofen

concentrations [1], [41]. Therefore, the concentrations in the stratum corneum of the lipophilic marker for most of the formulations, indicated a relationship between the skin and the lipophilic nature of the marker, which could have explained why the marker had been retained within this lipophilic region [42]. Accumulation of the marker could also have been as a result of its chemical structure, which could affect its solubility and partitioning characteristics. This may have ensured that flurbiprofen partitioned well between the hydrophilic and lipophilic regions of the stratum corneum. It partitioned easily into the stratum corneum, but due to the aqueous regions within the skin, it only slightly partitioned out of the stratum corneum into the epidermis-dermis [6], [43]. The high diffusion rate and accumulation displayed with formulation **(1)** could have been caused by the marker that was more soluble within the stratum corneum than within the hydrophilic formulation, therefore causing it to eagerly partition out of this formulation into the skin [44], due to the marker's lipophilic nature [36].

3.2.2.2 Concentration of flurbiprofen in the epidermis-dermis

Comparing the average concentration values of flurbiprofen (Figure 3) found in the epidermis-dermis following skin diffusion studies, formulation **(5)** achieved the highest concentration in the epidermis-dermis. Thus, the rank order was **(5)** ($16.14 \mu\text{g}/\text{cm}^2$) >>> **(1)** ($5.39 \mu\text{g}/\text{cm}^2$) > **(2)** ($4.56 \mu\text{g}/\text{cm}^2$) $\geq$ **(3)** ($4.28 \mu\text{g}/\text{cm}^2$) > **(8)** ($3.25 \mu\text{g}/\text{cm}^2$) > **(4)** ($2.98 \mu\text{g}/\text{cm}^2$) $\geq$ **(7)** ($2.46 \mu\text{g}/\text{cm}^2$) > **(9)** ($1.52 \mu\text{g}/\text{cm}^2$) $\geq$ **(6)** ($1.23 \mu\text{g}/\text{cm}^2$) > **(10)** ($0.97 \mu\text{g}/\text{cm}^2$).

No significant differences were noted (Figure 3) between the average and median concentrations of flurbiprofen. However, small variations in the data were seen for formulations **(3)**, **(4)** and **(8)**. It is thus advisable to rather use the median concentrations, since the outliers in the data would not affect them [30].

3.2.2.2.1 Effects of hydration on the concentration of the marker

Slight accumulation of the marker observed in the in the epidermis-dermis from formulations **(1)** and **(2)** may have been caused by the hydration of the stratum corneum, which caused a hydrophilic nature within this region [5]. Hydration may result from water diffusing from the underlying epidermal layers, which may have caused an increase in the penetration of the lipophilic marker by opening up the dense lipid layers of the stratum corneum [3]. Hydration could have made it more difficult to partition out of this hydrophilic surrounding by altering the structures within the intercellular domain through which it is believed flurbiprofen would partition through, thus causing fluidization of these layers. The resistance of this region decreased, and might have enhanced the partitioning of the marker within the skin layers [36], [45].

3.2.2.2.2 Effects of MUFAs and PUFAs on the concentration of the marker

High accumulation of the marker in the epidermis-dermis observed for formulation **(5)** could have been due to obvious scaling of the epidermis as a result of a small amount of palmitoleic acid (C16:1) and the high amounts of oleic- (C18:1) and linoleic fatty acids (C18:2) present within **(5)**. Scaling of the epidermis-dermis might have caused abnormal epidermal proliferation and differentiation (thickening) [46]. The small amounts of linolenic acid (C18:3) (PUFA) may have trapped the marker within the epidermis-dermis due to the degeneration of the skin appendages [36]. Therefore, unsaturated chains of C₁₈, appears to cause the most scaling of the skin with their bent *cis*-configurations as well as the disruption of the packing of the stratum corneum lipids [21]. The amount of the marker trapped within the skin is generally caused by the disruptive nature of oleic acid (C18:1) followed by linoleic acid (C18:2). These kinked shapes of the fatty acids cause them to insert themselves with difficulty into the lipid

layers of the stratum corneum. Hence, the epidermis-dermis accumulation could thus have been due to the enhancement of the marker reservoir within the epidermis-dermis region, due to this modification of the skin structures [36], [38], [46].

However, the high amounts of oleic acid (C18:1) remaining within the stratum corneum from formulations (3), (4), (6), (7) and (9) might have caused an increase in transepidermal water loss (TEWL) and morphological changes in this region. These changes might have resulted in small amounts of the marker diffusing through to the epidermis-dermis region. The formulations containing higher amounts of oleic acid (C18:1) caused more morphological changes in the skin and could therefore have resulted in a minor accumulation of the marker within the skin [38], [47].

3.2.2.2.3 Effects of SFAs on the concentration of the marker

The linear SFAs present within formulations (3), (8) and (10) might have contributed to the concentrations of the marker found within the epidermis-dermis for these formulations. Lauric- (C12:0) and palmitic SFAs (C16:0) have low solubilities [12], resulting in them being semisolid at the temperatures at which these experiments took place. These SFA have a modest effect on the packing of the lipids within the stratum corneum and consequently cannot insert themselves into the lipid regions, resulting in little or no effect(s) [37].

3.2.2.2.4 Effects of SFAs and MUFAs on the concentration in foam formulations

Low concentrations of the marker released from formulations (9) and (10) could have been caused by the formulation itself, which had trapped air inside the foam and might have prevented the formulation from having optimal direct contact with the available skin surface. The entrapped air in these formulations furthermore caused there to be less of the marker present within the donor phase of the Franz cells, due to the foam

formulation having less marker per volume compared to the semisolid emulgel formulation.

3.2.2.2.5 Concentration of the marker in the epidermis-dermis

Protein binding of the marker may have occurred within the stratum corneum with flurbiprofen's hydroxyl groups, and could have retarded the penetration of flurbiprofen within the epidermis [41]. The low concentrations of the marker in the epidermis-dermis region obtained from most of the formulations could have been due to the lipophilic nature of the marker that had lowered solubility within this aqueous environment of the epidermis-dermis region [48]. Due to the fact that the epidermis-dermis region is more hydrophilic than the stratum corneum-epidermis, it might have caused a barrier to the lipophilic marker [33]. Therefore, this viable layer of the epidermis-dermis presents a rather significant barrier to cross for the lipophilic marker [6].

3.2.3 Skin diffusion experiments

3.2.3.1 Comparison of the flux values obtained for the different formulations

A comparison of the median flux values (Figure 4) of the marker that had diffused through the excised human dermatomed skin after 12h showed that the highest flux was attained with formulation **(1)** ($23.79 \mu\text{g}/\text{cm}^2$). This was followed by **(7)** ($17.99 \mu\text{g}/\text{cm}^2$), **(2)** ($15.70 \mu\text{g}/\text{cm}^2$), **(8)** ($13.16 \mu\text{g}/\text{cm}^2$), **(4)** ($11.85 \mu\text{g}/\text{cm}^2$), **(3)** ($8.31 \mu\text{g}/\text{cm}^2$), **(6)** ($6.68 \mu\text{g}/\text{cm}^2$), **(9)** ($5.56 \mu\text{g}/\text{cm}^2$), **(5)** ($4.41 \mu\text{g}/\text{cm}^2$) and lastly **(10)** ($4.36 \mu\text{g}/\text{cm}^2$).

Average and median flux values did not differ significantly from each other. It could have been due to small variances between the data points as a result of the minimum outliers in the data generated. Since the median flux takes all of the data into consideration and is unaffected by distortion within the range of the obtained values, it provides a more accurate representation of the true flux values [30].

3.2.3.1.1 Effects of hydration on the flux of the marker

The high flux value observed from formulation **(1)** might have been due to its high water content that had caused hydration of the skin. Hydration causes swelling of the stratum corneum and opens up the dense lipid structures that could have caused an increase in the permeation of the marker [5], [49]. Water accumulation within the skin could have possibly resulted from occlusion of the skin with formulation **(2)** that could also have resulted in the swelling of corneocytes and an increase in the water content within the intercellular lipid region of the stratum corneum, preventing TEWL [10], [21], [50] with a subsequent increased flux of the lipophilic flurbiprofen [21]. Liquid paraffin preparations have been used as penetration enhancers before, but might cause early depletion of an API from a formulation [51]. In spite of this, it has successfully been used as an enhancer for another poorly absorbed anti-inflammatory [52].

However, El Magraby *et al.*, stated that an over hydrated skin could produce too much swelling of the corneocytes inside of the stratum corneum and could thus negatively affect the permeation of the marker [53].

3.2.3.1.2 Effects of UFAs on the flux of the marker

Within the fatty acids, the addition of double bonds (linolenic acid (C18:3) > linoleic acid (C18:2) > oleic acid (C18:1)) are thought to increase the flux of the marker, however more so for oleic acid. These fatty acids increase TEWL by decreasing the barrier function of the stratum corneum. Barrier function is decreased by the fatty acids packing inside of the skin that causes wider channels within, which subsequently could have increased the flux of the marker [38], [46], [47]. The more double bonds the UFAs consist of though, the more difficult it becomes for them to insert themselves within the lipid structure of the stratum corneum [37]. Formulation **(4)** containing an

average amount of oleic acid (C18:1), but high amounts of linoleic acid (C18:2), yielded a rather average flux for the marker. It is therefore believed that the “kink” within this fatty acid disrupted the organized lipid layers within the stratum corneum that mainly contains saturated, straight chain, fatty acids [54]. The flux of formulation **(4)** could also have been due to the wider channels formed within the skin for the permeation of the marker, due to the packing of flurbiprofen within the skin after the stratum corneum lipids have been disrupted [37].

3.2.3.1.3 Effects of MUFAs on the flux of the marker

The high oleic acid (C18:1) (MUFA) concentration in formulation **(7)** modulates the inter-cellular lipid area of the stratum corneum [23] and causes lipid fluidization [40], which might have been the reason for the high flux in comparison with the rest of the formulations [54]. The bulky “kink” conformation of oleic acid causes a disordering of the intercellular lipids, thereby decreasing the barrier function of the stratum corneum that could therefore have resulted in an increased flux of the marker [46].

3.2.3.1.4 Effects of PUFAs on the flux of the marker

Linolenic acid (C18:3) (PUFA) present within formulation **(3)** has three double bonds. Although formulation **(3)** contained a small amount of this fatty acid, it has been illustrated that an increase in the number of double bonds within fatty acids causes difficulty for them to insert themselves in the lipid layers of the stratum corneum [37], which could have been responsible for the low flux observed. Furthermore, the low flux values observed for formulations **(4)**, **(5)** and **(6)** could have been as a result of their high linoleic acid (C18:2) content and average levels of linolenic acid (C18:3). Therefore, it may be that an increase in the number of double bonds within the fatty acids did not enhance the flux of flurbiprofen within these formulations [24].

3.2.3.1.5 Effects of medium chain SFAs on the flux of the marker

The high levels of lauric acid (C12:0) (SFA) present within formulation **(8)** could have increased the flux of the marker through the skin [54]. A possible mechanism of action for lauric acid (C12:0) could have been due to a complex structure formed between the marker and the acid in the formulation, the dissociation of this complex into components within the skin between the stratum corneum-epidermis and epidermis-dermis might have caused the flux that was observed [24]. Medium chain lauric- (C12:0) and myristic acids (C14:0) present within formulation **(8)** have good penetration enhancing activities due to an optimal balance of the partition coefficient and their affinity for the skin [39]. This might indicate that the medium chain SFA may enhance the permeation of the marker [39].

3.2.3.1.6 Effects of longer chain SFAs on the flux of the marker

Although oleic acid (C18:1) has been shown to be a good enhancer [54], the lowered flux obtained formulation **(3)** could have been due to the SFAs, palmitic- (C16:0) and linolenic acid (C18:3) present. An increase in chain length ($> C_{14}$) does not always result in an enhanced flux [54], and as described previously, the more double bonds a fatty acid has, the more difficult it becomes to insert itself within the lipid matrix of the stratum corneum [39].

Increased flux values were expected for formulations **(5)** and **(6)** from animal origin, due to their similarities to human butter fat [16]. The melting points of palmitic- (C16:0) and stearic acids (C18:0) are higher [19] and might not have dissolved properly at the temperature at which the experiments were conducted [55]. The melting points of these linear SFAs range from 62.9 - 69.6 °C [19], resulting in lower solubilities of the fatty acids and less capability to disrupt the ordered lipids within the stratum corneum

through insertion within the layers [37]. Due to the fact that stearic acid (C18:0) is also present within the skin, it is believed that it will not cause disordering of these packed lipids of the stratum corneum. However, branched versions of the same chain length of stearic could upset the packing of the lipids in the stratum corneum to a more significant extent [55]. Formulation **(5)** contained the highest amounts of stearic acid (C18:0) compared to the other formulations, and therefore it is believed that the low flux value could have been due to the high melting point as indicated before. The stearic acid was probably a semisolid at the time of the skin diffusion studies [55]. Although SFAs depicted some enhancing activities during this study, Chi *et al.*, did not report any significant increase in diffused flurbiprofen with SFAs ranging from C₁₆ to C₂₀ [37].

3.2.3.1.7 Effects of SFAs and MUFAs on the flux in foam formulations

Low flux values were observed for formulations **(9)** and **(10)** and could have been attributed to the fact that these formulations had trapped air that probably reduced optimal contact with the available skin surface. It might have resulted in less formulation being present within the donor phase of the Franz diffusion cells, resulting in less of the marker present per volume applied compared to the emulgel formulations. The reason for the differences observed between these two formulations, although insignificant, could have been attributed to the oleic acid (C18:1) present within formulation **(9)**, which seemed to have increased enhancer properties compared to lauric acid (C12:0) present within formulation **(10)** [54].

3.2.4 Inferential statistical data analysis

3.2.4.1 Outcomes of membrane release studies for the different formulations

Statistical comparison of the flux values with one-way ANOVA showed that significant differences ($p < 0.05$) existed between the different formulations. The Tukey post-hoc test revealed twenty-eight different comparisons from the eight formulations used in the membrane studies. Therefore, the more conservative Dunn's, non-parametric, multiple comparison test was preferred and revealed less differences between most of the formulations. However, no statistical significant differences were observed between formulation **(4)** and **(1)** ($p = 1.000$), with statistical significant differences observed between the rest of the formulations ($p < 0.05$).

3.2.4.2 Outcomes of tape stripping for the different formulations

3.2.4.2.1 Stratum corneum-epidermis

From the possible forty-five comparisons, the Dunn's multiple comparison revealed only fourteen significant differences amongst the formulations. Formulation **(6)** revealed no significant differences with all the formulations, whereas formulation **(1)** depicted statistical significant differences when compared to formulation **(5)** ($p = 0.024$). The p-value for formulation **(3)** indicated statistical significant differences when compared to **(2)** ($p = 0.000$) and to **(9)** ($p = 0.000$) with a p-value of less than 0.05.

3.2.4.2.2 Epidermis-dermis

Of the possible forty-five comparisons, the Dunn's multiple comparison only revealed twelve significant differences. Formulation **(8)** showed no significant differences when compared to the rest of the formulations. Upon comparing formulation **(7)**, however, it does show statistical significant differences when compared to **(5)** ($p = 0.005$) with a p-value of less than 0.05.

3.2.4.3 Outcomes of skin diffusion studies for the different formulations

Statistical comparisons performed on the flux values obtained for the ten different formulations revealed forty-five different multiple group comparisons. The more conservative execution of the Dunn's post-hoc test revealed only twenty-two comparisons. This showed that formulation (3) had no significant differences with most of the formulations, it did however indicate statistical significant differences with formulation (1) ($p = 0.015$). The formulation (4), depicted statistical significant differences with formulation (10) ($p = 0.034$), but no significant differences were seen with any of the other formulations ($p\text{-values} > 0.05$).

4 Conclusions

Natural oils show properties of ideal penetration enhancers, without demonstrating any pharmacological activities. During the diffusion studies, the marker, flurbiprofen, was released from all of the formulations and some penetration enhancing activities between the different emulgel formulations were indicated. It became evident that the differences between the oils containing UFAs and those containing the same amount of carbons in the hydrocarbon chains of the fatty acids generated different enhancing strategies [11]. APIs with log P-values between 1 and 3 depicts noteworthy absorption within the skin [50]. A partition coefficient less than 1 would cause insufficient partitioning into the skin layers, whereas a coefficient higher than 3 might cause accumulation of an API within the stratum corneum [42]. It was therefore expected that the lipophilic flurbiprofen would partition easily into the stratum corneum *via* the intercellular route [36]. Suffice to say, as observed in the outcomes of most of the tape stripping data, the lipophilic flurbiprofen preferred to reside within the lipophilic stratum corneum. A study done by Fang *et al.*, showed rather similar results and they

found that UFAs slightly enhanced the reservoir of flurbiprofen within the skin [38]. It is suggested, that UFAs are more effective in the absorbing lipophilic APIs than SFAs into the skin[56].

Comparison of the data from the membrane and skin diffusion studies led to the belief that the skin itself played a major role in the permeation of flurbiprofen through the skin [36].

Low concentrations of flurbiprofen, obtained in the epidermis-dermis, could have been attributed to the fact that APIs present within oils or in other lipid soluble formulations penetrate the epidermis-dermis very slowly through the cell membrane layers of the stratum corneum [57]. The concentrations of the marker found within this region, might also have been as a result of its lipophilicity and its lowered solubility within the aqueous environment of the epidermis-dermis [48].

The enhanced flux for formulation **(1)** may be explained by the occurrence of hydration, which increased the skin's pliability and inhibited TEWL. Swelling of the stratum corneum could have increased the diameter of the sweat ducts and may have increased the permeation of the marker [6]. Flurbiprofen might also have had a low affinity for the aqueous hydrogel formulation, causing it to eagerly diffuse out of the formulation into the more lipophilic stratum corneum, resulting in the increased flux being demonstrated [52]. Penetration enhancers, similar to fatty acids, generally disorder the packing of the ordered lipids within the stratum corneum by interacting with the polar lipid head groups, or with the hydrophobic lipid tails within this region [8]. Due to the fact that the stratum corneum predominately consists of C₁₆ or longer chains, it is the branched or longer chain UFAs that disrupt these lipids within the stratum corneum to a greater extent than SFAs of the same chain length during permeation of the skin [55].

Alteration of the intercellular hydrocarbon chain lipids of the stratum corneum causes “kinks” within this pathway, thus, creating small openings and increasing the fluidity of the stratum corneum. This might have resulted in the increased diffusion of the marker across the skin [45]. The reason for the enhanced flux seen for formulation **(7)** could have been due to its high oleic acid (C18:1) content, which may explain the increase of the flurbiprofen concentration within the stratum corneum [54]. A study by Takahashi *et al.*, tested the enhancing effects of oleic acid (C18:1), found in formulation **(7)**, as well as the effects of liquid paraffin, found in formulation **(2)**. Since the results were similar to the results demonstrated in this study, it was concluded that liquid paraffin had slightly increased the penetration enhancing activity, however, the penetration enhancing activity of oleic acid (C18:1) was more significant [52].

The flux value obtained from formulation **(8)** was higher than the value obtained from formulations **(3)**, **(4)**, **(5)**, and **(6)**, which predominately contained UFAs; whereas formulation **(8)** mostly consisted of short-and medium chain SFAs. It is believed that the ordered regions of the stratum corneum contain C₆ to C₈ hydrocarbons, and when disrupted, it may enhance fluidity and increase the permeation of the marker, flurbiprofen. The alkyl chain of the fatty acid might penetrate to the same depth and thus induce fluidization [58].

Skin diffusion studies depicted low flux values for formulations **(9)** and **(10)** that could have been as a result of air being trapped in the foam formulations, hence causing the formulation to not have optimal direct contact with the available skin surface for maximum diffusion of the API, as well as to have less of the formulation present within the donor phase of the Franz diffusion cells. The collapse of the foam in the donor phase also decreased the volume of the formulation, due to the loss of air [59], and

therefore having less flurbiprofen per volume present when compared to the emulgel formulations. No significantly clinical advantage seemed to have been gained by utilizing foam formulations [59], compared to the emulgel formulations; although a study of Deng *et al.*, showed an increase in the rate at which the API was delivered from a foam, compared to a cream, solution and ointment formulation [60].

The high concentration of flurbiprofen observed within the stratum corneum for formulation **(9)** might have been as a result of the oleic acid (C18:1) found within the formulation [36], or as a result of the rapid evaporation of the volatile propellant from the foam, which might have caused an increase in the concentration of the marker within the remainder of the formulation [61].

Throughout this study, a common trend was noted in that the transdermal penetration of the lipophilic marker, flurbiprofen, was enhanced more by oils predominantly consisting of UFAs, and even more so for those oils high in oleic acid (C18:1) (MUFA). Oils that primarily contained SFAs also increased the flux of the marker to some extent, especially with chain lengths between C₁₀ and C₁₂ [55]. However, the delivery of flurbiprofen did not differ much from those oils containing high amounts of oleic acid. It was also concluded that oils containing a high concentration of SFAs, demonstrated increased enhancing action compared to oils having an almost equal combination of MUFAs and PUFAs. However this increase was not more significant than oil predominantly consisting of oleic acid (MUFA). Therefore, the results of this study demonstrated similarities to a study by Aungst, in that the enhancement effects observed for branched fatty acids, did not vary significantly from the effects shown for unbranched fatty acids of the same carbon chain lengths [55].

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Declaration of interest

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Tables:

Table 1: GC results of the fatty acid composition (%) of the selected natural oils.

	Capric	Lauric	Myristic	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	Linolenic	Arachidonic
	SFA ^a					MUFA ^b		PUFA ^c		
Oil or fat:	10:0	12:0	14:0	16:0	18:0	16:1	18:1	18:2	18:3	20:4
Avocado				15	1	6	68	10	1	
Grapeseed				6	4		27	61	0.5	
Emu		0.1	0.3	21	10	4	46	16	2	
Crocodile		0.1	1	21	5	6	40	24	2	1
Olive				12	3	1	76	7	1	
Coconut		52	21	9	3		8	3	0.2	

^a Saturated fatty acids

^b Monounsaturated fatty acids

^c Polyunsaturated fatty acids

Figure Legends:

Figure 1: Box-plot representation of the flux values for the different formulations in the membrane diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

Figure 2: Box-plot representation of the concentrations within the stratum corneum-epidermis for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

Figure 3: Box-plot representation of the concentrations within the epidermis-dermis for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

Figure 4: Box-plot representation of the flux values for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

Figures:

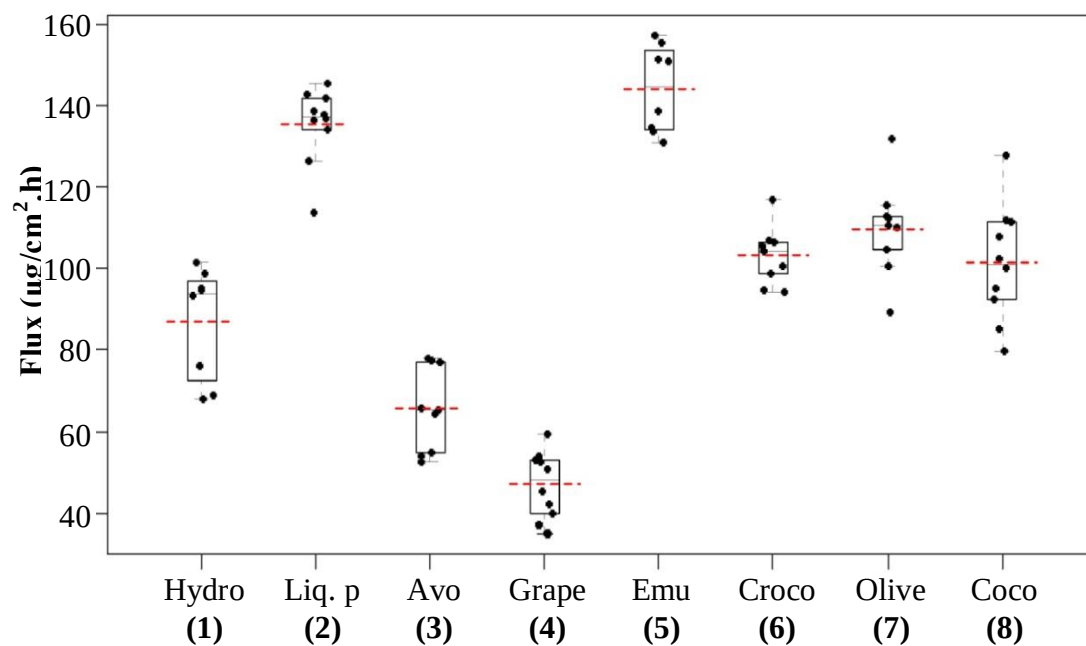


Figure 1: Box-plot representation of the flux values for the different formulations in the membrane diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

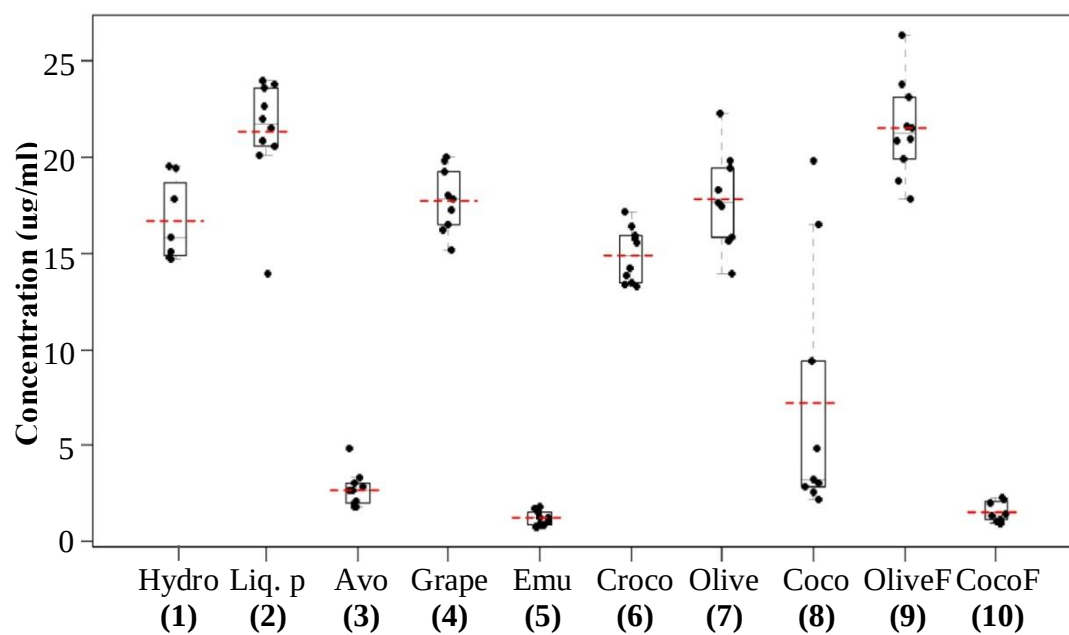


Figure 2: Box-plot representation of the concentrations within the stratum corneum-epidermis for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

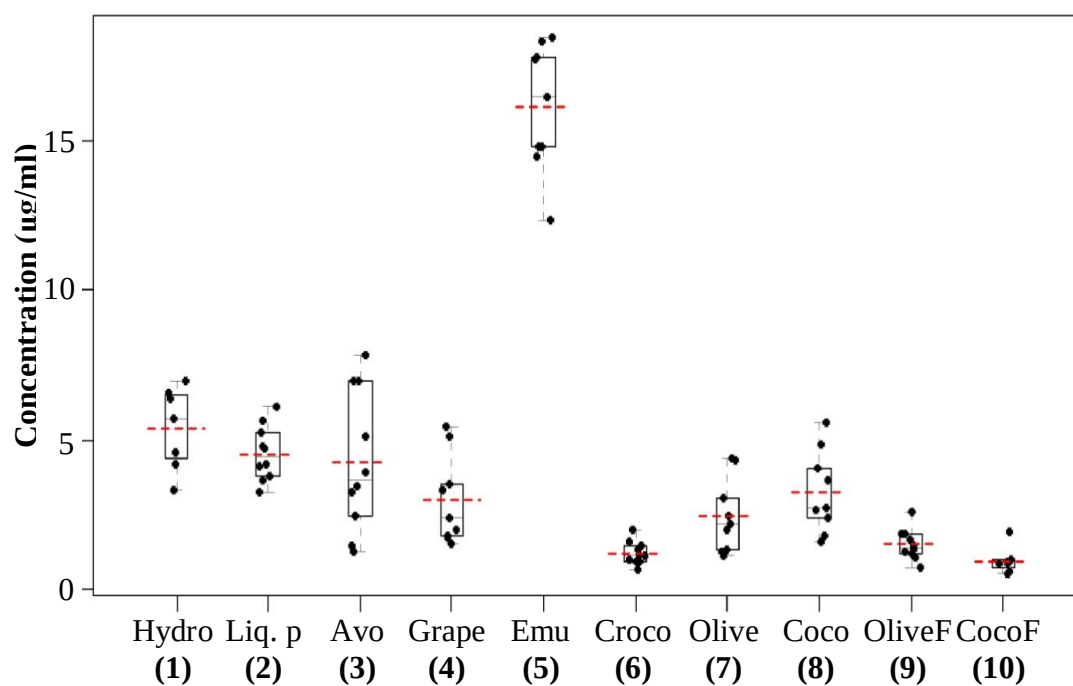


Figure 3: Box-plot representation of the concentrations within the epidermis-dermis for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

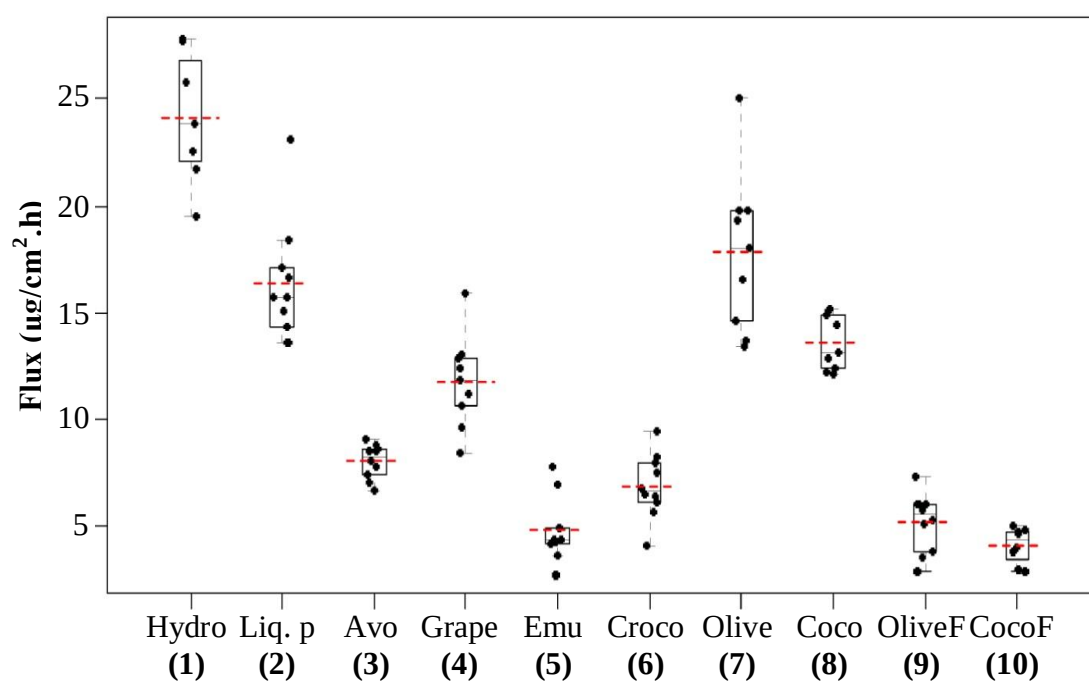


Figure 4: Box-plot representation of the flux values for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

CHAPTER 4

FINAL CONCLUSIONS AND FUTURE PROSPECTS

A topical delivery system is one that is applied directly onto any external body surface (Flynn, 2002:187). Topical formulations are applied to the skin in order to achieve its local effect directly underneath the area of application, by either targeting the specific site of application, or the deeper regions somewhat distant from the application site. Systemic uptake of drugs is thus not preferred or aimed by most topically applied formulations (Stanos, 2007:344). Transdermal absorption of an API is influenced by its physicochemical properties, as well as the formulation itself. The selection of an appropriate vehicle is therefore important in increasing the efficacy of a topically applied drug (Takahashi *et al.*, 1991:154). Chi *et al.* (1995:267) studied numerous vehicles that included ointments, gels and creams to enhance the intrinsically poor skin permeability of the lipophilic marker flurbiprofen, and found that penetration enhancers were needed. The role of penetration enhancers is to reversibly modify the barrier properties of the stratum corneum through improved fluidity of the structures within (Charoo *et al.*, 2008:300; Walker & Smith, 1996:299). Fatty acids have extensively been examined as potential penetration enhancers for the topical and transdermal delivery of numerous APIs (Babu *et al.*, 2006:144).

Therefore, the aim of this study was to investigate the penetration enhancing potential of several natural oils, based on their fatty acid contents, since fatty acids comprise a group of compounds frequently used to increase skin permeation. For the purpose of this study, lipophilic flurbiprofen was utilised as marker (Williams & Barry, 2004:609; Fang *et al.*, 2004:163).

The objectives of this study were to:

- develop and validate a HPLC method to quantify the flurbiprofen extracted receptor phase sampled during the membrane and *in vitro* diffusion studies; as well as the concentration of the diffused flurbiprofen in the different skin layers;
- determine the fatty acids percentage composition of each of the oils used in this study by GC;

- formulate six emulgel delivery systems, consisting of the selected natural oils, as well as two emulgels, one containing liquid paraffin and another without the inclusion of oil (hydrogel), for testing during comparative studies;
- formulate foams consisting of the natural oil emulgels that depicted the highest flux values;
- determine the release rate of the marker flurbiprofen from the formulated emulgels and hydrogel through membrane diffusion studies, prior to conducting the skin diffusion studies;
- determine whether the fatty acids have improved delivery into (topical) the skin by conducting tape stripping studies, using dermatomed excised, human skin; and to
- determine whether the fatty acids have improved delivery through (transdermal) the skin by conducting *in vitro* skin diffusion studies, using dermatomed excised, human skin.

The HPLC method used in this study to quantify the flurbiprofen in the extracted samples for each of the membrane and skin diffusion studies was successfully developed and validated at the North-West University, Potchefstroom Campus, South Africa.

Data generated during the fatty acids analyses on GC of the six natural oils, indicated that olive- (76%), avocado- (68%), emu- (46%) and crocodile oil (40%) presented with high levels of oleic acid (MUFA), whereas grapeseed- (27%) and coconut oil (8%) depicted lower levels thereof. High levels of linoleic acid (PUFA) were detected within grapeseed oil (61%), whereas the remainder of the oils yielded levels of less than 25%. The only oil high in SFA was coconut oil, consisting of predominantly lauric- (52%) and myristic acid (21%). Crocodile- (21%) and emu oil (21%), both of animal origin, portrayed average levels of palmitic acid (SFA); and avocado-, grapeseed-, olive- and coconut oil, from plants, presented with levels below 15%. All of the oils presented with levels below 10% for stearic acid (SFA), and the two oils of animal origin portrayed the highest values.

The aim of the study was not to deliver flurbiprofen in the skin for a specific indication, but only to serve as a marker in order to investigate the penetration enhancing effects, if any, of the chosen natural oils on this lipophilic API. For this reason, 1% of the flurbiprofen was included in all of the emulgel formulations, consisting of the six selected natural oils as the oil phase. These emulgels were compared to those of a hydrogel **(1)** without the inclusion of oil, and an emulgel containing liquid paraffin **(2)** as the oil phase. The semisolid emulgels contained one of the selected natural oils, i.e., avocado oil **(3)**, grapeseed oil **(4)**, emu oil **(5)**, crocodile oil **(6)**, olive oil **(7)** and coconut oil **(8)** were formulated. Olive- and coconut oil emulgels demonstrated the highest flux values during the skin diffusion studies compared to the other selected natural oils; and were subsequently incorporated into olive oil- **(9)** and coconut oil foam formulations **(10)**. The final foam product was prepared, by utilising previously prepared emulgel

formulations and including it under pressure with a propellant into a foam container after sealing.

Skin and membrane diffusion studies demonstrated that the flurbiprofen marker had penetrated through both the PTFE membranes and the dermatomed excised, human skin. The membrane diffusion studies performed prior to the skin diffusion studies indicated that the marker was released from all of the applied emulgel formulations. Results from these tests showed that those emulgels high in oleic acid (MUFA) [(**5**) (144.82 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), (**7**) (110.39 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) and (**6**) (104.28 $\mu\text{g}/\text{cm}^2\cdot\text{h}$)], released flurbiprofen to a more significant extent than formulation (**4**) (48.06 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), predominantly consisting of linoleic acid (PUFA). Formulation (**8**) (101.18 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), containing a majority SFA in the oil, showed a significant increased concentration released compared to the formulation of (**3**) (65.42 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) and (**4**) (48.06 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) that contained an average combination of MUFAs and PUFAs.

APIs with a log P between 1 and 3 should demonstrate good diffusion through the skin. Due to flurbiprofen's high log P value (4.16), indicative of its lipophilicity, it was expected that it would partition easily into the stratum corneum. The fact that this marker has a rather high log P was an indication of its inability to partition out of the stratum corneum into the viable epidermis (Charoo *et al.*, 2008:300; Craig, 1990:541; Wiechers & Watkinson, 2008:63).

Tape stripping data demonstrated that the lipophilic flurbiprofen had partitioned easily into the stratum corneum. It remained there after release from most of the formulations and probably preferred to stay within the stratum corneum due to the lipophilic nature of this region that would therefore result in minimal systemic absorption of this marker. Extremely lipophilic APIs can easily partition into the skin lipids, but because of the aqueous regions in the skin lipids, they do not easily partition out of the stratum corneum into the viable epidermis. The results of the tape stripping studies indicated poor permeability of the lipophilic marker into the epidermis-dermis regions. This could have been due to the hydrophilic nature of this area of the skin (Fang *et al.*, 2003a:319; Hadgraft & Finnin, 2006:365). The amount of flurbiprofen retained within the stratum corneum-epidermis experiments might have been an indication that the tissue concentrations are related to the flux across the skin from the applied formulations (Santoyo & Ygartua, 2000:247). A fraction of the available flurbiprofen might have supersaturated the stratum corneum by binding to the keratin or other tissue components and slowly diffusing downwards into the epidermis-dermis (Barry, 1983:117). A study by Fang *et al.* (2003b:156) concluded that a series of fatty acids slightly increased the drug reservoir within the skin, promoting transdermal absorption of flurbiprofen.

Formulation (**9**) yielded the highest concentration (21.47 $\mu\text{g}/\text{ml}$) of diffused flurbiprofen within the stratum corneum-epidermis, which may have been due to the high oleic (C18:1) acid

(MUFA) content of this formulation. Although it was insignificantly higher than the concentrations obtained from formulation **(2)** (21.29 µg/ml), it may have been that the flurbiprofen had absorbed and penetrated the skin further through the hair shafts (without leaving an oily residue). Rapid evaporation of the volatile components (i.e., propellant) from the foam formulation might have resulted in an increase in the flurbiprofen concentrations within the foam, with maximum drug transfer from this saturated formulation into the skin (Arzhavitina & Steckel, 2010:7, 11, 15; Green *et al.*, 1988:109; Purdon *et al.*, 2003:72, 75; Smith, 1990:25).

Low concentrations of diffused flurbiprofen found within the epidermis-dermis region for most of the formulations ranged between 0.97 - 5.39 µg/ml, except for formulation **(5)** (16.14 µg/ml). The higher accumulation of **(5)** might have been due to obvious scaling of the epidermis-dermis region by palmitoleic- (C16:1), oleic- (C18:1) and linoleic acids (C18:2) present in this formulation (Katsuta *et al.*, 2005:1009). The trapped marker within the epidermis-dermis region seemed to have been enhanced more by oleic- than by linoleic acid. It seemed that the more kinked shaped the fatty acids became, as a result of the double bonds present, the more difficult it became to insert themselves within the lipid layers of the skin. It therefore accumulated within the skin and enhanced the reservoir of the marker within the skin (Fang *et al.*, 2003a:318:319; Fang *et al.*, 2003b:156). The lowered concentrations observed for the remainder of the formulations were possibly due to flurbiprofen's lipophilic nature with reduced solubility within the aqueous region of the epidermis-dermis, therefore preventing the transport of the marker into the deeper tissues (Hadgraft, 1999:5; Ogiso & Shintani, 1990:1067).

For the Franz cell skin diffusion studies with the emulgel formulations, formulation **(1)** depicted the highest median flux (23.79 µg/cm².h) through the skin. This could have been as a result of the high water content of this formulation, causing hydration of the skin, and causing the stratum corneum to swell and open up its dense structures, with a subsequent increase in the penetration of flurbiprofen through the skin. Increased skin hydration appears to enhance the transdermal delivery of lipophilic and hydrophilic APIs (Benson, 2005:28; Williams & Barry, 2004:606). The liquid paraffin present within formulation **(2)** has shown to increase permeation of the API and the flux reached 15.70 µg/cm².h. Liquid paraffin is a lipophilic substance that can occlude the skin and increase the hydration of the skin. This might have resulted in an increase in the permeation of the marker by decreasing the barrier resistance of the stratum corneum through hydration (Barry, 1983:154; Barry, 2001:969; Barry, 2007:576,586; Benson, 2005:28; Leopold & Lippold, 1995:195; Ramchandani & Toddywala, 1997:550; Thomas & Finnin, 2004:699).

The second highest flux obtained for the natural oil formulations was observed for formulation **(7)** (17.99 µg/cm².h), which was probably as a result of its high oleic (C18:1) acid content. Oleic acid is believed to be the best enhancer of all the fatty acids. Santoyo & Ygartua (2000:247)

stated that when fatty acids were included in formulations, especially the C₁₈ UFA, the permeation of an API could be improved (Santoyo & Ygartua, 2000:247). The flux in the skin achieved with formulation **(4)** (11.85 µg/cm².h) might have been caused by additional double bonds of the fatty acids found within this oil. Oleic acid has, however, proven to be more successful than linoleic acid, and increases TEWL more effectively than linoleic acid by decreasing the barrier properties of the stratum corneum. This could have resulted in an increased flux of the marker through the skin (Fang *et al.*, 2003b:156; Katsuta *et al.*, 2005:1010; Tanojo *et al.*, 1999:102).

Lauric acid (C12:0) present in formulation **(8)** (13.16 µg/cm².h), may have had some influence on its flux value. A possible mechanism of action for lauric acid (C12:0) could have been due to complex formation between the fatty acid and the API. The latter dissociation of the formed complex into the interface between the stratum corneum-epidermis and the epidermis-dermis might have increased the flux of the marker within the skin (Santoyo & Ygartua, 2000:247). Lauric- (C12:0) and myristic acids (C14:0) have shorter chain lengths and thus better interaction with the skin, due to an optimal balance of the partition coefficient and their affinity for the lipids in the skin that may induce the flux of the marker (Ogiso & Shintani, 1990:1067).

The flux for formulation **(3)** (8.31 µg/cm².h) could have been due to the palmitic acid (C16:0) present. An increase in SFA of C₁₆ to C₁₈ has shown to not always increase the flux of the marker positively (Chi *et al.*, 1995:270). With formulations **(5)** (4.41 µg/cm².h) and **(6)** (6.68 µg/cm².h), both from animal origin, the flux was thought to be due to the palmitic- (C16:0) and stearic acids (C18:0) having higher melting points. These acids might not have dissolved at the temperature at which the experiments in this study were conducted. As a result, SFAs with low solubility have less potential to insert themselves within the skin lipids and therefore cause less disruption of these layers (Aungst, 1989:245; Chi *et al.*, 1995:270; O'Brien, 2009:266).

Skin diffusion studies indicated that foam formulations **(9)** (5.56 µg/cm².h) and **(10)** (4.36 µg/cm².h) had poor median flux values. This could have been due to the trapped air within these formulations, hence causing the formulation to be deprived of optimal direct contact with the available skin surface for maximum diffusion of the API; as well as to have less of the formulation present within the donor phase of the Franz diffusion cells. Foam formulations will effectively contain less of an API per volume due to the air bubbles within the foam. Thus, when the foam collapses as the trapped air escapes, the total volume which is in contact with the skin, will be significantly less compared to the formulations that do not contain any propellant.

The results of this study indicated that:

- The effects of the more branched fatty acids are not considerably different to those of the un-branched fatty acids of the same carbon number (Aungst, 1989:246). However, the effects of UFAs, especially of oleic acid, definitely increased the flux and concentration of the marker to a larger extent than the SFAs.
- SFAs with alkyl chain lengths of approximately C₁₀ to C₁₂ showed good flux enhancing properties, whilst the UFAs with alkyl chains of C₁₈ appeared to be most optimal in this regard due to the *cis* configuration of oleic acid. This configuration appears to disrupt the intercellular packing of the lipids within the stratum corneum to a higher extent and thus increases the flux of the marker within the skin (Williams & Barry, 2004:609, Aungst, 1989:246).
- Oils predominantly consisting of UFA proved some degree of accumulation of the lipophilic marker within the skin, due to the concentrations found within the stratum corneum-epidermis.

Future prospects for further investigation and aspects that need to be considered include:

- The activities of certain penetration enhancers (e.g. *cis*-unsaturated oleic acid), as well as the correct choice of a co-solvent are of utmost importance. For oleic acid (C18:1) to reach the polar surface of the lipid layers within the stratum corneum in larger amounts, it may require a co-solvent, such as propylene glycol (PG). The addition of PG could alter the polarity of the aqueous region and increase the solubility of lipophilic materials. Materials, for example Azone[®], which is not readily soluble in water, may be able to move into the internal region of the lipid in the skin by causing rapid disordering. This relationship may operate particularly well with an Azone[®]/PG mixture. Therefore, the polar head groups of oleic acid/Azone[®] could position themselves in-between the lipid head groups in the stratum corneum and increase the fluidity of the lipid domain. The PG could assist in the penetration of Azone[®] into the stratum corneum. Subsequently, Azone[®]/oleic acid may cause an increase in the flux of PG through the skin, which in turn increases the amount of Azone[®]/oleic acid in the tissue. These types of enhancers (i.e., oleic acid) together with an additive (i.e., PG) reach the polar surface of the lipid layers within the stratum corneum and increase the polarity of the aqueous regions, thereby increasing the solubility of lipophilic APIs (Ranade & Hollinger, 2004:213). Even though Fang *et al.* (2003a:316) did not find any enhancing behaviour with the addition of PG, however they did conclude that the vehicle in which an API is formulated has a significant influence on the efficacy of fatty acids. The enhancing effect of PG has shown to have a synergistic effect in the permeation across the skin and therefore needs to be explored further (Babu *et al.*, 2006:138; Nanayakkara *et al.*, 2005:129-130; Santoyo & Ygartua,

2000:247). Future prospects could hence include the investigation of the effects when a co-solvent, for example PG is added to topical formulations, in order to verify whether it would improve the enhancing effectiveness of fatty acids to an even higher extent.

- For a chemical to be a good penetration enhancer, it should not alter the biochemical and structural integrity of the skin. It is further important that the enhancer should increase penetration of a drug across the skin, but not cause irritation or sensitisation of the skin. Oleic acid, which has demonstrated good enhancing benefits in the permeation of APIs in the skin, unfortunately has notable skin irritation potential (Boelsma *et al.*, 1996:739; Williams & Barry, 2004:608-609; Shah, 1994:21). It is therefore advised that for future studies, when utilising oleic acid as enhancer, lower concentrations of this fatty acid within formulations should be used in order to reduce its irritancy level, without influencing its effectiveness, and could thus be advised to incorporate it with PG as described above.
- The stability of UFAs in natural oils could be subjected to peroxidation in the presence of air/light, making them more unstable than SFAs, due to their double bonds within their carbon structures (Nanayakkara *et al.*, 2005:130; O'Brien, 2009:269). Future prospects could therefore include the investigation of the stability of these oils due to their predominant fatty acids when exposed to light and air.
- APIs with solubility parameters comparable to that of the skin's surface lipids are generally the most suitable candidates for skin penetration studies; and fatty acids have mainly been employed as penetration enhancers for lipophilic drugs (Babu *et al.*, 2006:138, 154; Leopold & Lippold, 1995:195; Walters & Brain, 2009:478). It is suggested that fatty acids increase the polar pathway across the skin through interactions with polar and non-polar regions of the stratum corneum. Another future prospect would thus be to investigate the effectiveness of fatty acids on hydrophilic APIs, due to reports of an inverse relationship between an API's lipophilicity and the enhancement effects of a series of fatty acids (Nanayakkara *et al.*, 2005:129-130).
- Other natural oils high in oleic acid (C18:1) include canola-, mid-oleic sunflower-, peanut-, pistachio- and almond oils (Kris-Etherton, 2007:1600). The penetration enhancement properties of oleic acid in these oils could, in future, also be investigated.

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APPENDIX A

VALIDATION METHOD FOR FLURBIPROFEN

A.1 INTRODUCTION

In the pharmaceutical industry HPLC is an integral analytical tool. Chromatography is a physicochemical method for the separation of complex mixtures and was discovered at the beginning of the twentieth century by a botanist, Michael S Tswett. Chromatographic separations are based on a forced transport of the liquid or mobile phase that carries an analyte mixture through a porous media. Differences in interactions of analytes with the surface of this porous media result in different migration times for a mixture's components. This defines the presence of two different phases that states an interface between them. These phases are (Kazakevich & Lobrutto, 2007a:3-6):

- mobile phase: provides the analyte (liquid or gas); and
- stationary phase: the immobile phase (liquid or solid).

A mixture of components, or the analytes, is dispersed in the mobile phase at molecular level, allowing for uniform transport and interaction with the mobile and stationary phases. The nature of these phases, as well as the mode of transport through the column is the basis for the classification of chromatographic methods. According to Tswett (as cited in Kazakevich & Lobrutto, 2007a:3-6, 8), the fractional adsorption process explains that molecules of different analytes have different affinities or interactions with the adsorbent surface or stationary phase, whereas analytes with weaker interactions are less retained.

A typical HPLC system consists of the following main components:

- solvent reservoirs: storage of a sufficient supply of HPLC solvents for the continuous operation of the system (online degassing system);
- pump: provides a constant and continuous flow of the mobile phase through the system;
- injector: injects the analyte mixture into the stream of the mobile phase before it enters the column;
- column: separates the analytes in the mixture. This is where the mobile phase is in contact with the stationary phase and is it the heart of HPLC; and

- detector: registers specific physical or chemical properties of the column effluent, e.g. ultraviolet (UV) allows the monitoring and registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection) (Kazakevich & Lofrutto, 2007a:9).

Sample preparation plays an important part in method development for analytes and comprises centrifugation, filtration, sonication, extraction and dilutions. Factors that should be considered when developing a method for analytes include: (1) physicochemical properties (pK_a , log P and solubility), and (2) mode of detection suitable for the analysis (wavelengths for UV detection) (Lofrutto, 2007:361).

An analytical method validation is a basic requirement that ensures the quality and reliability of the results of an analytical application. The objective of this analytical procedure is to demonstrate that it is suitable for its intended purpose (Ermer, 2005a:3-4).

This method had been primarily developed and validated for use in transdermal and membrane diffusion studies at the Analytical Technology Laboratory (ATL) at the North-West University at the Potchefstroom Campus. Method validation was done according to the guidelines of the International Conference on Harmonisation (ICH, 1995:7; ICH 1996:8).

A.2 CHROMATOGRAPHIC CONDITIONS

Analytical instrument	The HPLC analysis of flurbiprofen was performed using an Agilent® 1100 Series HPLC system in a controlled laboratory environment at 25 °C. The instrument consisted of an Agilent® 1100 pump, diode array detector, autosampler injection mechanism and Chemstation Rev. A.10.03 software for data acquisition and analysis (Agilent Technologies, Palo Alto, CA).
Column	A high performance silica based, reversed phase C ₁₈ -2 column with a 5 µm particle size, endcapped, 150 x 4.60 mm (Venusil XBP Agela Technologies, Newark, DE) was used.
Mobile phase	A mixture of acetonitrile: HPLC grade (Milli-Q) water: acetic acid (70:30:1). It was degassed using an ultrasonic bath prior to use.
Flow rate	1.0 ml/min.
Injection volume	25 µl.
Detection	UV detector at 247 nm for the detection of flurbiprofen.
Run time	8 min.
Retention time	± 3.9 min.

Solvent Methanol obtained from Merck™.

A.3 STANDARD PREPARATION

The standard solution was prepared as follows:

1. Accurately weigh 10 mg of flurbiprofen in a 50 ml volumetric flask and fill to volume with methanol, to serve as the standard solution.
2. Transfer the standard solution into an autosampler vial and analyse using the chromatographic HPLC method described in Section A.2.

Inject 1, 2, 4, 6, 8, 10 and 14 µl of the standard solution in duplicate on the HPLC.

A.4 SAMPLE PREPARATION

Samples collected from Franz diffusion cells were transferred into HPLC autosampler vials and analysed without any further preparation.

A.5 VALIDATION PARAMETERS

A.5.1 Linearity

The purpose of a linearity test is to demonstrate that the analytical system exhibits a linear response and is therefore directly proportional to the relevant concentration range for the target concentration of the analyte. Acceptability of the linearity data is judged on examining (1) the correlation coefficient and the y-intercept of the linear regression line for the area response against concentration graph and (2) the residual standard deviation (RSD). This is also understood as the standard error compared to the calculated y-value at a certain target concentration level (Lobrutto & Patel, 2007:471-472).

The linearity of the marker (flurbiprofen) was determined by performing linear regression analysis on the graph of the peak area *versus* concentration (µg/ml). Flurbiprofen concentrations (X-axis) were plotted against the peak area (Y-axis) derived from integrating the peak on the chromatogram that should give a straight line on the graph. This data was best described by a linear equation (Equation A.1).

$$y = mx + c$$

Equitation A.1

Where:

- y = peak area of the analyte
- m = slope
- x = concentration of the analyte (µg/ml)
- c = y-intercept.

Standards were prepared in a concentration range of 20 - 285 µg/ml, as described in Section A.3 and injected in duplicate on the HPLC. Peak areas of flurbiprofen from the standard solution were integrated from the chromatogram, as summarised in Table A.1. Figure A.1 illustrates the linear regression obtained, whereas Figure A.2 shows the flurbiprofen peak area on an HPLC chromatogram.

Table A.1: Linearity results of flurbiprofen

Concentration (µg/ml)	Mean peak area (mAU)
20.4	846.2
40.8	1656.6
81.7	3300.6
122.5	4934.2
163.4	6611.8
204.2	8226.7
245.0	9872.5
285.9	11483.7

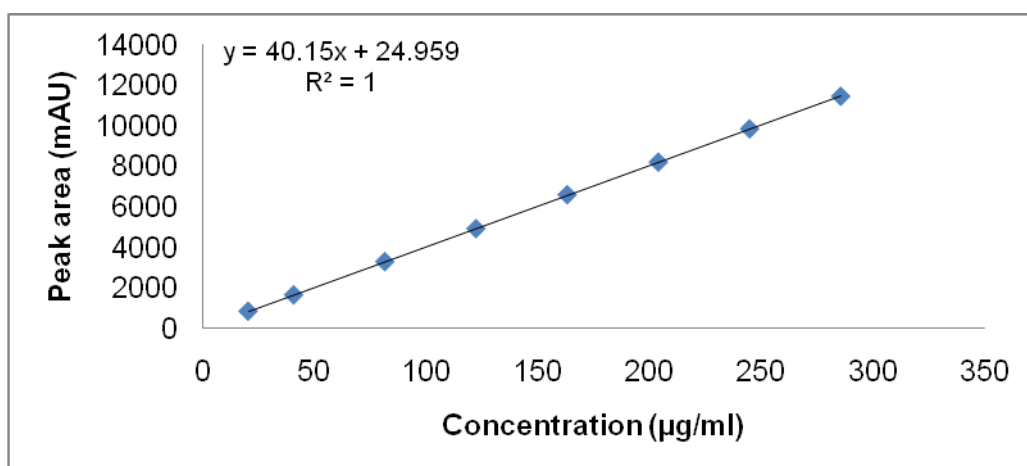


Figure A.1: Linear regression graph of flurbiprofen.

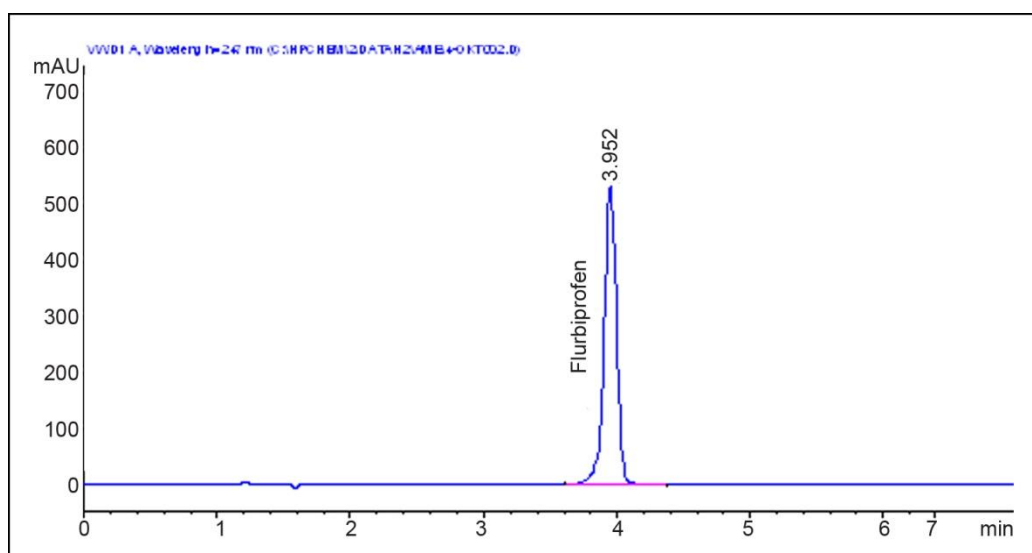


Figure A.2: Chromatogram of a flurbiprofen standard solution.

A.5.1.1 Acceptance criteria for linearity

Linear regression is the range being determined as the lowest and highest concentration between which the response remains linear, and where acceptable precision is obtained (Du Preez, 2010:4). Linear regression analysis should yield a regression coefficient (r^2) of ≥ 0.99 .

The method was linear for flurbiprofen over the concentration range of 20.4 - 285.9 $\mu\text{g/ml}$. The regression coefficient value ($r^2 = 1.0$) obtained for flurbiprofen indicated a high degree of linearity and demonstrated a stable analysis system.

A.5.2 Accuracy and precision

In an analytical procedure, precision expresses the closeness of agreement (the degree of scatter) between measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions. Precision considers three levels of analysis, i.e. repeatability, intermediate precision and reproducibility.

Accuracy and reliability is a crucial part of ensuring quality, safety and efficacy of the analytical result. The accuracy of an analytical procedure describes the closeness of agreement between the value, which is accepted as a conventional true value or an accepted reference value. Accuracy is thus determined by replicate analysis of samples containing known amounts of the analytes. It is also referred to as trueness (Ermer, 2005a:3-4; Ermer, 2005b:21).

Accuracy and precision were investigated on the same day, using the same method in terms of intra-day (repeatability) variations. This was done because no extra preparation of samples was required prior to analysis of the flurbiprofen diffusion samples. It was conducted as one experiment by weighing the following amounts of oil: 3 x 160 μl , 3 x 200 μl and 3 x 240 μl into

50 ml volumetric flasks. The oil samples were spiked with known amounts of a standard solution at concentrations of approximately 80, 100 and 120% of the expected sample concentration. These samples were filtered using 0.45 µm polyvinylidene fluoride (PVDF) syringe filters (Agela Technologies, Newark, Delaware), and transferred into HPLC autosampler vials for analysis, and injected on the HPLC in duplicate. Results are shown in Tables A.2 and A.3.

Table A.2: Accuracy and precision of flurbiprofen

Concentration spiked (µg/ml)	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
161.4	7381.7	7384.2	7382.9	163.8	101.5
161.4	7354.8	7340.8	7347.8	163.0	101.0
161.4	7347.2	7350.1	7348.6	163.0	101.0
201.8	9023.1	9031.5	9027.3	200.3	99.2
201.8	9041.4	9030.7	9036.0	200.5	99.3
201.8	9041.3	9035.5	9038.4	200.5	99.4
242.2	10714.9	10699.5	10707.2	237.5	98.1
242.2	10763.5	10731.3	10747.4	238.4	98.5
242.2	10752.5	10820.4	10786.5	239.3	98.8

Table A.3: Statistical analysis of flurbiprofen

Statistical analysis	
Mean	99.63
SD*	1.14
%RSD**	1.14

*SD refers to standard deviation.

**%RSD refers to relative standard deviation.

A.5.2.1 Acceptance criteria for accuracy and precision

Percentage recovery is an indication of the accuracy of the system. Recovery must be between 98 - 102% and inter-day precision should be below 2% (Du Preez, 2010:4). The method yielded (Table A.3) an acceptable mean recovery of 99.63% and precision (repeatability) of 1.14%.

A.5.3 Inter-day precision

The same homogenous samples were analysed in triplicate as described in Section A.5.2, to determine intra-day precision (at 100% of the sample concentration), as well as on two more occasions (Day 2 and Day 3) to determine the between-day variability of the method. On one

occasion (Day 3), a different analyst performed the analysis on a different set of equipment, with the purpose of verifying the reproducibility of the method. The results are summarised in Table A.4.

Table A.4: Inter-day precision of flurbiprofen

Flurbiprofen	Day 1	Day 2	Day 3	Between days
	99.2	100.5	99.1	
	99.3	99.8	99.9	
	99.4	101.0	100.6	
Mean	99.31	100.41	99.86	99.86
SD*	0.05	0.48	0.59	0.45
%RSD**	0.05	0.48	0.59	0.45

*SD refers to standard deviation

**%RSD refers to relative standard deviation

A.5.3.1 Acceptance criteria for inter-day precision

Inter-day precision must be less than 5% (Du Preez, 2010:5). The results, as shown in Table A.4, complied with acceptable pharmaceutical standards and met the requirements of the United States Pharmacopeia (USP). Inter-day precision was acceptable with a %RSD of 0.45%. Therefore, reproducibility was within acceptable limits. This meant that the method should perform well, even if carried out by other staff in a different laboratory.

A.6 RUGGEDNESS

A.6.1. Stability of sample solutions

A sample was prepared as described in Section A.4. To determine the stability of the sample, it was injected onto the HPLC and left in the autosampler tray to be reanalysed over a period of 24 h. Stability data are summarised in Table A.5.

Table A.5: The stability of flurbiprofen

Time (h)	Peak area of flurbiprofen	%Remaining
0	9391.23	100.0
1	9379.37	99.9
2	9419.76	100.3
3	9410.35	100.2
4	9435.45	100.5
5	9438.17	100.5
6	9499.98	101.2
7	9505.41	101.2
8	9513.04	101.3
9	9519.10	101.4
10	9546.28	101.7
11	9560.63	101.8
12	9588.63	102.1
13	9587.57	102.1
14	9615.12	102.4
15	9613.76	102.4
16	9620.21	102.4
17	9631.96	102.6
18	9650.23	102.8
19	9630.09	102.5
20	9623.85	102.5
21	9612.09	102.4
22	9589.57	102.1
23	9585.48	102.1
Mean	9541.52	101.60
SD*	85.01	0.91
%RSD**	0.89	0.90

*SD refers to standard deviation

**%RSD refers to relative standard deviation

A.6.1.1 Acceptance criteria for stability

Sample solutions should not be used for a period longer than it takes to degrade by 2%. However, if rapid degradation occurs, special precautions should be followed to compensate for the degradation (Du Preez, 2010:5). Flurbiprofen degraded with less than 1% over a period of 24 h.

A.6.2 System repeatability

To evaluate the repeatability of the peak area and retention time, a standard sample was injected six times. Table A.6 shows the variations in response (%RSD) of the detection system, when six determinations were made on the same day and under the same conditions.

Table A.6: System repeatability of flurbiprofen

Injection	Peak area (mAU)	Retention times (min)
1	9565	5.153
2	9528	5.150
3	9436	5.157
4	9411	5.171
5	9453	5.176
6	9412	5.178
Mean	9467.000	5.164
SD*	64.300	0.012
%RSD**	0.680	0.238

*SD refers to standard deviation

**%RSD refers to relative standard deviation

A.6.2.1 Acceptance criteria for system repeatability

The peak area and retention times should have a %RSD of 2% or less. System performance proved acceptable values, with a %RSD of 0.680% for peak area and 0.238% for retention time, respectively.

A.7 ROBUSTNESS

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, deliberate variations in the method parameters, and thus providing an indication of its reliability during normal usage.

In this procedure, deliberate changes were made to the flow rate, injection volume, wavelength and mobile phase composition and in turn the influences of these changes on the chromatographic results were determined.

Deliberate changes in mobile phase composition (Figure A.3), flow rate (Figure A.4) and wavelength (Figure A.5) still provided acceptable results. The influence of injection volume had already been investigated during the linearity testing. The method proved to be robust and able to function properly in spite of deliberate changes to the chromatographic conditions.

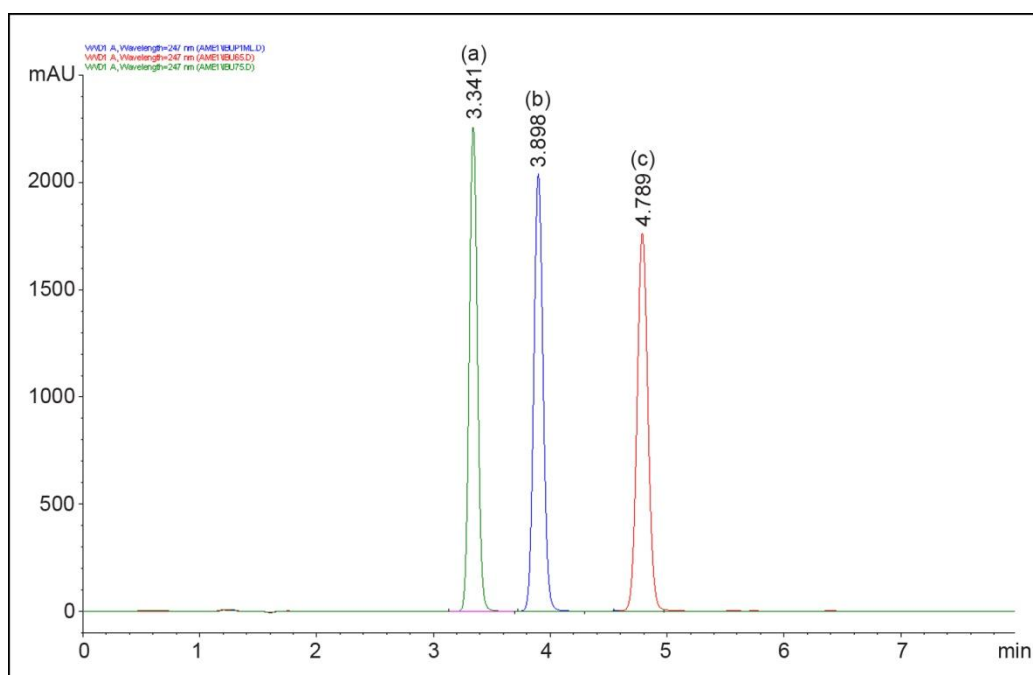


Figure A.3: Chromatogram of a flurbiprofen standard solution injected with 75% (a), 70% (b) and 65% (c) of acetonitrile in the mobile phase.

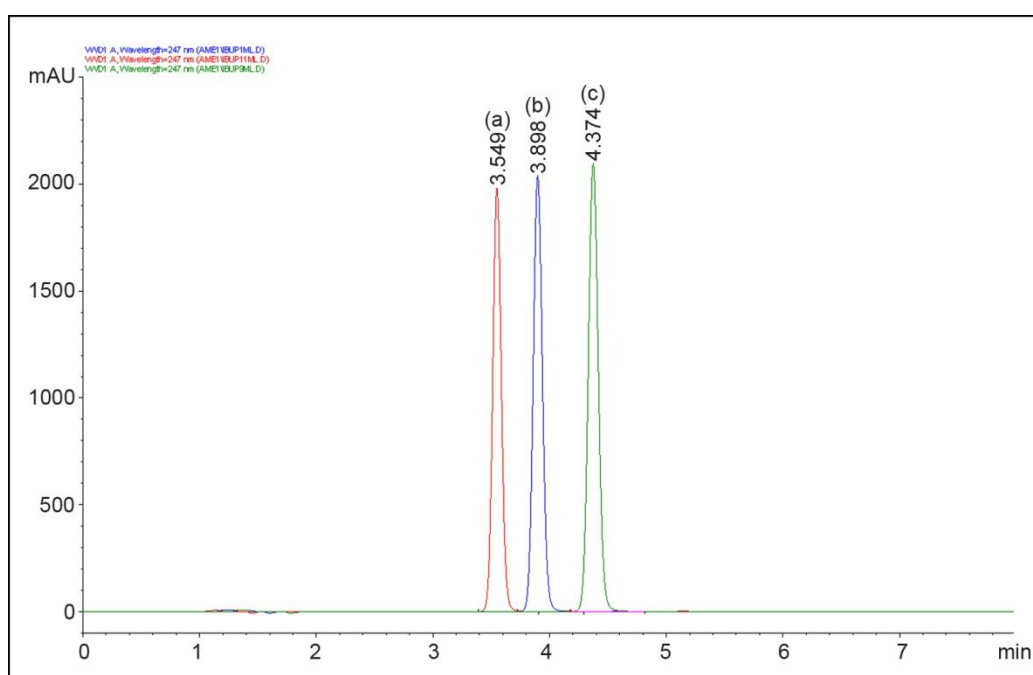


Figure A.4: Chromatogram of a flurbiprofen standard solution injected at a flow rate of 0.9 ml/min (a), 1.0 ml/min (b) and 1.1 ml/min (c), respectively.

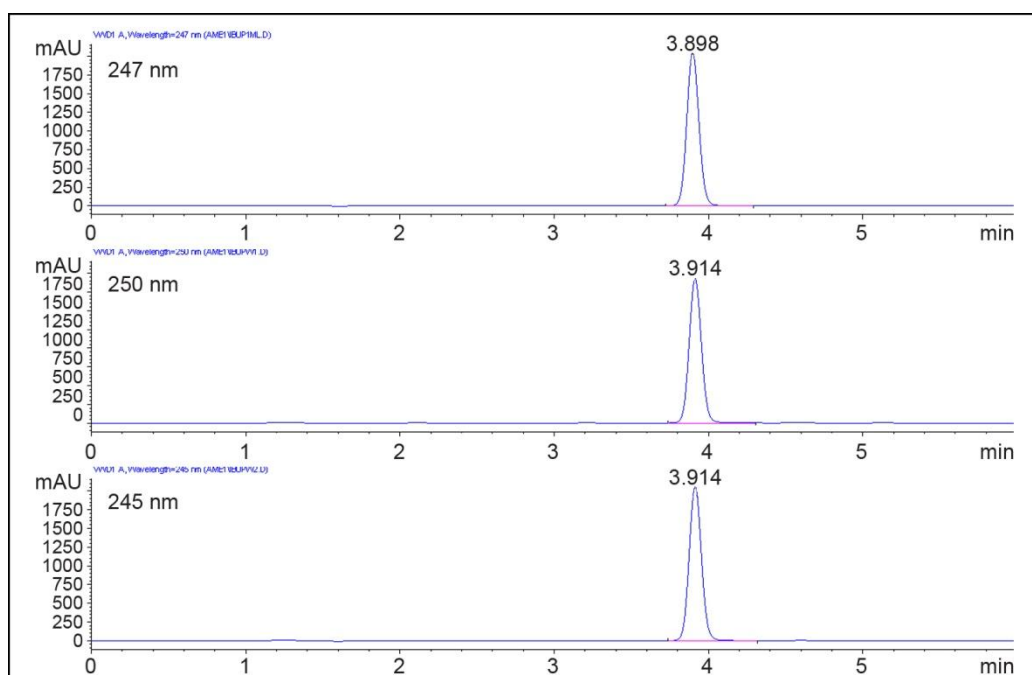


Figure A.5: Chromatogram of a flurbiprofen standard solution analysed at UV wavelengths of 247 nm, 250 nm and 245 nm.

A.8 SPECIFICITY

Specificity is the ability to assess the analyte in the presence of components that may interfere with analyte detection. The method is selective when no interfering peaks with the same retention times as the actives are detected (ICH, 2005:6). A standard solution was prepared as described in Section A.3, and 1 ml of this solution was placed in four test tubes. The standard solution was diluted in a ratio of 1:1 each with Milli-Q[®] water, 0.1 M hydrochloric acid (HCl), 0.1 M sodium hydroxide (NaOH), and 10% hydrogen peroxide (H₂O₂). These solutions were stored overnight at ± 40 °C in closed test tubes to allow for possible degradation. Samples were injected in duplicate onto the HPLC the following day. The run time was three times longer than the elution time of the flurbiprofen peak, in order to detect any possible additional peaks (Figure A.7). A blank injection of phosphate buffer solution was also injected (Figure A.6) in order to test for any solvent interference.

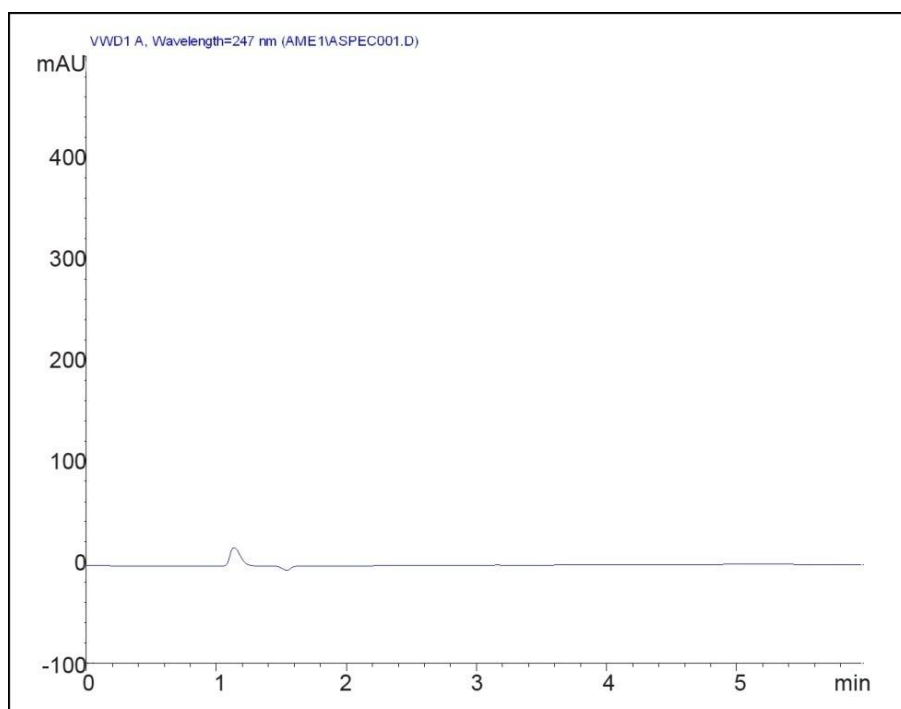


Figure A.6: Chromatogram of a phosphate buffer (pH 7.4) (blank solvent).

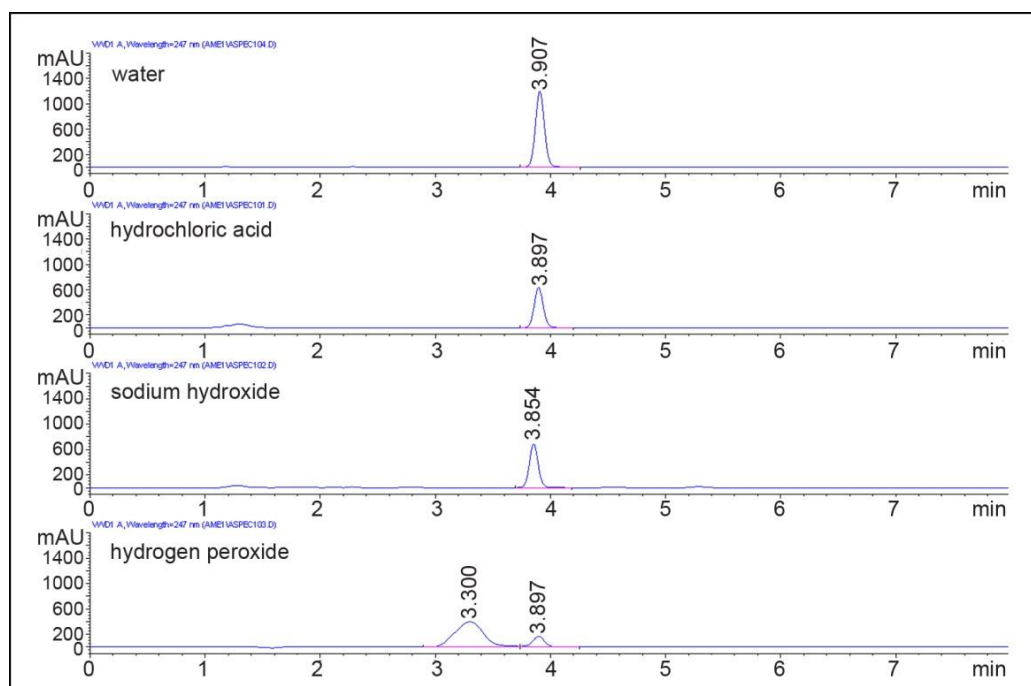


Figure A.7: Chromatograms of samples stressed in water, hydrochloric acid, sodium hydroxide and hydrogen peroxide in a ratio of 1:1.

The stressed samples showed additional degradation peaks (Figure A.7). However, none of these peaks interfered with the flurbiprofen peak, proving that this method was stable and specific.

A.9 SYSTEM SUITABILITY PARAMETERS

A.9.1 Chromatographic performance characteristics

Analytical instrument qualification is used to ensure that the instrument used is suitable for its intended purpose (USP, 2012a). For flurbiprofen, the USP monographs suggest that the tailing factor should not be more than 2.5 and that the run-time should be double the retention time (USP, 2012c). Peak tailing is a commonly observed result of sample overload (Kazakevich & Lubrotto, 2007b:125).

An analyte's retention is measured as the difference between the total retention time and the hold-up time within the stationary phase and is known as the retention volume. This ratio between the reduced retention volume and the void volume is called the capacity factor (retention factor) (Kazakevich & Lubrotto, 2007a:17).

The analytical instrument performed well during this study and the following performance characteristics were recorded during method validation:

Retention time (min):	3.952
Number of theoretical plates (N) plates/column (tangent method):	9655
USP Tailing factor (T):	0.897
Capacity factor (k'):	1.331

A.9.2 System suitability criteria

Accurate and reliable data is essential when validating data that is suitable for its intended purpose. Validation of procedures, the calibration of instruments, additional instrumentation checks, such as system suitability tests, as well as the analysis of in-process quality control checks ensure that the generated data will be reliable (USP, 2012a). The data obtained in Section A.9.1 can be applied as the minimum performance criteria that have to be met before starting an analytical batch (system suitability criteria). The following procedures were followed to acquire these chromatographic characteristics:

- inject a standard solution in triplicate;
- calculate the relative standard deviation of the peak areas obtained;
- calculate the number of theoretical plates for the flurbiprofen peak; and
- use the tangent method to calculate the parameters.

The system suitability test verifies whether the system would perform according to the set parameters for the specific procedure (USP, 2012a). The requirements for retention time, resolution efficiency and tailing factor are determined, based on prior method development experience. Resolution requirements (criteria for efficacy as well as the tailing factor) are added to ensure that the column performs adequately for achieving the desired separation (LoBrutto, 2007:389). The number of theoretical plates is an indication of the efficiency of the column (USP, 2012b).

The system is suitable to perform the analysis if the following criteria are met:

- %RSD of 3 injections is not more than 2% (Du Preez, 2010:5; USP, 2012b); and
- the column must have more than 7250 theoretical plates for flurbiprofen (75% of the value obtained during validation).

A.10 UNCERTAINTY OF MEASUREMENT

According to the “Co-operation on international traceability in analytical chemistry” (CITAC) guide, uncertainty of measurement is a parameter that is associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measured quantity (CITAC, 2000:4). Uncertainty of measurement does not necessarily imply that a result is doubtful. In fact, knowledge of the level of uncertainty implies increased confidence in the validity of the result (CITAC, 2000:4). It is compulsory to calculate the uncertainty of measurement in order to comply with the guidelines of ISO 17025 (SANAS, 2008:4-5).

The empirical calculation used to determine the level of uncertainty of measurements in this laboratory was as follows (Du Preez, 2011:1, 3):

Weighing of standard: 0.01 mg/10 mg x 100	= 0.10%
50 ml volumetric flask: 0.05 ml/50 ml x 100	= 0.10%
Injection inaccuracy (repeatability)	= <u>0.68%</u>
Total uncertainty	<u>0.88%</u>

The precision of the analysis is the agreement of the test results when the method is applied repeatedly to a homogenous sample. A decrease in precision (increase in variability) may lead to failed results being obtained in an analysis (USP, 2012a). According to the USP monographs for flurbiprofen, the relative standard deviation (SD) for replicate injections should not be more than 1% (USP, 2012c). Therefore, a total uncertainty of 0.88% is acceptable.

A.10.1 Validation data

Repeatability levels of uncertainty must be less than 2% when the same homogenous mixture is used for all of the precision experiments (Du Preez, 2010:4). The uncertainty estimation from the set parameters utilises (1) the best available estimate of the overall precision, (2) the best available estimate of the overall uncertainty or accuracy, and (3) the determination of any uncertainties associated with effects accounted for in the overall performance studies (Du Preez, 2011:3-4).

From the validation data obtained the percentage recovery was 99.6%, with an uncertainty level of 0.4%. Intra- and inter-day results showed a level of uncertainty $[(1.14 + 0.45)/2]$ of 0.795%. Therefore, a total level of uncertainty was calculated as $(0.40 + 0.795)$ being 1.195%.

A.11 CONCLUSION

The existing HPLC method developed was found to be reliable and adequately sensitive for detecting the concentration of the flurbiprofen present in the membrane diffusion and skin diffusion samples, thus, providing confidence that the specific method would test the data of acceptable quality (USP, 2012c). No interferences were encountered from stressed samples, or known related substances, therefore the method could be regarded as being stability indicating.

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APPENDIX B

FATTY ACID CONTENT AND DENSITIES OF SELECTED NATURAL OILS

B.1 INTRODUCTION

Fats and oils predominantly comprise of triglycerides of fatty acids and a glycerol (Mitsui, 1997:122). Oils have been used as penetration enhancers, since when they are metabolised by the skin, they release valuable free fatty acids (Lautenschläger, 2004:46; Takahashi *et al.*, 1991:154). To determine the fatty acid composition of the natural oils investigated during this study, GC was employed for the total quantitative analysis of the fatty acid methyl esters of:

- lauric acid (C12:0);
- myristic acid (C14:0);
- palmitic acid (C16:0);
- stearic acid (C18:0);
- palmitoleic acid (C16:1);
- oleic acid (C18:1);
- linoleic acid (C18:2);
- linolenic acid (C18:3); and
- arachidonic acid (C20:4).

Classic methods based on gas or liquid chromatography are commonly used to classify fats and oils. Trimethylsulphonium hydroxide (TMSH) is used as transesterification catalyst for triglycerides in fats and oils in the GC procedure. Fatty acids, present in fats and oils, are converted to their quaternary ammonium salts and then alkylated pyrolytically into their corresponding methyl esters (Beaten *et al.*, 1998:2363; Butte, 1983:142).

Density as a function of temperature was furthermore measured for these natural oils. The density or specific gravity (SG) of natural oils is an important factor in the fatty acid industry and especially in chemical engineering (Noureddini *et al.*, 1992:1184). A widely used method to predict the density of a wide variety of oils is called the Lund relationship, which makes use of

the specific gravity, saponification- and iodine levels of oils to predict its density. It further makes use of the fatty acid composition and its critical properties to determine density (Rondebush *et al.*, 1999:1415).

A range of chemometric methods can be employed to classify oils, and the aim of this experiment was to detect samples that deviated from the approved parameter values (Brodnjak-Vončina *et al.*, 2005:31).

B.2 METHODS

B.2.1 Fatty acid methyl ester (FAME) analysis with gas chromatography

The methodology for fatty acid analysis was developed by Professor J.L.F. Kock from the Department of Microbial, Biochemical and Food Biotechnology, Faculty of Natural and Agricultural Sciences at the University of the Free State, Bloemfontein, South Africa.

B.2.1.1 Preparation of samples

For GC it is necessary to produce volatile derivatives of the samples to be analysed (Mitsui, 1997:260). Samples were prepared by the transesterification of the oils, using TMSH and analysed by GC as described below. Oil samples were dissolved in 200 µL of chloroform to which 200 µL of a 0.2 mol/l solution of TMSH in methanol (50:50) was added. The samples were placed on a vortex to ensure complete mixture of the samples before being injected onto the GC. A 0.5 µl sample was injected in duplicate onto the GC, with a split ratio of 1:100 and an inlet port temperature of 230 °C.

B.2.1.2 Gas chromatography conditions

Analytical instrument The CG analyses of the natural oils were performed by using a Shimadzu 2010 gas chromatograph in a controlled laboratory environment at 25 °C. The instrument was controlled and results processed using Shimadzu GC solution software.

Column A SGE BP x 70 glass capillary column with an inner diameter of 0.32 mm, film thickness of 0.25 µm and length of 60 m was used. Column oven temperature was programmed at 180 °C and held for 1 min. It was then increased to 225 °C at a rate of 10 °C/min and held for 4 min, after which it was finally increased at a rate of 10°C/min to 256 °C and held for 1 min.

Gas phase Nitrogen (N₂) was employed as carrier gas.

Flow rate The carrier gas flow rate was 1.07 ml/min.

Detection Peaks were detected with a flame ionisation detector at a temperature of 310 °C.

B.2.2 Density determination

B.2.2.1 Preparation of samples

Specific gravity and density data measurements were performed using an ANTON PAAR DMA 38 (S/N: 254533) instrument (Anton Paar, Graz, Austria). This instrument is a compact, digital, density meter and is accurate to 0.001 g/cm³. It is easier to use than conventional hydrometers (Anton Paar, 2008). The volume of each of the six oil samples injected into the density meter measured approximately 2 ml per sample. Results are summarised in Table B.2. The water temperature was 25 °C, with the water density being 0.997 ± 0.001 g/ccm. Density of oils normally varies with each type and temperature and can range from 0.91 - 0.93 g/ccm for temperatures ranging between approximately 15 - 25 °C (Dorfman, 2000). It has been shown that the density of oils decrease linearly with an increased temperature (Rondebush *et al.*, 1999:1415, 1417).

B.2.2.2 Density verification

Density is the mass of a unit volume in one volumetric unit (i.e. cubic centimetre) of measurement. Contrary, specific gravity is closely related to density and measures the density of a material compared to the density of some standard, in this case, water (1 g/ccm), and is a unitless quantity (Science clarified, 2012a; Science clarified, 2012b; The MSDS hyper glossary, 2012). Purified water standards (25 °C = 0.997 ± 0.001 g/ccm) were tested after the density of each oil was determined in order to ensure a clean tube with every use. Water's density was measured as 0.997 ± 0.001 g/ccm. The density of the water hence stayed constant over the duration of the measurement. The density of water was measured before and after completion of the density verification experiments. No significant changes in these values were observed, as the density values for water remained constant at 0.997 g/ccm throughout the experiment.

B.3 RESULTS AND DISCUSSION

B.3.1 Fatty acid methyl ester (FAME) analysis

Identification of fatty acid methyl ester (FAME) peaks in the samples was done through comparison of the relative retention times of FAME peaks from samples with those of standards obtained from SIGMA, to ensure authenticity. Results of the percentage fatty acid composition from the GC for each of the oils are presented in Table B.1.

Table B.1: GC results of the fatty acid composition in percentage (%) of the selected natural oils employed in this study

	Capric	Lauric	Myristic	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	Linolenic	Arachidonic
	SFA ¹					MUFA ²		PUFA ³		
Oil or fat	10:0	12:0	14:0	16:0	18:0	16:1	18:1	18:2	18:3	20:4
Avocado				15	1	6	68	10	1	
Grapeseed				6	4		27	61	0.5	
Emu		0.1	0.3	21	10	4	46	16	2	
Crocodile		0.1	1	21	5	6	40	24	2	1
Olive				12	3	1	76	7	1	
Coconut		52	21	9	3		8	3	0.2	

1 Saturated fatty acids

2 Mono-unsaturated fatty acids

3 Poly-unsaturated fatty acids

B.3.1.1 Discussion

From the data collected, it was interesting to see that olive- (76%), avocado- (68%), emu- (46%) and crocodile oil (40%) presented with rather high levels of oleic acid, a MUFA, whereas lower levels of oleic acid were observed for grapeseed- (27%) and coconut oil (8%). Grapeseed oil (61%) was the only oil that portrayed high levels of linoleic acid, a PUFA, whereas the rest of the oils presented with levels below 25%. Coconut oil was the only oil that demonstrated high levels of lauric acid (SFA) (52%) and medium levels of myristic acid (SFA) (21%). Crocodile- (21%) and emu oil (21%) showed medium levels of palmitic acid (SFA), whilst levels smaller than 15% were observed for the rest of the oils. Stearic acid (C18:0) was present in levels below 10% for all the oils, with the animal oils containing the highest. An interesting observation was the similarity of the oleic- and palmitic acid levels obtained for crocodile- and emu oil. These results correlated well with results found in literature, stating that the fatty acid composition of herbivores and carnivores does not vary significantly, contrary to what would be expected (Kirschenbauer, 1960:25-26).

B.3.2 Density determination

Table B.2: Density (g/ccm) and specific gravity results of the natural oils employed in this study

Oils	Specific density (g/ccm)	Specific gravity determined	Specific gravity from literature ¹
Grapeseed oil	0.917	0.920	0.909 ¹
Coconut oil	0.922	0.924	0.917 ¹
Avocado oil	0.912	0.915	0.913 ¹
Olive oil	0.910	0.912	0.914 ¹
Emu oil	0.912	0.914	0.946 ²
Crocodile oil	0.912	0.914	-

¹ Kirschenbauer, 1960:190-198.

² MSDS, 2012.

B.3.2.1 Discussion

Density results measured (Table B.2), positively compared to results found in the literature. Emu oil differed slightly from that stated in the literature, but it could have been due to the palmitic- (C16:0) and stearic (C18:0) acids present in this oil. Since these fatty acids have higher melting points, they might not have dissolved properly at 25 °C (Aungst, 1989:245). An inability to dissolve at the temperature that this experiment where performed at, might have influenced the density of this oil.

B.4 SUMMARY

Results obtained for the fatty acid composition of the selected oils in this study compared well to the values found in literature (Kris-Etherton, 2007:1161e; Osthoff *et al.*, 2003:58; Shimizu & Nakano, 2003:58). Identification of the relative retention times of FAME peaks from samples were compared to those of standards obtained from SIGMA, to ensure authenticity of the oils. Furthermore, the results obtained from the density determinations correlated well with what was found in literature (Kirschenbauer, 1960:190-198; MSDS, 2012). This was a good indication of the reliability of the methods used and their suitability for the intended purpose.

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APPENDIX C

FORMULATION OF A COSMECEUTICAL SEMISOLID EMULGEL AND FOAM FOR TRANSDERMAL DELIVERY

C.1 INTRODUCTION

Cosmetics are part of our daily lives. It is used regularly by an ever increasing number of people, mainly for personal hygiene, for enhancing attractiveness (makeup), for improving self-esteem and serenity, for protecting the skin and hair from ultraviolet radiation and other harsh environmental factors, as well as to slow down the effects of aging on the skin (Mitsui, 1997:3, 1 21). The term “cosmetic” is derived from the Greek *Kosm tikos*, and early cave paintings of 30 000 years old indicate the use of body cosmetics in mating and hunting rituals (Milstein *et al.*, 2001:5). Contrary, “cosmeceutical”, a term that was first used in 1985, is described as a preparation with pharmaceutical properties (Merriam-Webster, 2012). The Pharmaceutical Affairs Law distinguishes between cosmetics (beautifying products), quasi-drugs (for teeth cleaning, hair colouring, etc.) and pharmaceuticals. Healthy people use cosmetics and quasi-drugs to sustain favourable personal appearance and for personal hygiene. Physiological activities of cosmetics in such cases must hence only be mild. In contrast, an API is used for the prevention and treatment of an illness and affects the structure and functioning of the body or parts thereof. APIs are only used over short periods of time to treat medical conditions, whereas cosmetics and quasi-drugs are often used on a daily basis over long periods of time, and should therefore be safe to use without any side effects (Mitsui, 1997:4).

Cosmetic aesthetic criteria for the formulation of dermatological formulations, with maximum bioavailability in mind, should still be acceptable to the patient. Therefore, in order to ensure patient compliance when manufacturing cosmetics and dermatological semisolid formulations, the design of any given formulation should be formulated with the ultimate consumer in mind. These criteria can be summarised to have (Barry, 2007:595; Ramchandani & Toddywala, 1997:542):

- an easily transferable formulation from the container to the skin;
- a smooth and pleasant feel on the skin;

- no unwanted, noticeable residues remaining on the skin;
- adhering properties at the site of treatment; and
- can easily be removed by the consumer (Barry, 2007:595).

Dermatological products are only to be applied to the skin or the scalp (Flynn, 2002:187). Topical pharmaceutical products can contain the following constituents in addition to the API, i.e. preservative, buffer, one or more co-solvent(s) (i.e. ethanol), emulsifying agents (surfactants), viscosity enhancing agent (xanthan gum) and a permeation enhancer (i.e. fatty acids). These formulations are applied to the skin to deliver the API to the local tissue and to the area directly under the site of application; therefore, these formulations are intended for intra-cutaneous drug penetration and deposition. Due to the fact that semisolid formulations are applied to the skin and remain intact to deliver an API over time, previous skin permeation studies formulated vehicles in which to apply the API to the membrane surface or skin. In modern-day pharmaceutical practice, these semisolid formulations are thus regarded as favoured vehicles for dermatological therapy. Therefore, we formulate topical delivery systems that are proposed to deliver APIs locally, rather than systemically. However, therapeutic compounds applied to the skin can be used for dermatological- (within the skin), local- (regional) and transdermal (systemic) delivery. Approximately 1 - 15% of an API in a dermatological formulation is bio-available from topical formulations (Ghosh & Pfister, 1997:7; Rahman & Gallo, 1992:251; Ramchandani & Toddywala, 1997:539; Walters & Brain, 2002:320, 322).

Topical, semisolid formulations that represent the most prevalent forms of topical dosage forms include creams, ointments, gels and solutions, and are mixtures of aqueous solvents, powders, oils, emulsifying agents, thickeners, buffers, anti-oxidants, preservatives, colorants and propellants in aerosols. A wide variety of approved excipients is available for use in these semisolid formulations and includes oils, surfactants and thickening agents, among others. It is therefore required to select an appropriate topical dosage form, API(s) and excipients for the production of a stable, safe and efficient formulation (Barry, 2007:593; Ramschandani & Toddywala, 1997:574).

Dermatological formulations often contain fixed oils of vegetable origin that contain mixtures of saturated and unsaturated fatty acids, with one of the most common oils used being olive oil (Barry, 2007:594). Oils control the evaporation of moisture from the skin and are generally used as components in cosmetics, because of their good feel on the skin (Mitsui, 1997:3,121).

The aim of this study was not to deliver a specific drug for a specific indication, and for this reason a marker API (flurbiprofen) was included in the topical formulations used in the experiments. Other *in vitro* studies also employed 1% flurbiprofen into formulations (Fang *et al.*, 2003a:154; Fang *et al.*, 2003b:314; Charoo *et al.*, 2005:343). Six emulgel formulations

prepared from a range of natural oils of plant and animal origin, were used to investigate the penetration effect(s) of these oils on the marker, flurbiprofen. Comparative studies were conducted on a liquid paraffin containing emulgel and a hydrogel without the inclusion of oil, in order to compare their penetration effects with the aforementioned six emulgel formulations containing natural oils. The oils investigated in this study were avocado-, grapeseed-, emu-, crocodile-, olive- and coconut oil, which functioned as the oil phase in the formulated emulgels.

C.2 VEHICLE SELECTION

An ideal formulation for cosmetic means should be well suited and pleasing to the consumer, and when designing a formulation it is important to consider the condition of the skin. Increased skin hydration has shown to increase absorption from topically applied formulations, whilst this moisturising technique enhances the absorption of both hydrophobic and hydrophilic APIs. The occlusive effect of oily formulations on the skin influences the rate and extent to which an API is absorbed, whilst it also alters permeability by softening and swelling of the skin (i.e. hydration) (Foldvari, 2000:421; Robinson *et al.*, 1997:60). Steps involved in transdermal delivery of an API are aimed at establishing the concentration gradient (API movement across the skin) that releases the API from the formulation (partition coefficient), and the diffusion of the API across the skin layers (diffusion coefficient) (Panwar *et al.*, 2011:334). A topical formulation should therefore improve product performance and deliver an API to the surface of the skin, or within the stratum corneum by (Rajadhyaksha *et al.*, 1997:493-494).

- assisting or manipulating the barrier function of the skin;
- attempting to breach the stratum corneum (at molecular scale, to direct drugs into the epidermis and dermis); and
- deliberately use the skin as a gateway of entry into the systemic circulation (Barry, 1983:23-25).

C.3 PRE-FORMULATION

Effective formulation of a topically applied product is therefore desired for the successful release of an API. Components within the formulation can have a huge impact on the rate at which the API is released from the formulation. It is important to determine the stability and physical characteristics of the formulated product to ensure a good quality product. Pre-formulation includes studies carried out before commencement of actual formulation. A selection procedure needs to be conducted beforehand to identify which ingredients should be included within the formulation, and if a dissatisfactory amount of the API is released from the formulation, extra research needs to be conducted before commencing with actual formulating. Release studies involve the measurement of diffusion out of a formulation into a receiving medium (receptor

phase) that is separated from the formulation by a synthetic membrane, and are referred to as membrane diffusion studies (see Appendix D). However, API release data cannot predict skin permeation and therefore skin diffusion studies need to be performed additionally (Ademola, 1997:517; Walters & Brain, 2002:321, 335-336). Pre-formulation usually indicates the viability of a range of possible dosage forms and indicates any possible problems regarding instability, poor dissolution and bioavailability. HPLC (as described in Appendix A) is acknowledged as a useful technique in the pharmaceutical analysis and method development in pre-formulation assessments, to determine the release of the marker from the formulations (Wells & Aulton, 2007:337, 350).

C.4 SEMISOLID FORMULATIONS

When formulating a topical delivery system, it requires a decision based upon the type of dosage form and is dependent on numerous factors, namely the physicochemical properties of the API, the stability of the API, the manufacturing equipment, costs involved, preferred dosage form by consumers, as well as the stability of the API within such a formulation (Ramchandani & Toddywala, 1997:542). In the formulation of transdermal delivery systems, the API should be incorporated into a structure that serves as a reservoir and that provides a delivery system between the drug and the skin surface. This structure can consist of a liquid, semisolid, three-dimensional non-flowing material (rubbery gel), or a combination of all three (Ranade & Hollinger, 2004:214).

Classic semisolid dosage forms used in the pharmaceutical industry include ointments, water-in-oil (w/o) emulsions, pastes, oil-in-water (o/w) creams, gels and foams. Of all the formulations available on the market, semisolid dosage forms are distinctly used for topical application. Semisolid formulations perform a specific topical need, as they remain on the skin surface, irrespective of where they are applied, but can easily be washed or worn off. These dosage forms have a distinctive rheological character and are therefore allowed to spread evenly over the skin and cling persistently (Flynn, 2002:213-214). An important characteristic of semisolids is that they are often too viscous to flow, but are known as “wet” products, due to the sufficient amount of water they contain. Semisolid products should effectively be preserved, seeing that “wet” products have the potential problem of water loss, as well as microbial contamination (Taylor, 2007:630).

C.4.1 Gels

Gels are classified as semisolids. They consist of a liquid phase trapped within a matrix of natural or synthetic gum. Provided that the API does not bind to the polymer (natural gum) in the formulation, these gels release an API rather well (Barry, 2007:593). Some gels are as transparent as water, while others are cloudy, because of colloidal aggregation from the

polymer present. A polymer disperses light and can range from slightly hazy to a white, semi-transparent semisolid (Flynn, 2002:216). Polymers used in pharmaceutical gels include some natural gums (i.e. xanthan gum). These gums are also classified as gelling (thickening) agents and are responsible for the consistency of gels (Buchmann, 2001:155). Depending on the gelling agent, gels may show signs of newtonic-, plastic-, or pseudoplastic-behaviour. The concentration of this gelling agent and the molecular weight of the polymer influence the consistency of gel formulations (Ramchandani & Toddywala, 1997:547; Walters & Brain, 2009:500). The preparation of a gel involves high-temperature processing seeing that it is easier to disperse a gum in hot than in cold water. The polymer goes into the solution and thickens it as the temperature is lowered, after which the hot polymer solution is dispersed in ice water, where it promptly reaches its equilibrium state. Gels normally contain high viscosities and it is vital to select the correct equipment to manufacture it. Mixing equipment should thus be able to uniformly mix the ingredients and remove any air bubbles (Flynn, 2002:216; Mitsui, 1997:353).

The general purpose and function of gels are to produce a moisturising feeling on the exterior of the skin, and to ensure that it appears as a uniform product. Gel products generally contain a gel basis, humectants, surfactants, preservatives, the API and perfumes (Mitsui, 1997:351). Dermatological gels are generally non-greasy, spreads easily on the skin, are non-staining and are easily removed (Mohamed, 2004:1).

C.4.1.1 Emulgels

An emulgel is classified as an emulsion of either a dispersed hydrophobic-based o/w, or a dispersed aqueous based w/o phase. Upon the addition of a gelling agent to the water phase, it converts into an emulgel. Emulsions usually contain an oil phase, water phase and emulsifier. In a hydrophilic emulsion, water is the continuous phase and forms the o/w emulsion, whereas in a lipophilic emulsion oil is the continuous phase forming the w/o emulsion. Therefore, emulsions are dispersions of two immiscible phases, with the one being dispersed in the other. A typical w/o emulsion is composed of at least 20% lipid components, 1% thickening agent, water, preservatives and emulsifying systems with a favourable hydrophilic-lipophilic balance (HLB) of 3 - 8. Emulsifying systems or surfactants are added to minimise the interfacial tension and reduce the separation of phases. Preparation of emulsions requires a mechanical shear i.e. homogeniser, and heat is essential to melt the oil components if they are in solid form. It is advised that the oil and aqueous phases are heated to the same temperature. Other solid phases should be dissolved before the emulsion is phased with the homogeniser. If the API is soluble in water it may be added to the emulsion. In this study, the marker flurbiprofen was insoluble in pure water and was added in the form of a solution. If an API on the other hand is heat sensitive, it should be added after the semisolid has cooled down. Emulgels are highly

acceptable by the consumer, due to advantages of both gels and emulsions (appears as elegant products, easily washed off with high penetration abilities). They are created to overcome some limitations of gels regarding the delivery of lipophilic APIs, (i.e. flurbiprofen) (Buchmann, 2001:154; Mitsui, 1997:174-175, 167-168; Mohamed, 2004:1; Panwar *et al.*, 2011:334; Ramchandani & Toddywala, 1997:542, 544, 550; Walters & Brain, 2002:327).

C.4.1.2 Hydrogels

Hydrogels (water based) usually consist of approximately 85 - 95% water, or aqueous-alcoholic mixtures, combined with a gelling agent. Xanthan gum is normally used as the gelling agent (Buchmann, 2006:110; Ramchandani & Toddywala, 1997:547).

C.4.2 Foams

The operation whereby a formulation is turned into foam is considered to be a well established manufacturing procedure (Arzhavitina & Steckel, 2010:2). A topical aerosol can be dispensed as sprays, foams or semisolid formulations. Aerosols have the advantage of preventing contamination of the formulation if it is used frequently (Ramchandani & Toddywala, 1997:549). Topical foams usually include an API, solvents, co-solvents and surfactants in a sealed container under pressure. These pressurised pharmaceutical dispersion systems represent a rather new concept in topical delivery of APIs and are gaining increasing popularity (Zhao *et al.*, 2010a:278; Zhao *et al.*, 2010b:228).

Foams are classified as a system where air/gas/propellants are emulsified within a liquid/semisolid phase to the point of stiffening (Flynn, 2002:217). A foam is a coarse dispersion of gas in a liquid, which is a colloidal lamellae dimension between gas bubbles (Attwood, 2007:97). Foams are thus elastic systems with a gas phase trapped in the foam bubbles that can be compressed. It is considered to be a “transition state” between the device used for foam production (aerosol can) and the skin. In other words, if the foamable formulation in the aerosol can is an emulgel, it would evolve into foam upon the release from the can, and when administered to the skin surface, will return to an emulgel (Arzhavitina & Steckel, 2010:2).

An aerosol package consists of a can, valve and actuator, which is filled with a formulation. Special foaming valves are used that are crimp sealed onto the aerosol can. Valves (Figure C.1) consist of a stem that is usually made from a polymer (polyethylene), whereas a valve seat is generally produced from aluminium (Arzhavitina & Steckel, 2010:2-5).

The foam actuator is fixed to the valve and disperses the aerosol foam. It should also be noted that the type of nozzle plays a significant part in the quality of the dispersed foam (Arzhavitina & Steckel, 2010:2-5).



Figure C.1: The aerosol package consisting of a can, valve and actuator.

Upon activation of the actuator, this system uses propellant evaporation to produce a semisolid creation, which is expanded with air. The formation of the foam is produced by the dispersion of a gas phase (propellant) into an immiscible liquid or solid phase (emulgel) (Zhao *et al.*, 2010b:228). The propellant used in this study was norflurane (HFA 134a), also known as Solkane[®] 134a pharma.

C.5 FORMULATION OF DIFFERENT SEMISOLIDS CONTAINING FLURBIPROFEN

In this study, 1% semisolid emulgels and a 1% hydrogel formulation were formulated. The liquid paraffin and hydrogel formulations were compared to the natural oil formulations and their effects on the permeation of the flurbiprofen marker was compared. The emulgels were formulated by incorporating the natural oils or liquid paraffin, while the hydrogel was created using Milli-Q water. The w/o emulsions and hydrogel were gellified and thickened with the use of xanthan gum, and a Elix-5 advantage system delivered HPLC grade water through reversed osmosis, followed by further purification with a Milli-Q[®] (Millipore Corporation, Milford, MA, USA) water purification system. Ambade *et al.* (2008:33) also used Milli-Q water throughout their study to prevent the integration of surface-active impurities.

C.5.1 Materials used in the manufacturing of the formulations

All chemicals were commercially available. The suppliers and batch numbers of the materials used during the formulation in this study are listed in Table C.1 and the six natural oils used in this study, their suppliers, batch numbers and toxicological information are summarised in Table C.2.

Table C.1 Ingredients used in the formulations

Ingredient	Supplier	Batch number
Flurbiprofen	DB Fine Chemicals	FBPN 100707
Liquid paraffin	Merck Chemicals	1035428
PEG ¹ 400	Saarchem	1014595
Span [®] 60	Merck Chemicals	55361721034
Tween [®] 80	Merck Chemicals	1035460
Xanthan gum	Warren Chem Specialties	4450902790
Methyl paraben	Merck Chemicals	K42067957 117
Propyl paraben	Merck Chemicals	K41917527 123
Ethanol	Associated Chemical Enterprises	23796
Solkane [®] 134 pharma	Solvay Chemicals	A0150N0066

1 Polyethylene glycol

Table C.2 Natural oils used in this study

Oil	Batch number	Supplier	Toxicological information
Avocado oil	WFA 10049	Nautica Organic Trading	Not expected to be an irritant ¹ .
Grapeseed oil	27083-11	Nautica Organic Trading	No hazard ²
Emu oil	549-08-02-10	Emuphoria	Is expected to be non-irritating to most skin types ³
Crocodile oil	23-8-2010	Croc City	Rarely allergenic ⁴
Olive oil	OIEV 1654	Nautica Organic Trading	Not expected to be an irritant ⁵
Coconut oil	15-7-2010	ENCO Fuels CC	Is expected to be non-irritating to most skin types ⁶

1 MSDS, 2008a:3

2 MSDS, 2008b:1

3 MSDS, 2010:4

4 Lotter, 2010:1

5 MSDS, 2008c:3

6 MSDS, 2008d:1

C.5.1.1 Liquid paraffin

To manufacture liquid paraffin, solid paraffin is removed from the petroleum fraction above 300 °C. Liquid paraffin is classified as a hydrocarbon oily material, with a mixture of saturated hydrocarbons that can have up to 15 - 30 carbons. It is a liquid at room temperature, colour- and odourless, and chemically inactive. In topical formulations it easily forms emulsions and is classified as occlusive agents to control moisture loss from the skin, as well as enhances the feeling of use (Mitsui, 1997:124).

C.5.1.2 Tween[®] 80 and Span[®] 60

The surfactants, Tween[®] 80 (sorbitan mono-oleate) and Span[®] 60 (sorbitan mono-stearate), are classified as non-ionic surfactants that do not dissociate into ions (Mitsui, 1997:132; Ramchandani & Toddywala, 1997:545). A surfactant's role in a formulation is to aid in drug

molecule solubilisation as well as water wash-ability for cosmetic reasons. Concentrations used within these formulations will most probably have little or no effect on the skin permeability (Barry, 1983:170; Foldvari, 2000:421). Mixtures of surfactants generate a more stable emulsion than individual surfactants (Walters & Brain, 2002:331).

The factor that determines whether a surfactant is hydrophilic or lipophilic is the HLB. The HLB number is an effective indicator of the properties of a surfactant and balances the hydrophilic and lipophilic characteristics of the objects used. It is suggested that the HLB dictates the stability of emulsions (Mitsui, 1997:167-168; Ramchandani & Toddywala, 1997:544, 550, 553-554). Most oils used in this study had HLB-values of between 7 and 8 (The herbarie, 2011). Therefore an emulsifier with an HLB number of approximately 7 - 8 is required to emulsify the oily components and the emulgel formulations were adjusted accordingly.

C.5.1.3 Propyl- and methyl parabens

Propyl- and methyl parabens are widely used as antimicrobial preservatives in cosmetics and pharmaceutical formulations. The paraben activity may be improved when used in combination in topical formulations and are therefore combined within the emulgel formulations. In cosmetics though, methyl paraben are the most commonly used antimicrobial preservative. Propyl and methyl parabens are effective over a wide pH-range and are known as broad spectrum antimicrobial agents, as they exhibit activity between pH 4 - 8. The parabens are more effective against yeast and moulds than bacteria and show more activity against gram-positive than gram-negative bacteria. Parabens occur as white, odourless, crystalline and tasteless powders and are non-mutagenic, non-teratogenic and non-carcinogenic (Pharmaceutical excipients, 2009a; Pharmaceutical excipients, 2009b).

C.5.1.4 Xanthan gum

Xanthan gum is a polymeric thickening agent used in cosmetics and its concentrations within a formulation may range from 0.5 - 10% (Walters & Brain, 2002:325; Walters & Brain, 2009:498). It is an acidic polysaccharide with outstanding usage characteristics, due to low temperature dependence and stability over a wide pH-range. Xanthan gum is used to ensure the stability of certain foundations and prevent the separation of emulsified particles, and is further used to adjust the viscosity of products. This is a natural gum that is obtained by fermentation of glucose with *Xanthomonas campestris* and usually water soluble polymers. Xanthan gum improves the feeling on use in topical formulations, however, when manufacturing a semisolid with natural gum one should bear in mind that too much in the formulation could create a sticky coating on the skin surface (Barry, 2007:595; Buchmann, 2001:152; Mitsui, 1997:139-140).

C.5.1.5 Polyethylene glycol 400 (PEG 400)

Liquid PEG is a colour- and odourless humectant and a liquid at room temperature. It has an average molecular weight ranging between 200 - 600 Da, whereas the semisolid forms have increasing molecular weights. Humectants are water soluble materials with high water absorption abilities and are important components in the aqueous phases of topical dosage forms. Humectants play an important role in topical formulations by mimicking the NMF of the skin, but at the same time also maintain the moisture content and stability the formulation itself. These formulations therefore need to possess bacteriostatic and fixative activities. PEG is a mixture polymer with various degrees of polymerisation (Mitsui, 1997:134-136). It is a stable hydrophilic substance and is generally non-irritating to the skin. PEG does not readily penetrate the skin and is easily removed from the skin by washing. Liquid PEG can be used to enhance dissolution characteristics of weakly soluble compounds (i.e. flurbiprofen). The PEG 400 used in this study occurred as a clear, slightly yellow-coloured, viscous liquid (Pharmaceutical excipient, 2011).

C.5.2 Formulation process of an emulgel containing flurbiprofen

The final formulation for the flurbiprofen emulgel, used during this study for the donor phase in the Franz diffusion cells, is summarised in Table C.3.

Table C.3: Formulation for the flurbiprofen emulgels and foams

Ingredient	% m/m	Activity of ingredient
A: Flurbiprofen	1.00%	Active ingredient/marker
Ethanol / PEG 400	6.00%	Polar solvents for flurbiprofen ¹
B: Xanthan gum	1.00%	Polymeric thickeners and gelling agent ¹
dH ₂ O	qs	Polar solvent ¹
C: Natural oil / liquid paraffin	20.00%	Oil phase or emollients ^{1,2}
Span 60	3.55%	Emulsifier and non-ionic surfactant
Tween 80	1.35%	Emulsifier and non-ionic surfactant
Methyl paraben	0.40%	Preservative ¹
Propyl paraben	0.08%	Preservative ¹

¹ Walters & Brain, 2009:498

² Walters & Brain, 2002:552

The above table shows all the ingredients used to formulate the semisolid emulgels, hydrogel and foam formulations. The procedure followed in the manufacture thereof is described next, while the dH₂O used in this formulation was Milli-Q water. During early pre-formulation, difficulties were experienced dissolving the lipophilic flurbiprofen. The solubility of flurbiprofen was tested in numerous organic materials, due to its poor water solubility (British

Pharmacopeia, 2012). However, flurbiprofen depicted increased solubility in a thoroughly mixed polar mixture of 5 ml PEG and 5 ml ethanol (50:50).

C.5.2.1 Preparation of 1% flurbiprofen emulgel with natural oils and liquid paraffin

The following method was used to prepare the formulations:

1. Accurately weigh 1 g of flurbiprofen and use 6 ml of the ethanol/PEG 400 (50/50) to dissolve the flurbiprofen **(A)**;
2. Weigh approximately 66 g of dH₂O and heat to 80 °C;
3. Weigh the xanthan gum and slowly add to the heated water (80 °C) in small quantities, while stirring with a glass rod **(B)**;
4. In another container, heat the mixture of natural oil/liquid paraffin, Span[®] 60, Tween[®] 80, methyl paraben and propyl paraben, to a temperature of 80 °C **(C)**;
5. Subsequently, add the mixture containing the API **(A)** to the heated oil phase **(C)**;
6. Immediately add the heated mixture containing flurbiprofen **(A + C)** to the xanthan gum mixture **(B)** and homogenise at 13 500 rpm, until it reaches a temperature of approximately 40 °C;
7. Place the container with the final product in ice water and allow to cool to room temperature; and
8. Leave overnight at 2 - 8 °C.

C.5.2.2 Preparation of 1% flurbiprofen hydrogel

The formulated hydrogel consisted of approximately 86.62% water with no oil phase and xanthan gum as gelling agent. The oil phase was replaced with Milli-Q water. To increase the viscosity of the hydrogel, approximately 1.5 g xanthan gum was added to the formulation. The same procedure was followed as described in Section C.5.2.1 in order to prepare the 1% flurbiprofen hydrogel. However, Milli-Q water was added to Phase **C**, thus replacing the oil.

C.5.2.3 Method for preparation of 1% flurbiprofen foam

Pre-formulated emulgel was combined with a propellant (HFA 134a) during the manufacturing of the foam. The emulgel was measured into new open containers and sealed by crimping an aluminium valve seat onto the container. The final foam product was produced after forcing the propellant under pressure into the sealed containers. Most topical foam formulations are generally produced by using this method of sudden pressure reduction (Zhao *et al.*, 2010b:228). To ensure equal amounts of gas used during manufacturing of the foam, the containers were

weighed before and after filling with the propellant gas and previously prepared emulgel formulations, which are summarised in Table C.4 and were prepared in duplicate.

Table C.4: Weight (g) of foam containers before and after filling with HFA 134a in duplicate

Emulgel formulations	Weight of empty container	Amount of emulgel (g)	Weight after sealing	Weight after gassed	Weight of gas
Olive oil foam	19.78	6.10	29.60	32.90	3.40
Olive oil foam	19.76	6.03	29.50	32.98	3.48
Coconut oil foam	19.79	6.00	29.45	32.85	3.40
Coconut oil foam	19.78	6.03	29.50	32.90	3.40

From the data generated it was evident that there were no significant differences in weight between the duplicate containers filled with the same emulgel formulation and propellant. A container is assembled with a foam actuator (Figure C.2). Upon activation of the actuator, rapid evaporation of the propellant causes the aired semisolid to proceed outwards. As it comes into contact with a topical surface, it often collapses and the foam excipients converge onto the skin surface (Zhao *et al.*, 2010b:228).



Figure C.2: The final foam formulation sealed in the container under pressure.

Emulsions can produce foams that can be aqueous, or non-aqueous in nature. It could also be a stable or fast-breaking system. Due to its apparent stability, the previously prepared emulgel formulation was incorporated into foam (Barry, 2007:595).

C.6 RESULTS AND DISCUSSION

C.6.1 Outcome of the flurbiprofen emulgel containing natural oils and liquid paraffin

The prepared 1% flurbiprofen emulgels containing natural oils and liquid paraffin formulations were visually inspected for their colour, spreadability, feel and consistency. The emulgel

formulations were viscous, white and creamy and had an elegant appearance that was not too oily or watery. It was easily spreadable and had a smooth feel and homogenous form.

The pH of each emulgel was measured with a Mettler Toledo Seven Multi pH-meter and found to be between 5.5 and 6.0. These levels indicated that no skin irritation would occur (Barry, 1983:32; Mohamed, 2004:3, Ramchandani & Toddywala, 1997:557).

C.6.2 Outcome of the formulated flurbiprofen hydrogel

The final hydrogel product portrayed a watery consistency, but appeared stable and spread well over the skin. It had a whitish colour, which might have been due to the Span[®] 60 included into the formulation. The product had a sticky feeling that could possibly be attributed to the amount of xanthan gum added; however, it had an overall homogenous presentation. The pH of the hydrogel was measured and also found to be within the same range as the emulgel formulations.

C.6.3 Outcome of the formulated flurbiprofen foam

Pressurised foams are unique topical dosage forms in terms of their structural properties, as they appear different before and after dose actuation. Upon the actuation of the valve, the emulgel mixes with air and generates air bubbles. It is the surfactants inside the formulation that rearrange the surface tension and stabilise the air bubbles. A small amount of surfactant is required for foam production and stability, as well as to maintain adequate coverage. A stable foam was formed, due to the surfactant present, which adsorbs to the air/water interface when it is ejected from the container. The two foams appeared stable upon actuation of the valve and appeared to be drip-free, with slowly diminishing properties. The foams had a uniform distribution and almost no residue was left when rubbed onto the skin. These foams appeared smooth and homogenous and felt less sticky than the emulgel (Zhao *et al.*, 2010b:229-230).

C.7 CONCLUSION

Ten topical preparations of 1% flurbiprofen were formulated, i.e. six formulations using natural oils, one utilising liquid paraffin and one containing water to form a hydrogel. Furthermore, two foams were prepared from previously prepared natural oil emulgel formulations that were transferred into a new foam container and sealed under pressure to create a foam. All of the emulgel formulations were successfully formulated and stable. They were placed in suitable amber glass containers, refrigerated overnight and used the next day in Franz diffusion cell studies.

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APPENDIX D

DIFFUSION STUDIES UTILISING FRANZ CELLS

D.1 INTRODUCTION

The use of *in vitro* methods to calculate the release of an active from a semisolid formulation is of high importance to the pharmaceutical industry, as the adequate release from these formulations is a prerequisite for therapeutic efficacy. Currently, no compendial method for the determination of the *in vitro* release of an API from a semisolid formulation is available. The Franz diffusion cell has become a recognised method for studying the release, or diffusion of an API in transdermal delivery systems (Chattaraj *et al.*, 1995:119-120).

The stratum corneum consists of dead keratinised cells, and it is believed and recognised that the *in vitro* skin penetration results are an excellent illustration of the *in vivo* situation (Behl *et al.*, 1990:2). The most important benefit of *in vitro* investigations is that experimental conditions can be controlled, with the only variables being the skin and the test material (Brain *et al.*, 1998:162). *In vitro* studies require a formulation applied to intact skin on a diffusion cell that simulates the passive diffusion from one side of the membrane/excised skin to the other (allowed to permeate). At certain points in time, samples are taken from the receptor compartment of these cells and analysed for the presence of the API. The amount determined in the receptor compartment is the transdermal delivery of the API (Bronaugh, 1990:62; Wiechers, 2008a:17). Flux (J) is the amount of API that crosses the skin, or enters the systemic circulation and is given in units of mass/area.time ($\mu\text{g}/\text{cm}^2\cdot\text{h}$). A potential factor to keep in mind is the depletion of the API from the applied formulation (vehicle). If the active is poorly water soluble and delivered from a saturated/sub-saturated aqueous formulation, the amount of active present in the formula is low. A lipophilic API will enter the stratum corneum faster, resulting in rapid diminishing of the active from the formulation (Williams, 2003:30, 37).

In this study, *in vitro* diffusion was conducted on dermatomed, excised, human skin to determine the transdermal permeation of the lipophilic marker, flurbiprofen. The vertical Franz diffusion cell method was used to conduct the skin diffusion studies; and a total of 10 diffusion studies were performed utilising skin samples from a single donor per study in order to minimise biological variability. A comparative study was done with the hydrogel **(1)** and liquid paraffin **(2)** formulations in order to determine whether the formulated emulgels, containing six natural oils, i.e. avocado- **(3)**, grapeseed- **(4)**, emu- **(5)**, crocodile- **(6)**, olive- **(7)** and coconut oil emulgel **(8)**

would enhance the permeation of flurbiprofen to a larger extent. The two oil emulgels, which yielded the best flux values, were then formulated into foams and comparative *in vitro* diffusion studies were also performed on them.

Membrane studies were done beforehand to determine whether flurbiprofen had indeed been released from the formulations. The results of all these studies are discussed in Section D.3, together with the statistical analysis used to analyse the data collected from the diffusion studies.

The reason for preparing a 1% flurbiprofen semisolid, emulgel formulation, was based on the results obtained from a series of previously performed studies. Ambade *et al.* (2008:32) studied the transdermal delivery effects of microemulsions containing 1% flurbiprofen, formulated with fatty acid containing isopropyl myristate and ethyl oleate as the oil phase (British Pharmacopoeia, 2012). The study concluded that a microemulsion could be a promising method for delivery of topically applied flurbiprofen. Fang *et al.* (2003b:154) also used a 1% flurbiprofen formulation in their study to investigate the enhancing effect of saturated and unsaturated fatty acids, using flurbiprofen as marker. Chi *et al.* (1995:268) prepared a 1% flurbiprofen formulation in their study in order to investigate the penetration enhancing effects of certain fatty acids through rat skin. The results indicated that plasma concentrations of flurbiprofen in oral delivery have to be between 0.04 - 0.47 mg/kg to achieve a pharmacological effect. During a study by Lee *et al.* (1992:431) using a different enhancing technique, a 2% flurbiprofen gel was prepared. Flux levels obtained in rat skin were four times higher than the levels achieved in human skin experiments.

D.2 MATERIALS AND METHODS

D.2.1 Sample analysis of flurbiprofen by HPLC

The HPLC analysis was developed and validated by Prof. J.L. du Preez from the Analytical Technology Laboratory at the North-West University, Potchefstroom Campus, South Africa (Appendix A). This method was used to analyse flurbiprofen in a controlled laboratory environment at 25 °C. Samples collected from the receptor compartments during permeation experiments were analysed using an Agilent® 1100 Series equipped with a pump, autosampler injection mechanism and UV-detector. The analysing software used was Chemstation Rev. A.10.03 for data acquisition. High performance Venusil XBP silica based reversed phase C₁₈ - 2 column (150 x 4.6 mm) with a 5 µm particle size was used.

The degassed mobile phase consisted of a mixture containing acetonitrile, Milli-Q water and acetic acid (70:30:1). The UV-detector was set at 247 nm for the detection of flurbiprofen and its retention time was $\pm$ 3.9 min. Run time was 8 min and flow rate was set at 1 ml/min whereas

the injection volume was 25 μ l. Phosphate buffer solution was used as solvent for the diffusion studies.

At the start of each analysis on the HPLC, a standard solution was prepared to act as control for the derived samples from the receptor phase of the Franz cells in the membrane and skin diffusion studies. This standard was prepared by weighing approximately 10 mg flurbiprofen in a 100 ml volumetric flask made up to volume with phosphate buffer solution (pH 7.4). A further 5 ml of this solution was diluted to 50 ml with phosphate buffer solution to provide a concentration of 10 μ g/ml. Approximately 2 ml of this standard solution was placed in an autosampler vial. The standard solution was injected in duplicate at volumes 5, 10, 15, 20 and 25 μ l into the HPLC to establish a concentration range. Afterwards, the average values of the obtained peak areas were plotted against the spiked concentrations to achieve a linear regression line. This was used to calculate the concentration of the marker that diffused through the membrane/skin from the extracted samples from the receptor phase of the Franz cells, as a percentage of the amount of the marker originally applied to the skin.

D.2.2 Preparation of skin

Caucasian female skin was obtained after cosmetic abdominoplastic surgery from ten healthy volunteers. Human abdominal skin is used since it has an intermediate permeability (Hawkins, 1990:67). Ethical approval for the use of biological material obtained from human subjects in experiments was provided by the Research Ethics Committee of the North-West University (NWU), (reference no NWU-00114-11-A5). Informed consent was obtained from each patient before the surgery and the identities of all patients were kept confidential. Donated skin was obtained directly after surgery from the theatre, placed on ice and transported in biological waste containers to the transdermal laboratory at the NWU. To decrease "specimen-to-specimen" variability in the skin penetration studies, each of the ten studies was performed on dermatomed harvested abdominal skin from a single donor (Aungst, 1989:246). A dermatome can be used with hairless and haired skin without negatively affecting the viability of the skin (Bronaugh, 1990:63). The obtained skin sections were dermatomed before it was frozen within one week from harvesting, by using an electric dermatome (Zimmer[®] LTD, Swindon, Wiltshire, UK). The excised skin were prepared by cleaning the surface of the skin with a small amount of Milli-Q water and tapping it dry with paper tissue, with the stratum corneum facing upwards. Thereafter, the skin was wiped once with an ethanol-soaked paper tissue to remove any possible traces of residual subcutaneous fat and lipids. The skin tissue with adipose fat still attached to it was pinned down and twelve layers of 5.0 x 2.5 cm and a thickness of 200 - 400 μ m were removed per diffusion study using the dermatome. The removed samples included the stratum corneum, viable epidermis and upper dermis. Each harvested skin sample was examined for imperfections (scars, stretch marks or extremely dry skin). The harvested

skin samples were placed on Whatman® filter paper with the stratum corneum facing upwards. Subsequently, it was covered with aluminium foil and carefully placed in airtight Ziploc® bags and kept frozen at -20 °C until it was ready to be used. Prior to the diffusion study, the skin was thawed at room temperature and punched into circles of approximately 15 mm in diameter by making use of a punch and hammer. These skin samples were mounted on the diffusion cells as soon as possible.

D.2.3 Preparation of receptor phase solution

Phosphate buffer solution was prepared by accurately weighing 13.62 g potassium dihydrogen ortho-phosphate in a 500 ml volumetric flask and filling it up to volume with Milli-Q water. Subsequently, 3.14 g sodium hydroxide pearls was weighed and dissolved in 768.80 ml Milli-Q water; and the two solutions were mixed. The pH of the solution was adjusted to 7.4 utilising a Mettler Toledo Seven Multi pH-meter, by either adding 2.0 M orthophosphoric acid (20% m/v) or 2.0 M sodium hydroxide (8% m/v) solution, whilst being stirred on a magnetic stirrer.

D.2.4 Preparation of the flurbiprofen emulgel and foam for the donor phase

The donor phase in this study, for both the membrane and skin diffusion studies contained an emulgel formula with 1% flurbiprofen as the marker API and 20% natural oil (oil phase). Two other formulations were formulated, one with 20% liquid paraffin (oil phase), and another semisolid containing 20% Milli-Q water (hydrogel). The liquid paraffin (oil) and hydrogel (Milli-Q water) were used to compare the activity of the natural oils, containing the fatty acids. The emulgel formulations, containing a natural oil, were prepared by accurately weighing the ingredients in two separate glass containers.

- Container 1: Phase **A** contained the ethanol/PEG 400 mixture, which was used to dissolve flurbiprofen and which was added to Phase **C** directly after heating.
- Container 2: Phase **B** contained the xanthan gum and Milli-Q water mixture.

Phase **C** contained the oil, Span® 60, Tween® 80, methyl- and propyl paraben, which were heated to 80 °C. Phase **A** was added to Phase **C**, directly after Phase **C** reached the acquired temperature. Phases **(A + C)** was immediately added to Phase **B** and homogenised at 13 500 rpm until it cooled down to ± 40 °C and left to cool down to room temperature. A placebo emulgel was also formulated, exactly as the aforementioned except for the fact that no marker (flurbiprofen) was added and the oil phase contained liquid paraffin.

Preparation of the liquid paraffin emulgel was conducted in the exact same manner as was done for the natural oil emulgel formulations. The only exception was that the natural oil was substituted with liquid paraffin. As for the hydrogel formulation, exactly the same steps were taken, but because there was no oil phase present, the xanthan gum used as thickener, was

adjusted to create a more viscous mixture. Phase **B** contained Milli-Q water as well as Phase **C**. They were mixed together after heating and homogenised at 13 500 rpm until it reached 40 °C.

After completion of the skin diffusion studies, the two emulgel formulations that presented with the highest flux values were chosen in order to incorporate them into foam formulations. These freshly produced emulgels were placed into new, open foam containers and sealed. A propellant was then forced under pressure into the containers in order to produce the final foam product.

All the formulations for the donor phase were refrigerated at 2 - 8 °C until it was used in the Franz cell diffusion studies.

D.2.5 Transdermal Franz cell diffusion studies

In vitro permeation studies were conducted using vertical Franz diffusion cells. A single Franz cell has a receptor capacity of ± 2 ml and a diffusion area for exposed skin of ± 1.075 cm². These Franz cells consist of a donor (top) and receptor (bottom) compartment. One of the semisolid formulations (1 g) or foams containing the 1% flurbiprofen was inserted into the donor compartment, and the phosphate buffer solution (pH 7.4) was placed in the receptor compartment. Depletion of the donor phase with the foam experiments was avoided by adding fresh foam to the donor phase periodically as needed for the concentration to be more or less constant. The prepared skin pieces (stratum corneum facing upward) or membranes were placed between the donor and receptor compartment and Dow-corning[®] vacuum grease was used to seal the cells and prevent any leakage in or out of the cells. The donor and receptor compartments were then secured with a horseshoe clamp. Receptor compartments were filled with pre-heated (32 °C) phosphate buffer solution (pH 7.4) and care was taken to avoid entrapment of air bubbles under the surface of the skin or membrane sample. Any air bubbles that formed during the experiment were removed by tilting the Franz cells. To maintain stirring of the phosphate buffer solution throughout the experiment, a small magnetic stirring bar was placed into the receptor compartment of each individual Franz cell. The cells were placed in a tray on a Variomag[®] stirrer plate at 750 rpm. All ten skin diffusion experiments were conducted in a Grant[®] water bath and maintained at 37 ± 1 °C, in order to attain a skin temperature of 32 °C (Smith, 1990:28). A total of 12 Franz cells were used in each of the individual skin and membrane diffusion studies; 10 cells received the emulgel formulations with 1% flurbiprofen and 2 cells were included as the negative control containing the placebo emulgel formulations that contained no marker. The donor compartments were covered with a piece of Parafilm[®] and secured with a plastic cap that prevented evaporation or leakage of the semisolid. The entire volume of the saturated phosphate buffer solution in the receptor compartment was withdrawn

from the receptor phase at specific time intervals and immediately replaced with fresh preheated (32 °C) phosphate buffer solution (pH 7.4). All samples obtained from the receptor phase was placed into autosampler vials and directly analysed on the HPLC without any further processing.

D.2.5.1 Membrane diffusion studies

The aim of the membrane studies were to determine whether or not flurbiprofen was released from the formulation. The entire volume of the receptor phase was withdrawn and replaced hourly with fresh preheated phosphate buffer solution (pH 7.4) for 6 h.

Similar methods as discussed in Section D.2.5 were used during the membrane diffusion studies. Membrane diffusion studies preceded the skin diffusion studies; and were conducted with a hydrogel **(1)**, liquid paraffin **(2)**, avocado oil **(3)**, grapeseed oil **(4)**, emu oil **(5)**, crocodile oil **(6)**, olive oil **(7)** and coconut oil **(8)** formulation. As a skin substitute, 0.45 µm polytetrafluoroethylene (PTFE) membranes that were obtained from PALL Corporation (Ann Arbor, Michigan), were used.

D.2.5.2 Skin diffusion studies

The method discussed in Section D.2.5 was also used for the skin diffusion studies. The aim of the skin diffusion studies was to determine the flux of the marker flurbiprofen from formulations containing a selected natural oil in order to determine if these oils had any penetration enhancing effects. All skin diffusion studies were conducted with previously harvested human skin subsequent to the membrane diffusion studies and were performed using the aforementioned emulgel formulations and foams. The entire volume of the receptor phase was withdrawn and replaced with the fresh preheated phosphate buffer solution (pH 7.4) after 20, 40, 60, 80, 100 and 120 min, as well as at 2, 4, 6, 8, 10 and 12 h. Tape stripping was performed directly after the 12 h withdrawal (also see Section D.2.6).

D.2.6 Tape stripping

The aim of the tape stripping experiments was to determine the concentration of the marker flurbiprofen found within the stratum corneum-epidermis and epidermis-dermis. The skin was removed from the cells after the last 12 h receptor withdrawal and placed on Whatman® filter paper. The removed skin was dabbed dry with tissue paper, stratum corneum facing upwards in order to remove any residual semisolid. Thereafter the diffused area of the stratum corneum was partially removed by tape stripping the skin fifteen times using pieces of 3M Scotch® Magic™ tape strips. All the strips were cut to size as not to overlap the areas outside the diffusional area. The first tape strip was discarded as part of the cleaning procedure, after which the following fifteen strips were placed in a polytop filled with 5 ml phosphate buffer solution (pH 7.4). Glistening of the epidermal layer indicates that the stratum corneum was

completely removed (Pellet *et al.*, 1997:94). The diffused area of the remaining epidermis-dermis was cut out and extra skin was trimmed away from the protuberant imprints into small pieces and placed in different polytops containing 2 ml phosphate buffer solution (pH 7.4) (Pellet *et al.*, 1997:94). Polytops containing the samples were kept overnight at 4 °C for 12 h. Preparation of the samples involved sonification thereof and subsequent extraction from the polytops utilising 0.45 µm syringe filters. Extracted samples were transferred into autosampler vials and analysed on HPLC to determine the concentration of the marker flurbiprofen.

D.2.7 Data analysis

D.2.7.1 Transdermal data analysis and calculation of flux values

Before analysis of the samples in the diffusion-studies could commence, a standard solution was prepared to determine linearity as described in Section D.2.1. Subsequently the calibration curve of flurbiprofen was used to determine the concentrations of each individual Franz diffusion cell.

For the analysis of the diffusion studies the cumulative concentration ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that permeated the skin was plotted against time and the flux of flurbiprofen was determined by calculating the slope of the straight line. This was performed for each of the formulations and each individual Franz cell. The profiles exhibited a characteristic clear steady-state flux between 2 - 6 h for the membrane diffusion studies and 2 - 12 h for the skin diffusion studies. Average amounts of flurbiprofen that permeated through the membranes and the skin in the Franz-diffusion cells of each formulation, were represented as a percentage of the applied flurbiprofen in the formulations. For the membrane studies the average percentage yield after 6 h was determined and with the skin studies it was determined after 12 h.

Initially, all ten Franz-diffusion cells containing flurbiprofen were used for each formulation, but outlier cells were removed from the data. These outlier cells include cells that leaked, cells that depleted prior to the completion of the experiment, and cells that showed abnormal drift in the cumulative concentrations. The actual number of cells used to do the statistical inference is reported in subsequent summary tables (Section D.3).

D.2.7.2 Statistical data analysis for Franz cell diffusion studies and tape stripping

To analyse the data, descriptive as well as inferential statistics were employed. The descriptive statistics used involved calculation of the mean (usually accompanied by the standard deviation) and median (measures of central location), as well as the standard deviation (SD) or error bars (measure of spread) (Lang & Secic, 1997:267, 287-288). The data was graphically presented using scatter- and box plots.

A schematic diagram of the statistical analysis followed in this study is shown in Figure D.1. The statistical inference used involves analysis of variance (ANOVA) as well as non-parametric hypothesis testing. To investigate whether the flux values differed significantly between the different formulations, a one-way analysis of variance was employed followed by the post-hoc Tukey test. The post-hoc Tukey test was used to identify those formulations which differed from each other; and this analysis was performed for both the membrane and skin diffusion studies.

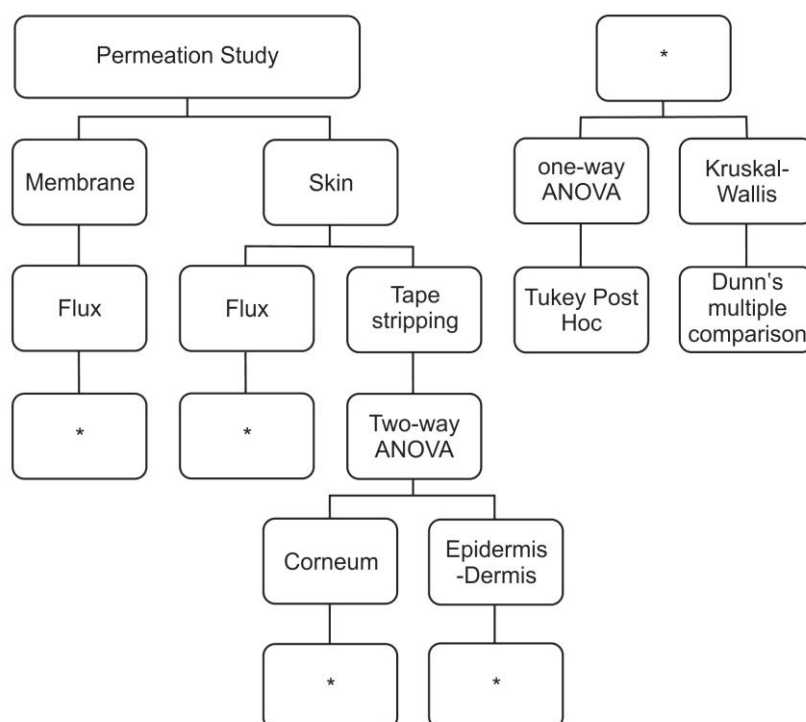


Figure D.1: Illustration of the statistical methods used in this study.

The median or statistical centre point is useful in describing the central tendency of abnormally distributed data. Therefore, the median (statistical centre point) is preferred because it is less affected by extreme values that could skew the distribution and thus having a disproportionate effect on the mean value. If data is normally distributed the mean will be useful (Lang & Secic, 1997:244, 267-269). Gerber *et al.* (2008:190) suggest that the median flux is a more exact method to compare flux values of formulations. For this reason the non-parametric Kruskal-Wallis test (gives group medians, actual p values and test statistics) was also performed, followed by Dunn's multiple group comparison (Juneau, 2007:131, 135; Lang & Secic, 1997:265).

Results obtained from the tape strips, provided information on the amount of marker that diffused into the stratum corneum-epidermis and the epidermis-dermis of the skin. Hence, a two-way analysis of variance was performed to first investigate whether knowledge of both the cell location (i.e. stratum corneum-epidermis and epidermis-dermis) and the formulation was

important to model the accumulation of the marker in the tape strips. Significant interaction between location of the skin and the formulations was found. In case of a statistically significant interaction, a one-way ANOVA and non-parametric Kruskal-Wallis test were applied to each of the individual skin locations (stratum corneum-epidermis and epidermis-dermis). These tests were followed by the appropriate multiple comparison test as described earlier.

For the statistical group comparisons, a significance level of 0.05 was used to do statistical inference, therefore, inferential statistics were performed at the 5% level of significance and it was determined that differences were significant when the p value was less than 0.05 (Fang *et al.*, 2003b:155; Lang & Secic, 1997:272).

It should be noted that the Dunn's test, reported less significant differences, without contradicting the Tukey results, and therefore, it was preferred to interpret the more conservative Dunn's test for further pharmaceutical interpretation, as discussed in Section D.3.4.

All statistical analysis was performed using Statistica (version 10), SPSS (version 18) and the R statistical software package (version 2.13) by the Statistical Consultation Services, North-West University, Potchefstroom Campus.

D.3 RESULTS AND DISCUSSION

D.3.1 Membrane diffusion studies

In all eight of the membrane studies average cumulative concentrations for flurbiprofen were detected in the receptor compartments of the Franz cells. This was an indication that flurbiprofen permeated through the membranes and therefore released a portion of flurbiprofen from all the formulations.

Summary statistics as well as comparative box-plots are presented in Table D.1 and Figure D.2, respectively, for the flux of the membrane experiments. Table D.1 also contains summary statistics concerning the percentage flurbiprofen diffused for from each semisolid formulation.

Comparing the median flux values from Table D.1, the following was observed through the membranes after 6 h: Formulation **(5)** (144.82 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) achieved the highest median flux value followed by formulation **(2)** (137.42 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), **(7)** (110.39 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), **(6)** (104.28 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), **(8)** (101.18 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), **(1)** (93.95 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), **(3)** (65.42 $\mu\text{g}/\text{cm}^2\cdot\text{h}$) and lastly **(4)** (48.06 $\mu\text{g}/\text{cm}^2\cdot\text{h}$).

Table D.1: Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) and average percentage (%) diffused flurbiprofen from different emulgels through membranes after 6 h (n = number of Franz cells used)

Emulgel formulation	n	Flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)		% Diffused
		Average	Median	Average
Hydrogel (1)	8	87.06 ± 13.78	93.95	2.03 ± 0.306
Liquid paraffin (2)	10	135.41 ± 9.26	137.42	4.88 ± 0.470
Avocado oil (3)	9	65.48 ± 10.39	65.42	1.73 ± 0.304
Grapeseed oil (4)	10	46.89 ± 8.17	48.06	1.19 ± 0.208
Emu oil (5)	8	144.18 ± 10.69	144.82	4.18 ± 0.363
Crocodile oil (6)	9	103.18 ± 7.13	104.28	2.99 ± 0.529
Olive Oil (7)	9	109.69 ± 11.59	110.39	3.24 ± 0.347
Coconut oil (8)	10	101.39 ± 14.15	101.18	2.81 ± 0.145

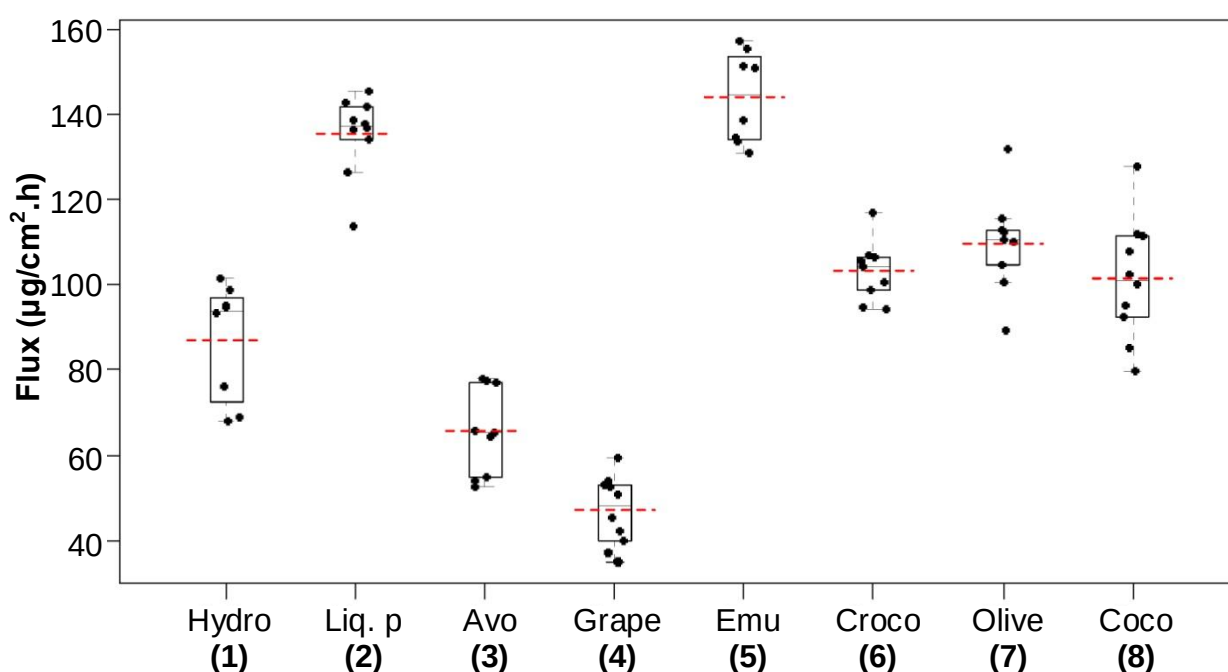


Figure D.2 Box-plot representation of the flux values of flurbiprofen for the different formulations in the membrane diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

The parameters shown in Table D.1 indicate that formulations **(5)**, **(7)** and **(6)** formulated from the natural oils, which presented with high amounts of MUFAs (oleic acid) showed higher concentrations flurbiprofen released than from formulation **(4)** containing more of the branched linoleic acid (PUFA). Furthermore, formulation **(8)** containing high amounts of lauric acid (SFA) released the flurbiprofen to a more significant extent than formulation **(3)** and **(4)** that contains an almost equal mixture of both MUFAs and PUFAs.

In the aforementioned table, minor differences were observed between the average and median flux values. This was an indication that there were not many outliers in the data collected; and for this reason both the average and median values could be used to determine the flux values. However, formulation **(2)** showed a small difference and formulation **(1)** a more significant difference between the average and median flux value, and for this reason the median flux would be a more accurate representation of the true flux value, because it is unaffected by the outliers in the data (Gerber *et al.*, 2008:190).

Concerning the flux values, it is clear that there are differences in the expected flux values of some of the formulations. This finding was further explored quantitatively (see Section D.3.4), using the statistical methods described in Section D.2.7.2.

D.3.2 Tape stripping

Results for the tape stripping data are presented in Table D.2. Summary statistics are presented in Table D.2, for both the stratum corneum-epidermis and epidermis-dermis, whereas box-plots are presented in Figures D.3 and D.4, respectively. The emulgel formulations **(7)** and **(8)** depicted on average the best flux values (see Section D.3.3) and were subsequently manufactured into foams **(9)** and **(10)** in that order.

From Figures D.3 and D.4, differences between the performance of the various formulations on the stratum corneum-epidermis and the epidermis-dermis were observed. In addition, the response appeared to be different depending on whether it was measured on the stratum corneum-epidermis or the epidermis-dermis. This was an indication of an interaction between the formulation and place of measurement (i.e. stratum corneum-epidermis and epidermis-dermis). Lastly, the tape stripping concentration measurements on the stratum corneum-epidermis seemed to be higher compared to those measured on the epidermis-dermis for most formulations. These findings will be further explored quantitatively (see Section D.3.4), using the statistical methods, as described in Section D.2.7.2.

As summarised in Table D.2 and Figure D.3, the highest average concentration within the stratum corneum-epidermis was detected within formulations **(2)** (21.29 µg/ml) and **(9)** (21.47 µg/ml), followed by formulations **(7)** (17.82 µg/ml), **(4)** (17.78 µg/ml), **(1)** (16.73 µg/ml), **(6)** (14.89 µg/ml), **(8)** (7.18 µg/ml), **(3)** (2.72 µg/ml), **(10)** (1.57 µg/ml) and lastly **(5)** (1.25 µg/ml).

Table D.2: Average and median concentrations ($\mu\text{g/ml}$) of flurbiprofen present in the stratum corneum-epidermis and epidermis-dermis for formulations **(1)** - **(10)**. (n = number of Franz cells used)

Formulation	n	Concentration ($\mu\text{g/ml}$)			
		Stratum corneum-epidermis		Epidermis-dermis	
		Average	Median	Average	Median
Hydrogel emulgel (1)	7	16.73 ± 2.156	15.84	5.39 ± 1.381	5.71
Liquid paraffin emulgel (2)	10	21.29 ± 2.925	21.74	4.56 ± 0.917	4.46
Avocado oil emulgel (3)	10	2.72 ± 0.897	2.67	4.28 ± 2.359	3.68
Grapeseed oil emulgel (4)	9	17.78 ± 1.689	17.81	2.98 ± 1.476	2.40
Emu oil emulgel (5)	9	1.25 ± 0.375	1.21	16.14 ± 2.118	16.48
Crocodile oil emulgel (6)	10	14.89 ± 1.412	14.90	1.23 ± 0.388	1.38
Olive oil emulgel (7)	9	17.82 ± 2.513	17.68	2.46 ± 1.234	2.20
Coconut oil emulgel (8)	9	7.18 ± 6.650	3.27	3.25 ± 1.365	2.71
Olive oil foam (9)	10	21.47 ± 2.476	21.24	1.52 ± 0.516	1.41
Coconut oil foam (10)	8	1.57 ± 0.545	1.40	0.97 ± 0.419	0.91

D.3.2.1 Concentration of flurbiprofen in the stratum corneum-epidermis for all the formulations

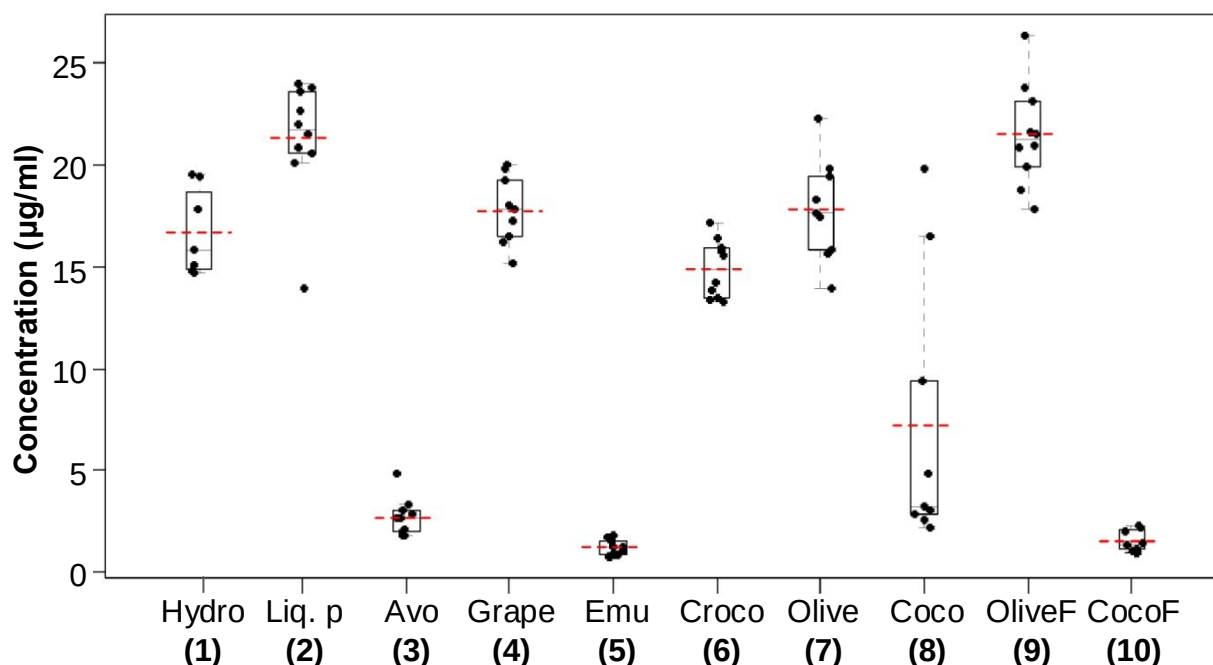


Figure D.3 Box-plot representation of the flurbiprofen concentrations within the stratum corneum-epidermis for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

Small differences were noted between the average and median stratum corneum-epidermis concentration values for all the formulations ((1) - (10)). However, significant differences between the average and median concentrations were seen for formulations (1), (2) and (8). This could be avoided by using the median concentrations as it gives a more precise depiction of the true concentration, due to the fact that it is not affected by a distortion in the spread of the data (Gerber *et al.*, 2008:190).

D.3.2.1.1 Effects of hydration on the concentration of flurbiprofen

Fats and oils present in a formulation can act as occlusives, and the fatty acids incorporated in formulations (3) - (10) are classified as lipophilic material. These prevent water loss from the skin and may produce full hydration of the skin with a marked increase in the permeation of flurbiprofen. It is also believed that formulation (2) could enhance the permeation of the marker due to the thick layer of oily material or lipophilic substances within, which could occlude the skin and increase hydration thereof (Barry, 1983:154; Barry, 2001b:969; Barry, 2007:576-577, 586, 595; Ramchandani & Toddywala, 1997:550). Therefore, occlusion of the skin might have increased the diffusion through a swelling process (hydration) of the stratum corneum and enhanced the absorption of the marker (Robinson *et al.*, 1997:62; Smith, 1990:28). Hydration caused by formulation (1) of the stratum corneum, is an important aspect when it comes to increasing the flux of most substances into the skin as water opens up the packed horny layer of the stratum corneum (Benson, 2005:28).

D.3.2.1.2 Effects of MUFAs and PUFAs on the concentration of flurbiprofen

Formulations (9), (7) and (6) predominantly contained oleic acid (C18:1), a MUFA, which has been shown to be a good penetration enhancer (Fang *et al.*, 2003a:319). Formulation (4) did not depict very high oleic acid (C18:1) values, but rather a high linoleic acid (C18:2) content. The main fatty acids present in formulation (4) are MUFAs and PUFAs. The number of double bonds present in the chemical structure of oleic- (C18:1), linoleic- (C18:2) and linolenic acid (C18:3) causes these fatty acids to have a kinked shape. The more kinked shaped *cis*-double bond numbers present, especially in formulations (4), (6), (7) and (9), the more difficult it become for these acids to insert themselves into the lipid bilayers within the stratum corneum. Therefore, this might have been the reason why a higher accumulation of flurbiprofen was observed in the stratum corneum with formulations containing oleic- (C18:1) and linoleic acid (C18:2) than with the other formulations. Due to the packing within the skin (skin modification), higher flux values of flurbiprofen were obtained, due to wider channels produced by these fatty acids, with resulting accumulation of flurbiprofen within the stratum corneum for formulations (4), (6), (7) and (9) (Fang *et al.*, 2003a:319; Chi *et al.*, 1995:270). A study of Fang *et al.* (2003b:163) indicated a disruption and fragmentation caused by oleic acid (C18:1) within the

stratum corneum, and this might have contributed to the enhancing effect on the permeation of the marker as observed. The same study obtained rather similar results with a slight enhancement of the flurbiprofen reservoir within the skin as a result of the UFAs incorporated into their formulations (Fang *et al.*, 2003b:156, 163).

D.3.2.1.3 Effects of longer chain SFAs on the concentration of flurbiprofen

The low flurbiprofen concentrations depicted within formulations **(3)**, **(5)**, **(8)** and **(10)** may have been as a result of the palmitic acid (16:0) present within these formulations. It is believed that due to an increase of hydrocarbon chain lengths ($> C_{14}$) the enhancement effect within the skin decreases. SFAs present in these formulations are from the medium to long chain series, and based on the believe that the higher chain acids have more affinity toward the lipids within the stratum corneum; it could have hindered the penetration of flurbiprofen through the skin (Ogiso & Shintani, 1990:1067). It is therefore understood that, based on the hydrophobic interactions within the stratum corneum, longer chain fatty acids depict higher affinity for the lipids within the stratum corneum, due to their lipophilicity, that might have caused some retardation of the marker within the skin (Ogiso & Shintani, 1990:1067).

D.3.2.1.4 Effects of the foam formulation high in SFAs and MUFAs on the concentration of flurbiprofen

With regards to the poor permeability of the foam formulation **(10)**; the dosage form could have had an effect on the poor flurbiprofen concentration values obtained as demonstrated by both the stratum corneum-epidermis and epidermis-dermis, which could have been due to the air bubbles within the foam. Another reason could have been as a result of the short chain fatty acids present within formulation **(10)** that might not have had adequate lipophilicity to actively penetrate the stratum corneum; hence accumulation of the marker was thus not observed (Ogiso & Shintani, 1990:1067). Contrary, a higher flurbiprofen concentration was observed for formulation **(9)**, which predominantly contain the UFA oleic acid (C18:1), which is indicated to have good penetration enhancing effects as described before (Fang *et al.*, 2003a:319).

D.3.2.1.5 Concentration of the lipophilic flurbiprofen found in the stratum corneum

A lipophilic API will automatically partition into, and thus accumulate in the stratum corneum, which is thought to be the reason for the high marker concentrations of within the stratum corneum for most of the formulations applied. It is however a problem keeping a hydrophilic API within the stratum corneum (Ghafourian *et al.*, 2004:115; Wiechers, 2008a:11). Amounts of the marker that had succeeded in partitioning into the stratum corneum might have had a high tendency to be taken up into the hydrophilic viable epidermis, as had been observed for some of the formulations with lower concentrations (Walters & Brain, 2009:479; Wiechers, 2008a:11-

12). The penetration of the marker from all of the formulations seemed to have indicated a relationship between the skin and the lipophilic nature of flurbiprofen; and therefore it might have been that the amount of marker retained in the skin could have been due to its lipophilic nature (Wiechers & Watkinson, 2008:81-82). An assumption for the accumulation of flurbiprofen within the stratum corneum could possibly be due to the chemical structure of flurbiprofen that could affect its partitioning and solubility characteristics. This ensures that flurbiprofen would partition reasonably well between the hydrophilic and lipophilic domains of the stratum corneum. Extremely lipophilic actives can easily partition into the skin lipids, but due to the aqueous regions in the skin lipids, they will not easily partition out of the stratum corneum into the viable epidermis. It appears that the lipid-soluble flurbiprofen segregated and diffused through the lipid-regions of the stratum corneum. It was possibly retarded within the stratum corneum by the lipid barrier and aqueous boundaries (Barry, 1983:109-113; Hadgraft & Finnin, 2006:365).

High flurbiprofen concentration values were obtained during the diffusion studies when formulation **(1)** was tested. The reason could have been that flurbiprofen was more soluble within the stratum corneum than within this hydrophilic formulation. The increased permeation of flurbiprofen might thus have been due to its low solubility in formulation **(1)** relative to that in the stratum corneum (Wiechers, 2008b:94). Due to its lipophilicity it would have partitioned easily into the skin (Fang *et al.*, 2003a:319). Therefore, choosing a formulation where the API is less soluble than in the stratum corneum, will enhance the partitioning into the stratum corneum. On the other hand, choosing an aqueous gel for a lipophilic ingredient and a lipophilic oil for a water-soluble active, will create a high driving force for diffusion into the stratum corneum (Wiechers, 2008a:11-12; Williams, 2003:37). The lipophilic marker will, however, automatically partition into the skin and accumulate within the stratum corneum. Protein binding with the hydroxyl groups of the penetrating marker might retard penetration thereof within the epidermis (Wiechers, 2008a:11).

D.3.2.2 Concentration of flurbiprofen in the epidermis-dermis for all the formulations

The fact that the stratum corneum is such a lipophilic section of the skin the lipophilic flurbiprofen tends to remain within this section and refrain from moving into the deeper layers of the skin i.e. epidermis (Ogiso & Shintani, 1990:1067). It is also possible that the amount of flurbiprofen retarded within the stratum corneum could have been as a result of the degree of ionisation of the flurbiprofen. The ionised (polar form) species cross the stratum corneum to a limited degree, due to their aqueous solubilities and only unionised (non-polar form) APIs are absorbed well. Therefore, this might have been a reason for the high flurbiprofen concentration present within the lipophilic stratum corneum. As the polarity of a molecule increases, its oil-

water partition coefficient decreases (Barry, 2007:576; Cooper, 1984:1153; Panwar *et al.*, 2011:336).

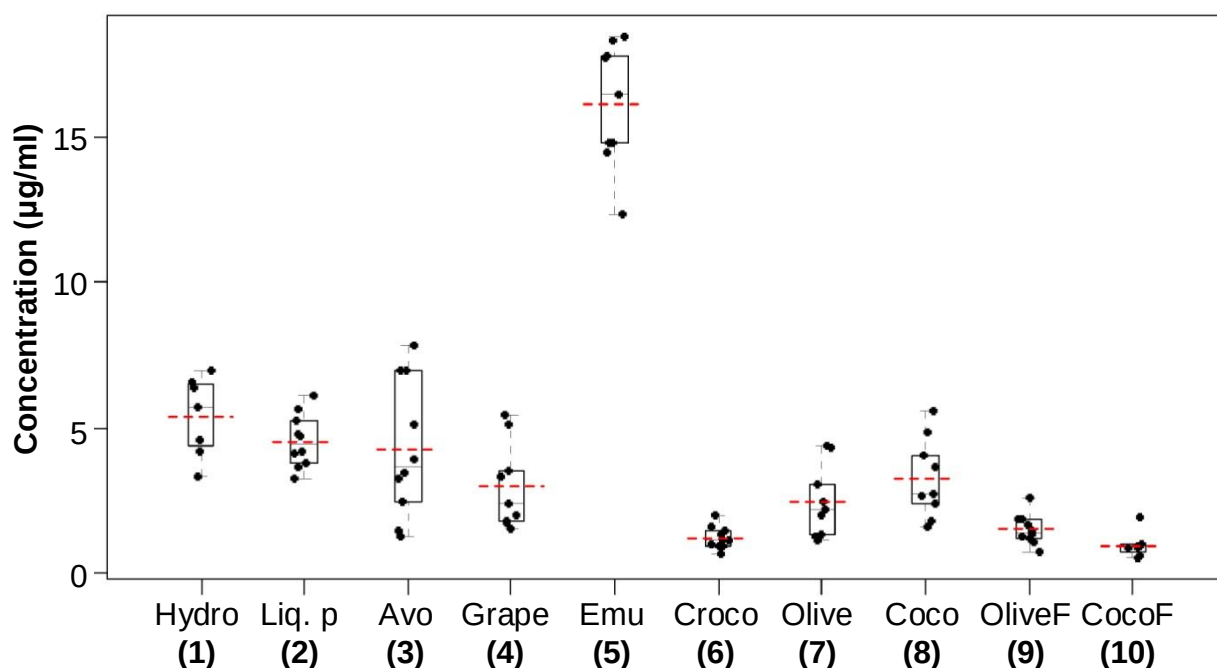


Figure D.4 Box-plot representation of the flurbiprofen concentrations within the epidermis-dermis for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

Upon comparing the average concentrations inside the epidermis-dermis, given in Table D.2 and illustrated in Figure D.4, the following ranking order was observed: formulation **(5)** (16.14 µg/ml) >>> **(1)** (5.39 µg/ml) > **(2)** (4.56 µg/ml) > **(3)** (4.28 µg/ml) > **(8)** (3.25 µg/ml) > **(4)** (2.98 µg/ml) > **(7)** (2.46 µg/ml) > **(9)** (1.52 µg/ml) > **(6)** (1.23 µg/ml) > **(10)** (0.97 µg/ml).

Comparing the average and median concentrations (Figure D.4), no major differences were noted. Though small variations between formulations **(3)**, **(4)** and **(8)** were observed, and for that reason it is advisable to rather use the median concentration since it is not affected by outliers in the data (Gerber *et al.*, 2008:190).

D.3.2.2.1 Effects of hydration on the concentration of flurbiprofen

The slight flurbiprofen reservoir concentration of flurbiprofen observed for formulations **(1)** and **(2)** within the epidermis-dermis could have been explained by the fact that these formulations hydrated the stratum corneum. Hydration generally facilitates the permeation of hydrophilic compounds, but makes the partitioning through the hydrated stratum corneum more difficult for lipophilic APIs i.e. flurbiprofen (Fang *et al.*, 2003b:156). Hydration causes an increase in the partitioning of the marker within this region by disrupting the lipid layers within the stratum

corneum, with subsequent accumulation of the lipophilic marker (Benson, 2005:28). It might also have made it more difficult for the marker to diffuse out of this lipophilic surrounding area. Hydration alters the structures within the intercellular lipid domain of the skin causing structural defects of the lipid pathway, which is said to represent small mobile free volumes and this might have resulted in an increased fluidity of these lipid structures and increased permeation of the marker. The resistance of this newly formed liquid lipid phase is less than the structured lipid matrix. Therefore, it is believed that a slight increase in the marker observed here could have been due to the fact that it followed this intercellular, newly disrupted pathway (Fang *et al.*, 2003a:319; Marjukka Suhonen *et al.*, 1999:152).

D.3.2.2.2 Effects of MUFAs and PUFAs on the concentration of flurbiprofen

The high accumulation of flurbiprofen within the epidermis-dermis observed from formulation **(5)** could have been caused by obvious scaling of the epidermis as a result of the small amount of palmitoleic acid (16:1) and high oleic- (18:1) and linoleic acids (C18:2) present in this oil (Katsuta *et al.*, 2005:1009). Penetration enhancers that contain unsaturated alkyl chains (C₁₈) appear to cause optimum scaling and the bent *cis*-configuration of their chains is believed to disrupt the intercellular lipid packing of the stratum corneum (Williams & Barry, 2004:609). In terms of the drug amount trapped within the epidermis-dermis, oleic acid seemed the most potent fatty acid followed by linolenic- (C18:3) and linoleic acid (C18:2) considering their penetration enhancing effects. These acids demonstrate that the more kinked shaped the fatty acids, the more difficult it is for them to insert themselves into the lipid matrix of the stratum corneum and that might have accumulated in the epidermis-dermis region by creating a drug reservoir within the skin (Fang *et al.*, 2003a:318-319; Fang *et al.*, 2003b:156). Another possibility could have been epidermal proliferation, with a thickening of the epidermis with oleic- and linoleic acid (Fang *et al.*, 2003b:163). It is thought that due to this abnormal epidermal proliferation and differentiation, flurbiprofen's accumulation was detected within this region (Katsuta *et al.*, 2005:1011). The amounts of flurbiprofen obtained within the epidermis-dermis with regards to formulation **(5)** could also have been as a result of skin appendages degeneration, due to the small amounts of linolenic acid (C18:3) present (Fang *et al.*, 2003a:323).

Oleic acid (C18:1) is thought to cause hyperplasia of the stratum corneum and epidermis-dermis as result of an inflammatory response with resulting epidermal proliferation (Fang *et al.*, 2003b:163-164). However, in the *in vitro* studies, the higher amount of oleic acid (C18:0) remaining in the stratum corneum when released from formulations **(3)**, **(4)**, **(6)**, **(7)** and **(9)** caused an increase in TEWL and could have resulted in a concentration of flurbiprofen diffusing through into the epidermis-dermis region. The reason for the levels detected in the epidermis-dermis, could have been as a result the morphological changes in the skin, due to the oleic acid

(C18:1) present (Tanojo *et al.*, 1999:101). Thickening of the epidermis was more clearly observed with oleic acid (C18:1) than with linoleic acid (C18:2) (Fang *et al.*, 2003b:163-164).

D.3.2.2.3 Effects of SFAs on the concentration of flurbiprofen

In formulations **(3)**, **(8)** and **(10)** the SFAs present could have contributed to the marker concentrations obtained in the stratum corneum, as well as the epidermis-dermis. SFAs of linear shape and low solubility have little effect on the packing of the lipids within the stratum corneum. They cannot insert themselves into the lipid layers, and consequently showed little or no effect (Chi *et al.*, 1995:270).

D.3.2.2.4 Effects of the foam formulation high in SFAs and MUFAs on the concentration of flurbiprofen

Low concentrations demonstrated by formulations **(9)** and **(10)** might have something to do with the foam formulation itself as described previously. The air bubbles inside the foam could have contributed to the low concentrations obtained in the stratum corneum. It resulted in less contact with the stratum corneum surface as well as less product present per volume when compared to the emulgel formulations inside the donor phase of the Franz diffusion cells.

D.3.2.2.5 Concentration of the lipophilic flurbiprofen found in the epidermis-dermis

Low marker concentrations obtained within the epidermis-dermis region that was observed for the majority of the formulations could have been as a result of the marker's lipophilic nature, which had a lowered solubility within the aqueous environment of the viable epidermis-dermis (Hadgraft, 1999:5). Once flurbiprofen crossed the stratum corneum, it had to partition into the underlying layers of the epidermis and dermis. Due to the fact that these tissues are more hydrophilic than the stratum corneum, they might have presented as a barrier to the lipophilic (hydrophobic) marker flurbiprofen (Smith, 1990:25). Another reason for the low concentrations within the epidermis-dermis region could have been due to unsaturated levels of flurbiprofen within the formulations (Pellet *et al.*, 1997:97). It is also believed that APIs in oils or in other lipid-soluble carriers penetrate the epidermis very slowly through the layers of the cell membranes in the stratum corneum (Martini, 1998:153). This viable layer of the epidermis-dermis is a relatively more significant barrier to the penetration of the lipophilic marker (Barry, 1983:105).

In an aqueous surrounding, flurbiprofen has to diffuse out of the hydro-formulation first before it can permeate through the skin, and partitioning of the flurbiprofen between the skin and formulation **(1)** might have been the cause of the reduction of the drug reservoir within the skin (Fang *et al.*, 2003a:316). The resistance shown to flurbiprofen within the epidermis-dermis

could thus be as a result of its lipophilicity. Therefore, absorption within the stratum corneum-epidermis possibly took place via the intercellular lipid route (Fang *et al.*, 2003a:319).

D.3.3 Franz cell skin diffusion studies

The average cumulative amount of flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) that penetrated the skin, was plotted against time to illustrate the average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) for the emulgels ((1) - (8)) in Figures D.5, D.7, D.9, D.11, D.13, D.15, D.17, D.19; and for the foams ((9) - (10)) in Figures D.21 and D.23, respectively. The cumulative amount of flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell that penetrated the skin was plotted against time for the emulgels ((1) - (8)) in Figures D.6, D.8, D.10, D.12, D.14, D.16, D.18, D.20; and for the foams ((9) - (10)) in Figures D.22 and D.24, respectively.

As in the case of the membrane diffusion studies, clear differences (see Figure D.25) amongst the flux values for the different formulation were observed, and these were quantitatively (see Section D.3.4) compared using the methods described in Section D.2.7.2. The box-plot presented in Figure D.25 was used to illustrate the differences observed between the average and median flux values for each individual formulation containing 1% flurbiprofen in the skin diffusion studies.

Summary statistics concerning the percentage flurbiprofen diffused through each formulation as well as comparative box-plots are presented in Table D.3 and Figure D.25, respectively for flux in the skin diffusion experiment.

D.3.3.1 Hydrogel (1)

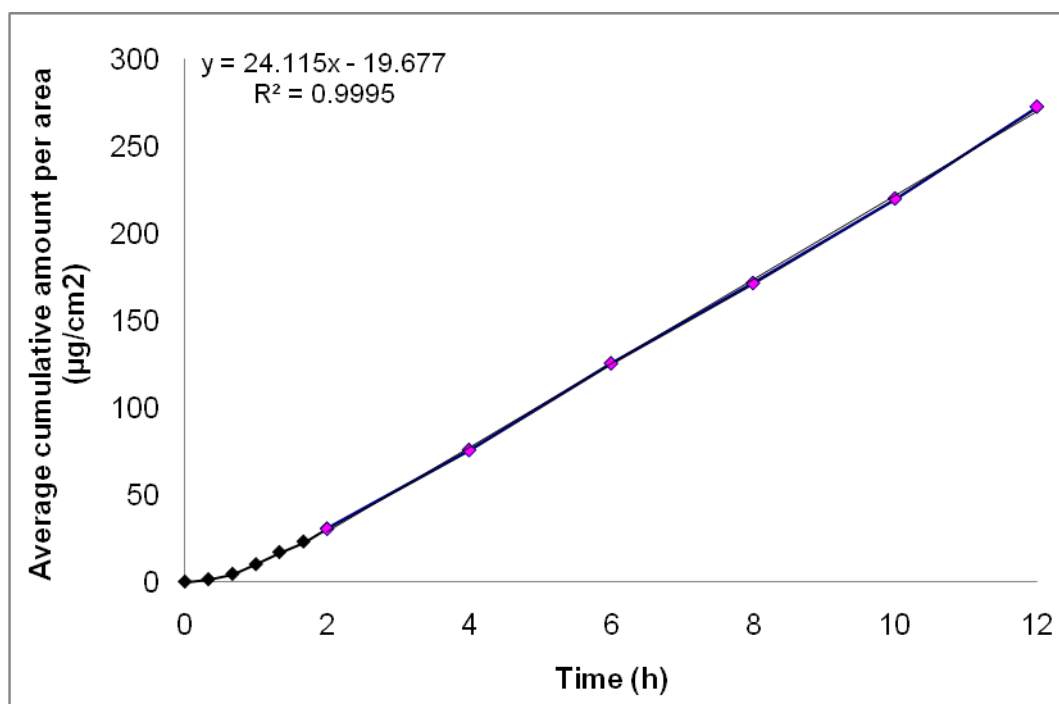


Figure D.5: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for **(1)** from 2 - 12 h.

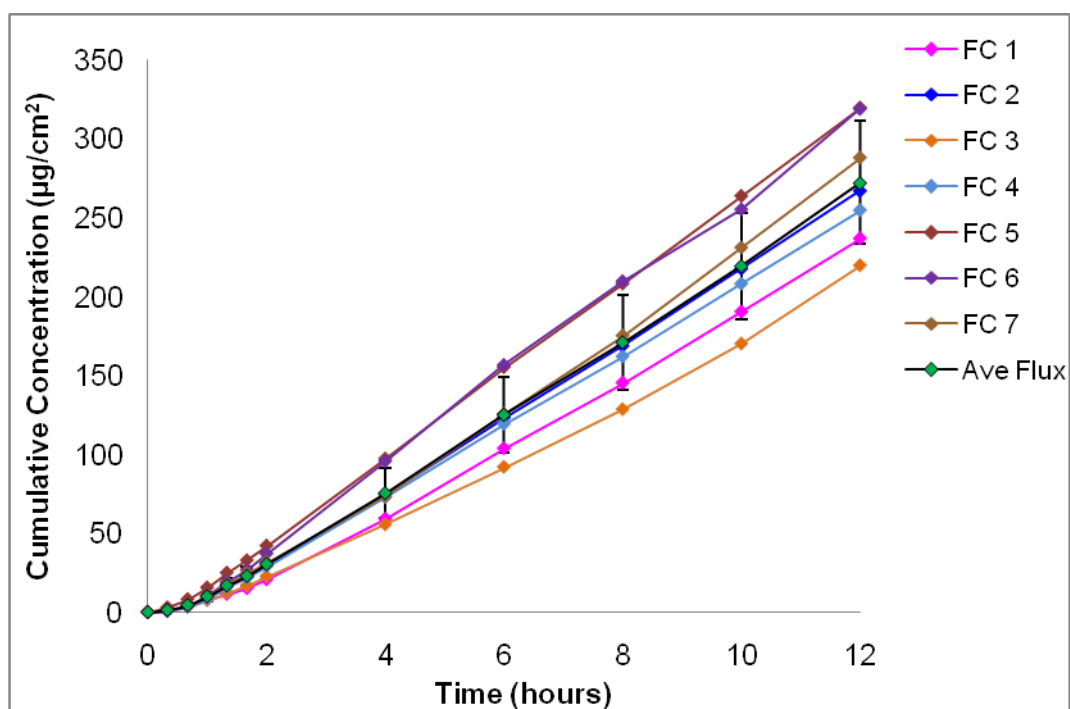


Figure D.6: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of **(1)** that penetrated through the skin as a function of time.

D.3.3.2 Liquid paraffin (2)

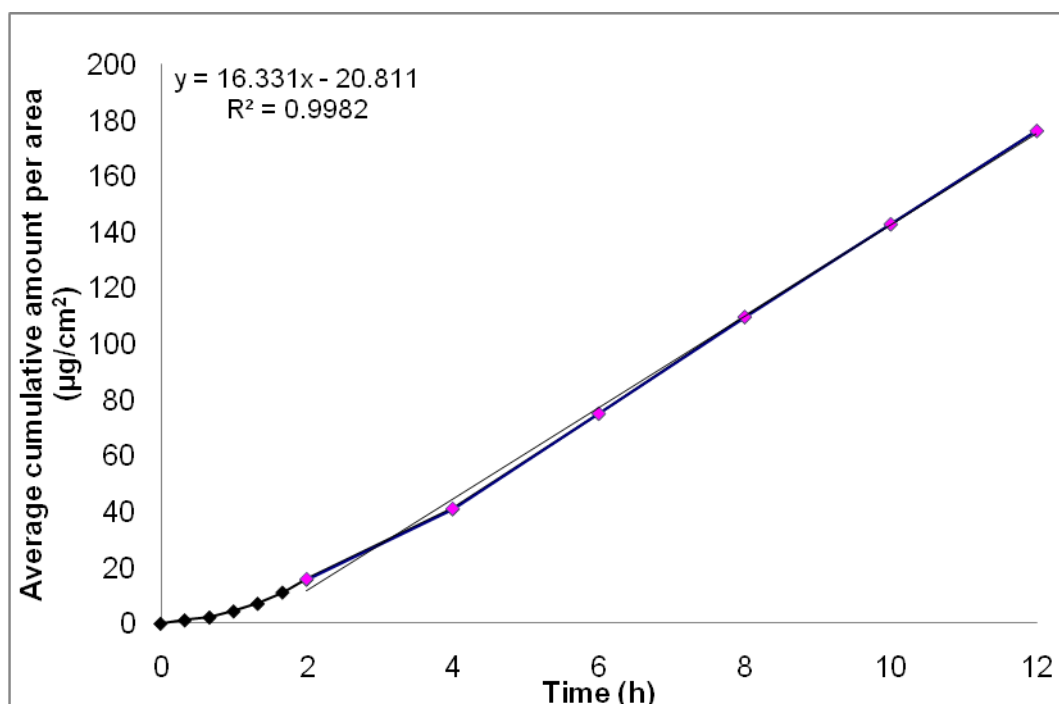


Figure D.7: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (2) from 2 - 12 h.

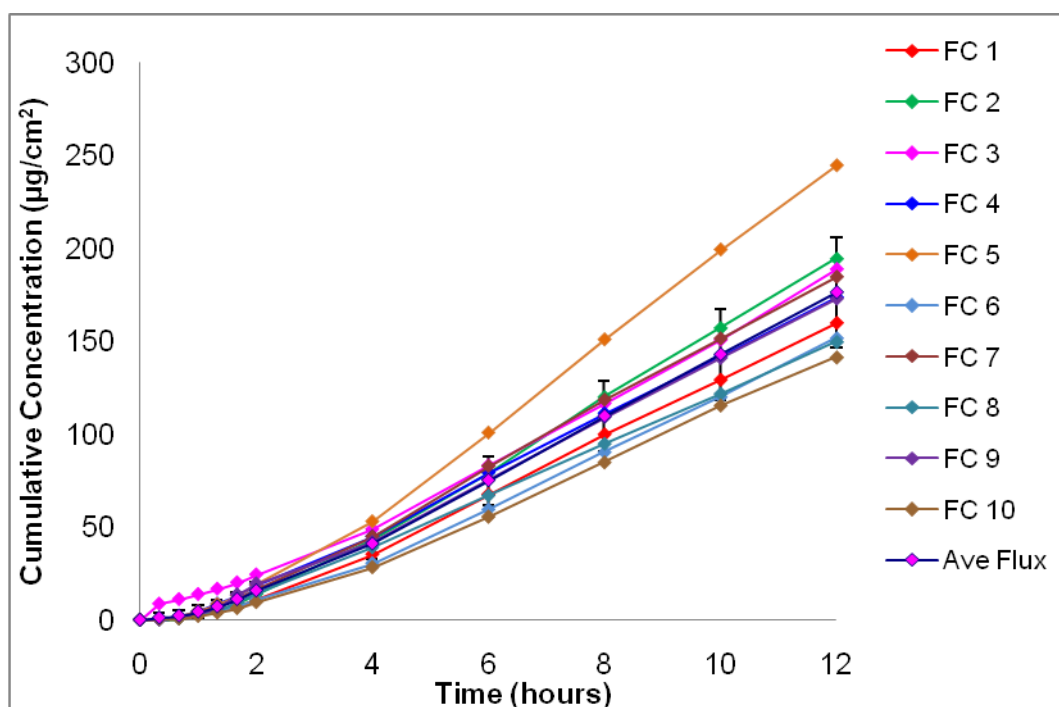


Figure D.8: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (2) that penetrated through the skin as a function of time.

D.3.3.3 Avocado oil (3)

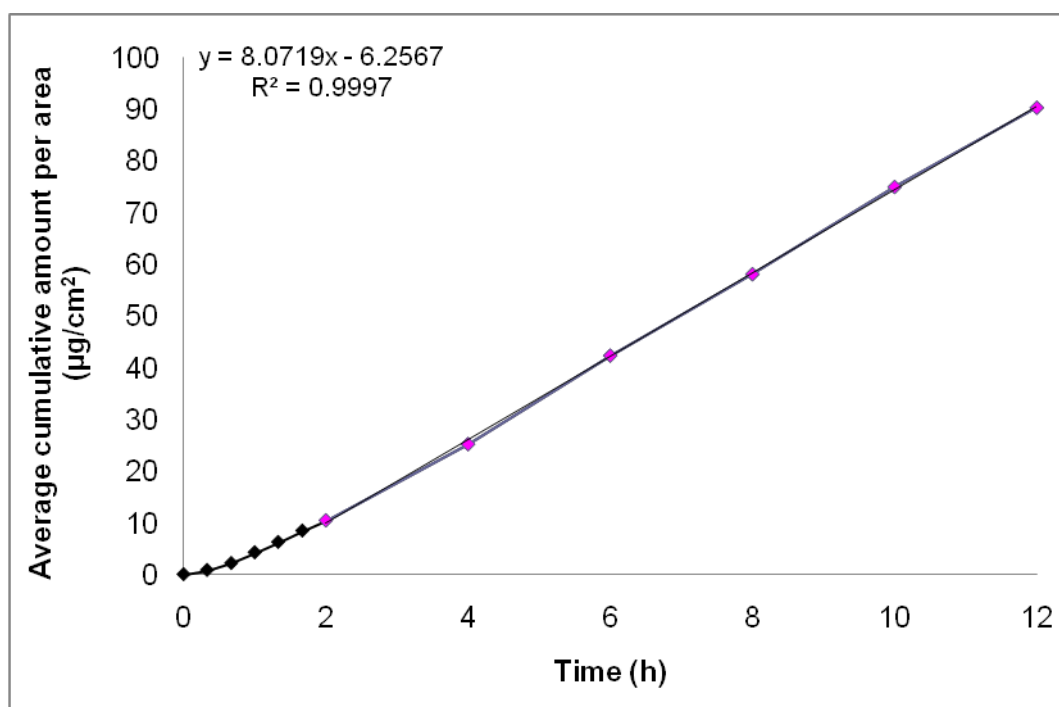


Figure D.9: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for **(3)** from 2 - 12 h.

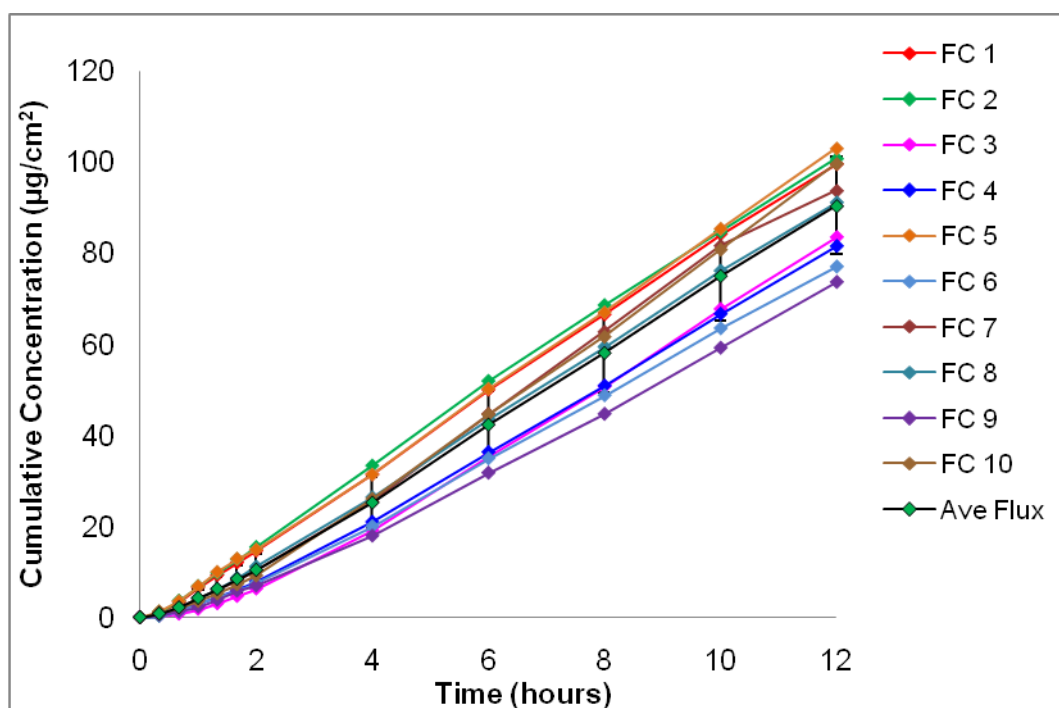


Figure D.10: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of **(3)** that penetrated through the skin as a function of time.

D.3.3.4 Grapeseed oil (4)

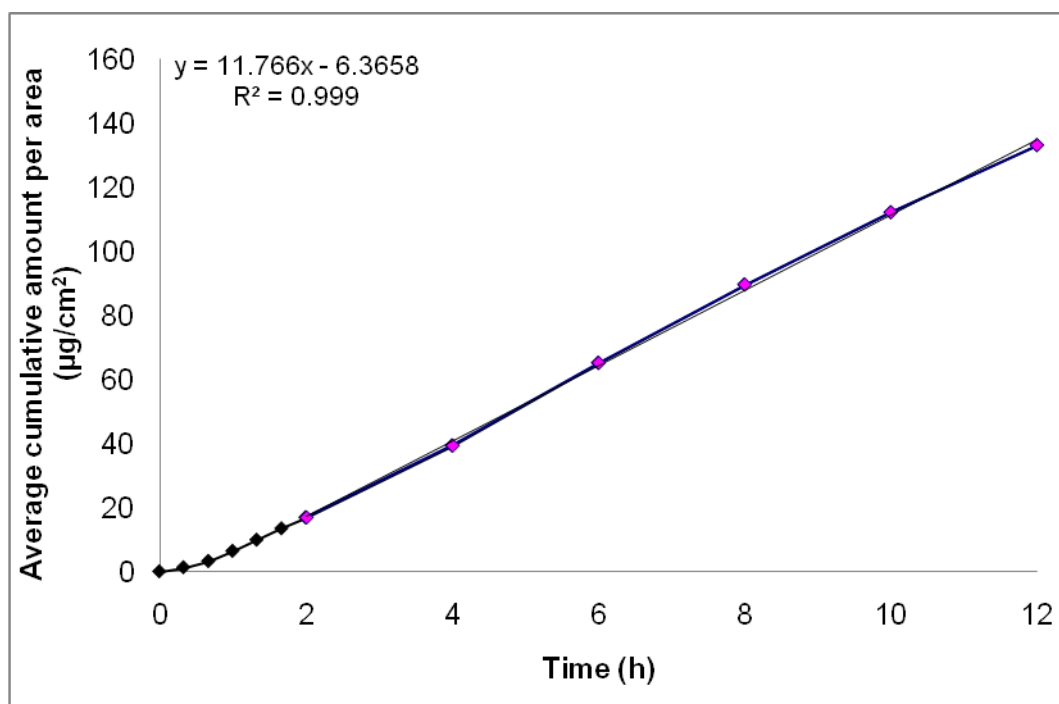


Figure D.11: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (4) from 2 - 12 h.

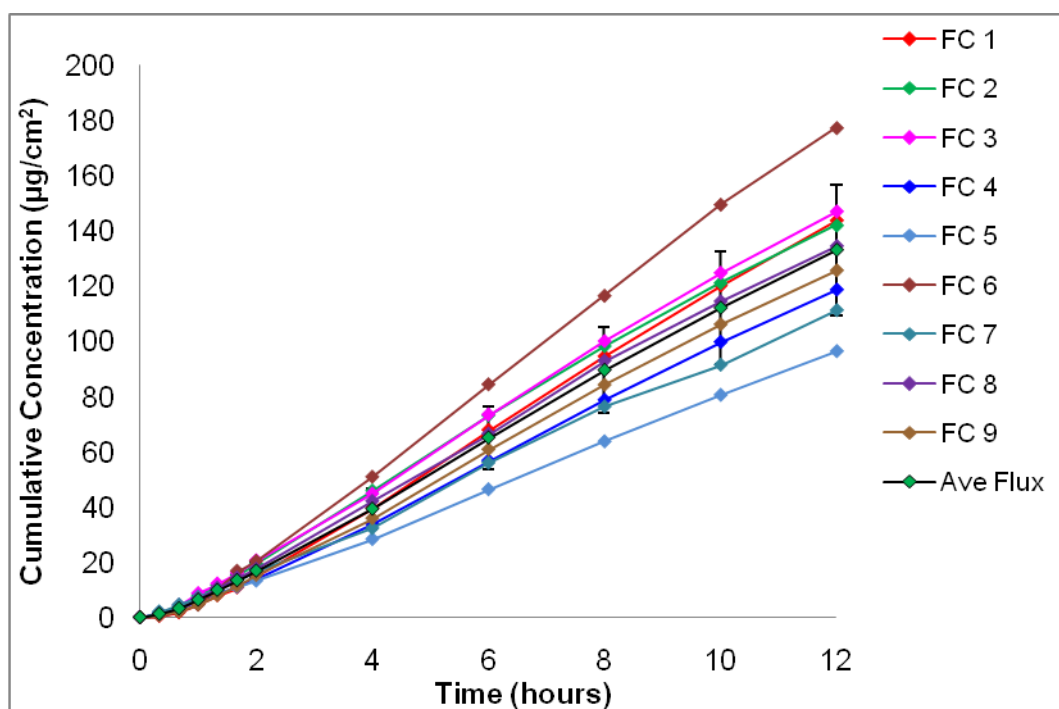


Figure D.12: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (4) that penetrated through the skin as a function of time.

D.3.3.5 Emu oil (5)

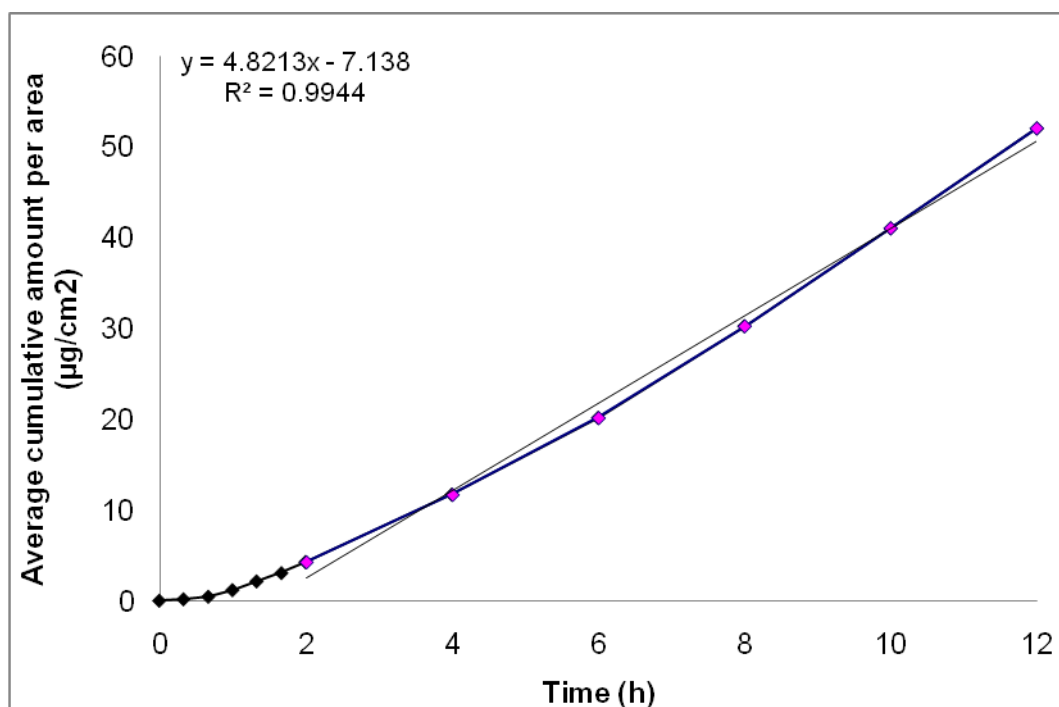


Figure D.13: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (5) from 2 - 12 h.

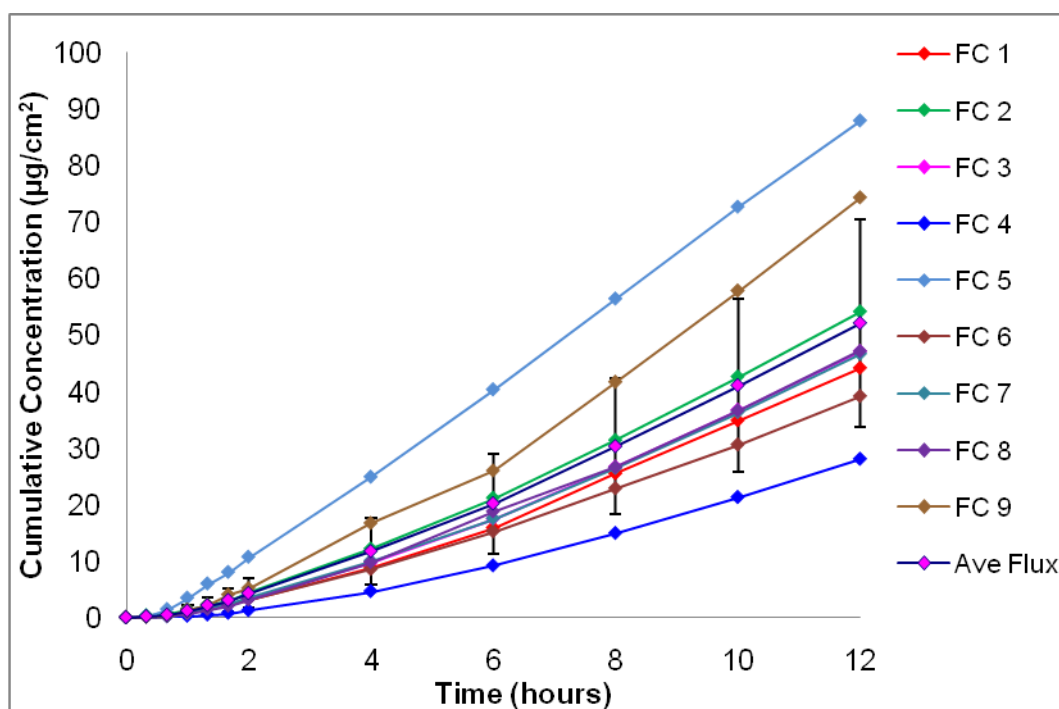


Figure D.14: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (5) that penetrated through the skin as a function of time.

D.3.3.6 Crocodile oil (6)

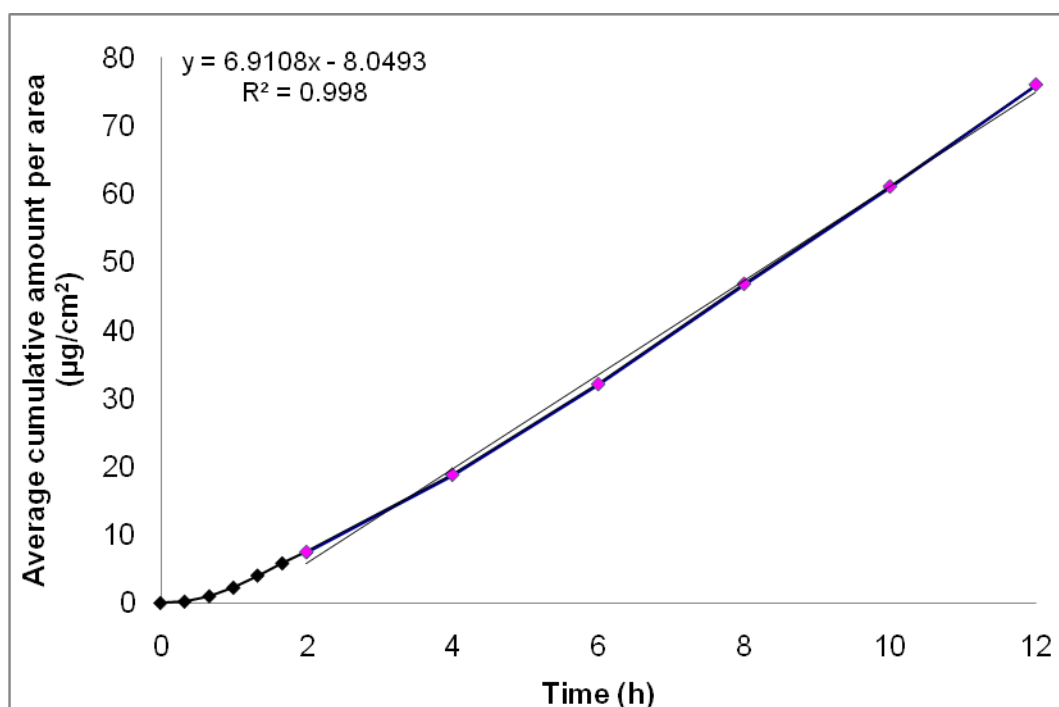


Figure D.15: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for **(6)** from 2 - 12 h.

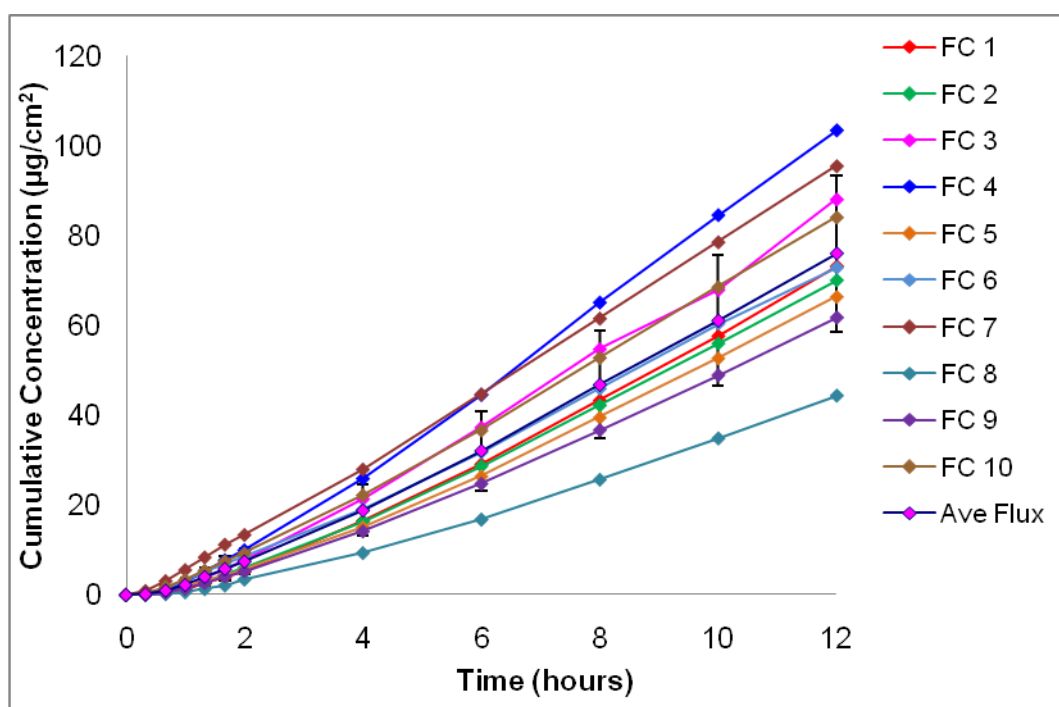


Figure D.16: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of **(6)** that penetrated through the skin as a function of time.

D.3.3.7 Olive oil (7)

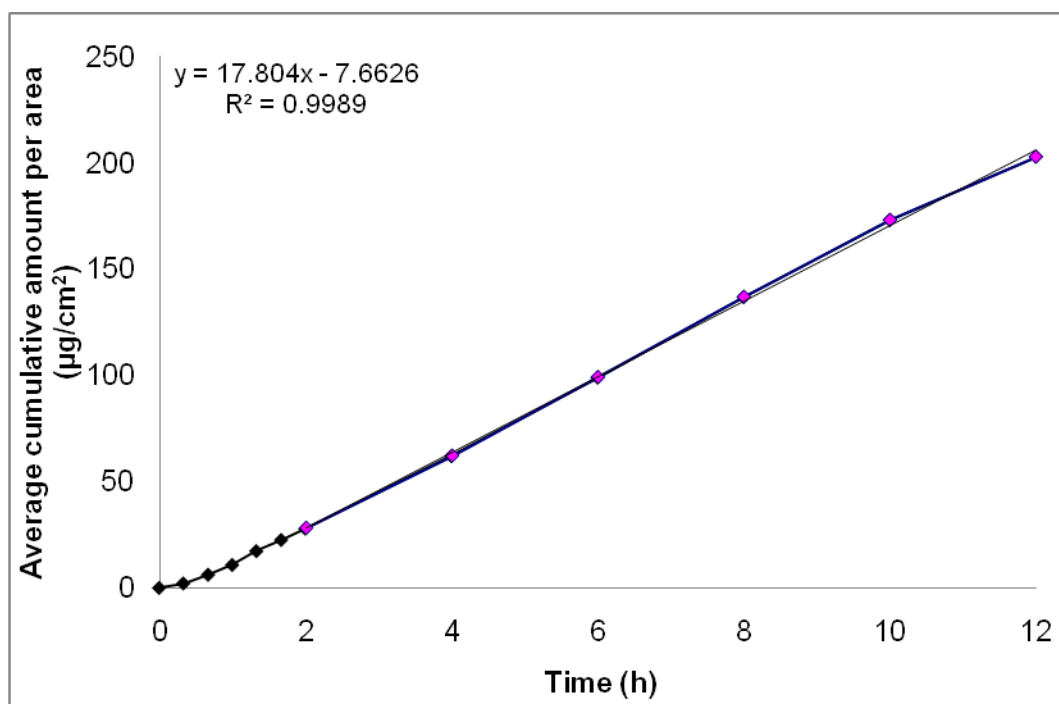


Figure D.17: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (7) from 2 - 12 h.

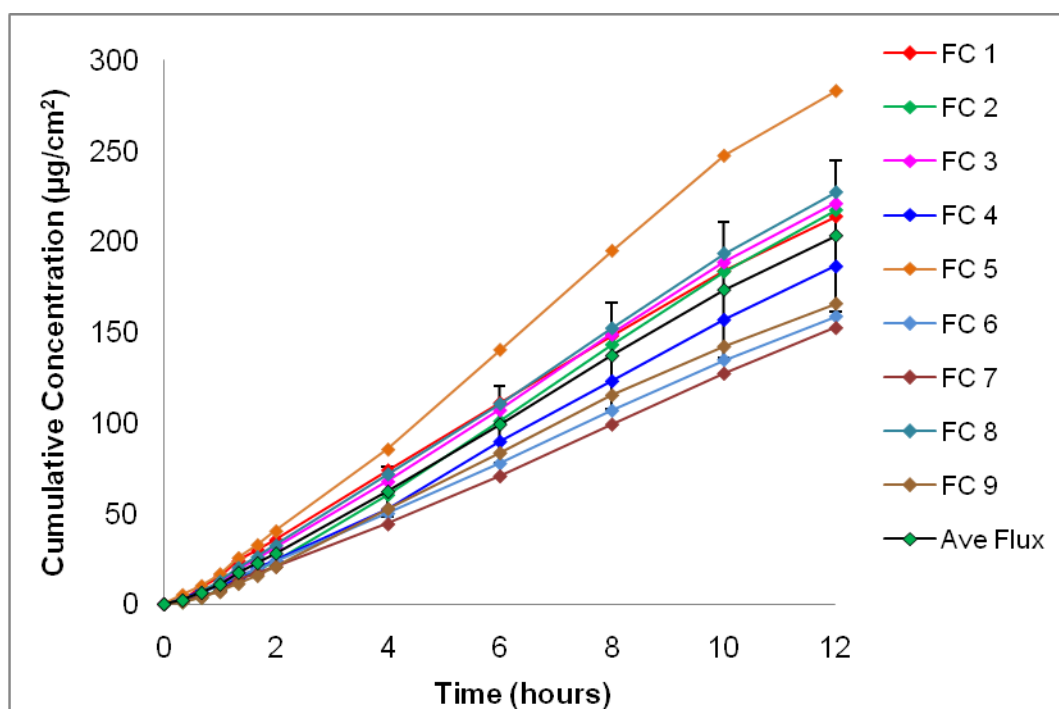


Figure D.18: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (7) that penetrated through the skin as a function of time.

D.3.3.8 Coconut oil (8)

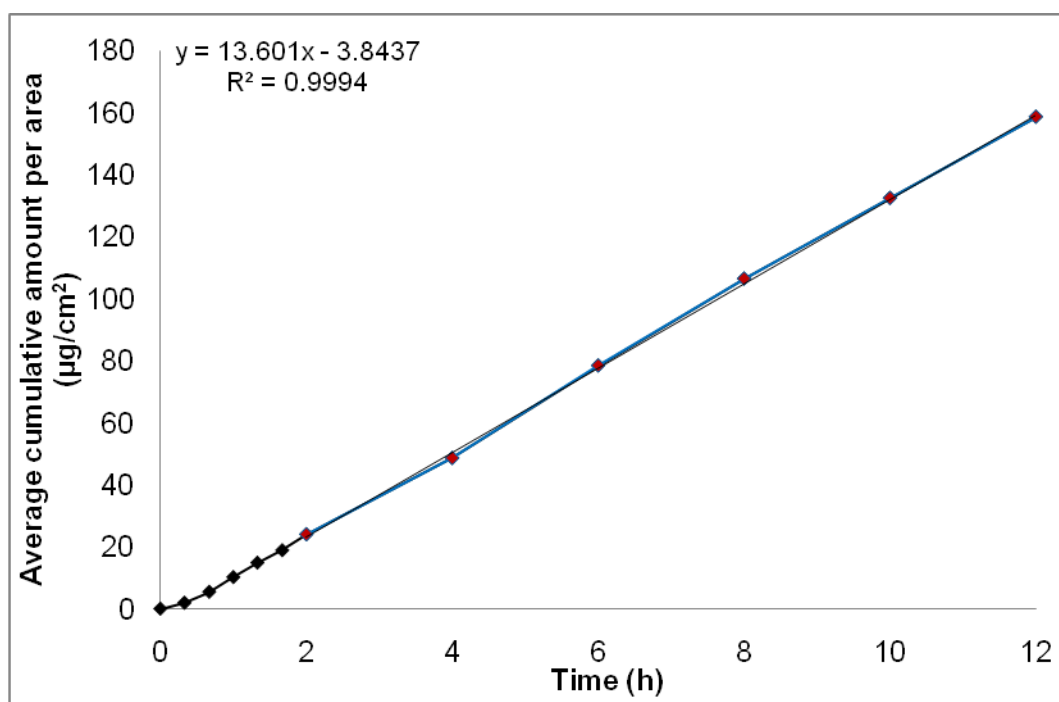


Figure D.19: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (8) from 2 - 12 h.

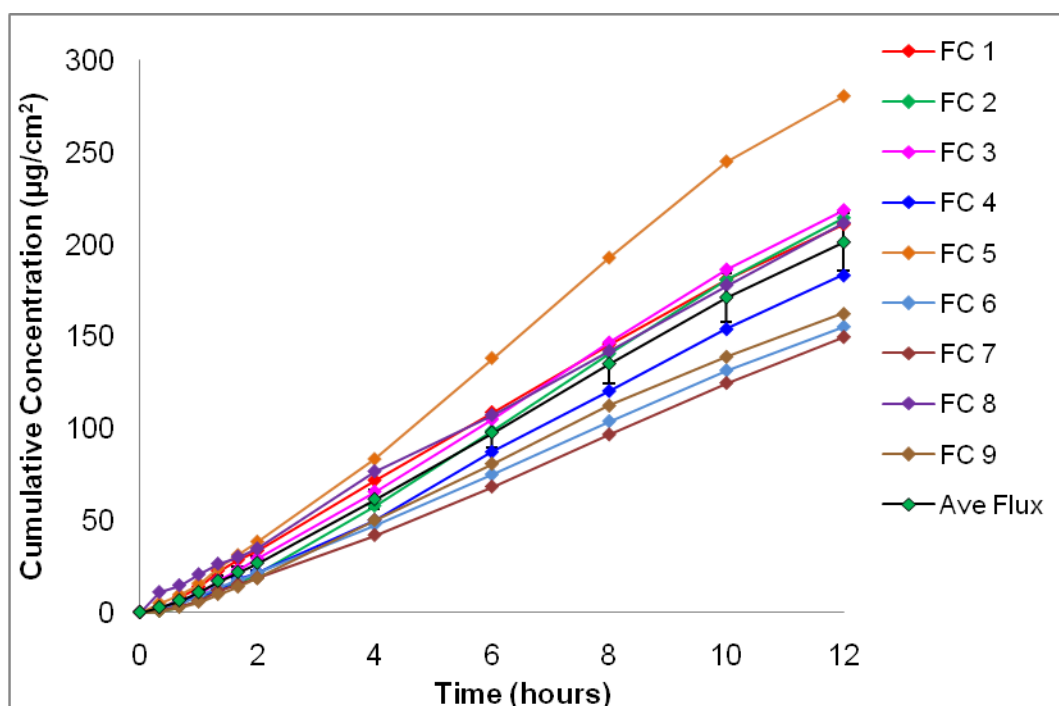


Figure D.20: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (8) that penetrated through the skin as a function of time.

D.3.3.9 Olive oil foam (9)

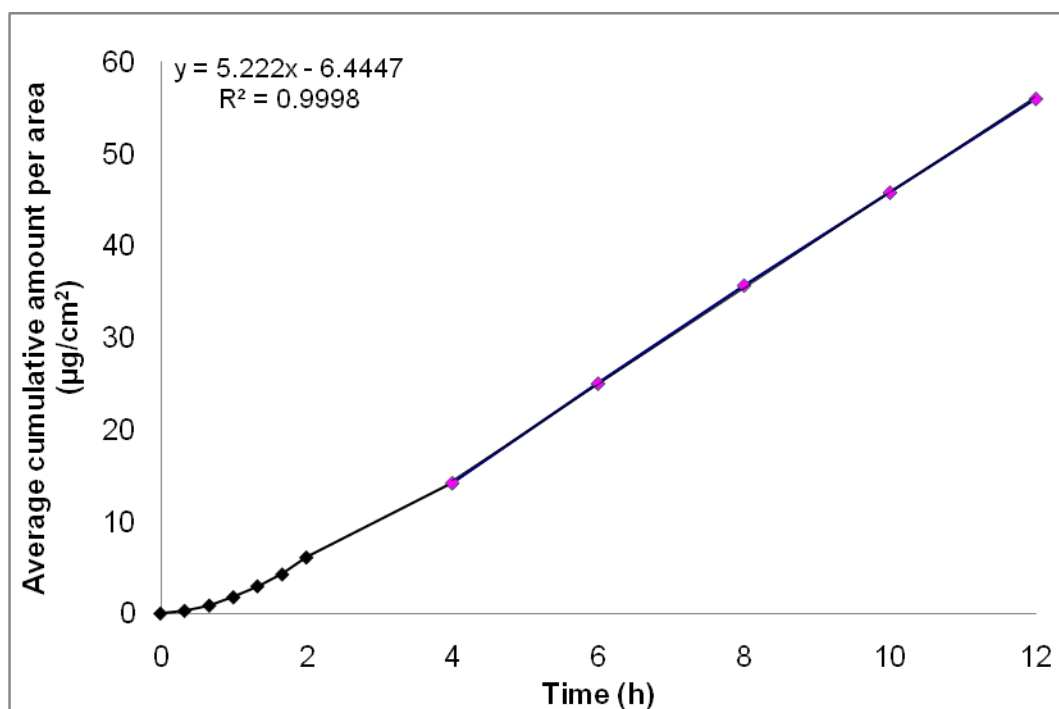


Figure D.21: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (9) from 4 - 12 h.

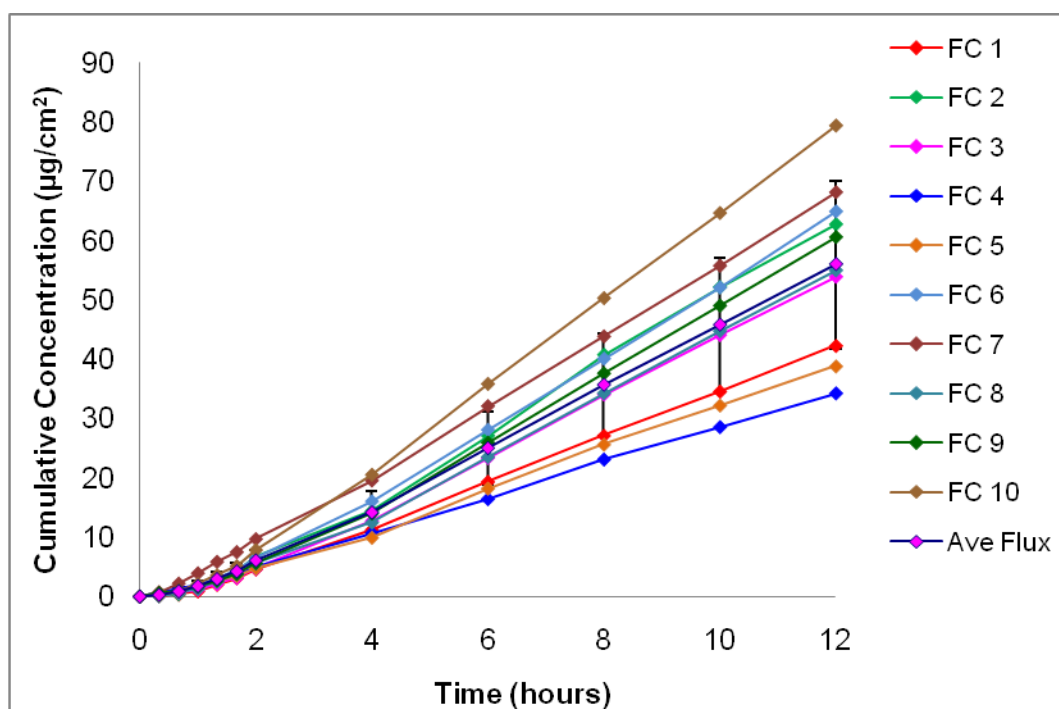


Figure D.22: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (9) that penetrated through the skin as a function of time.

D.3.3.10 Coconut oil foam (10)

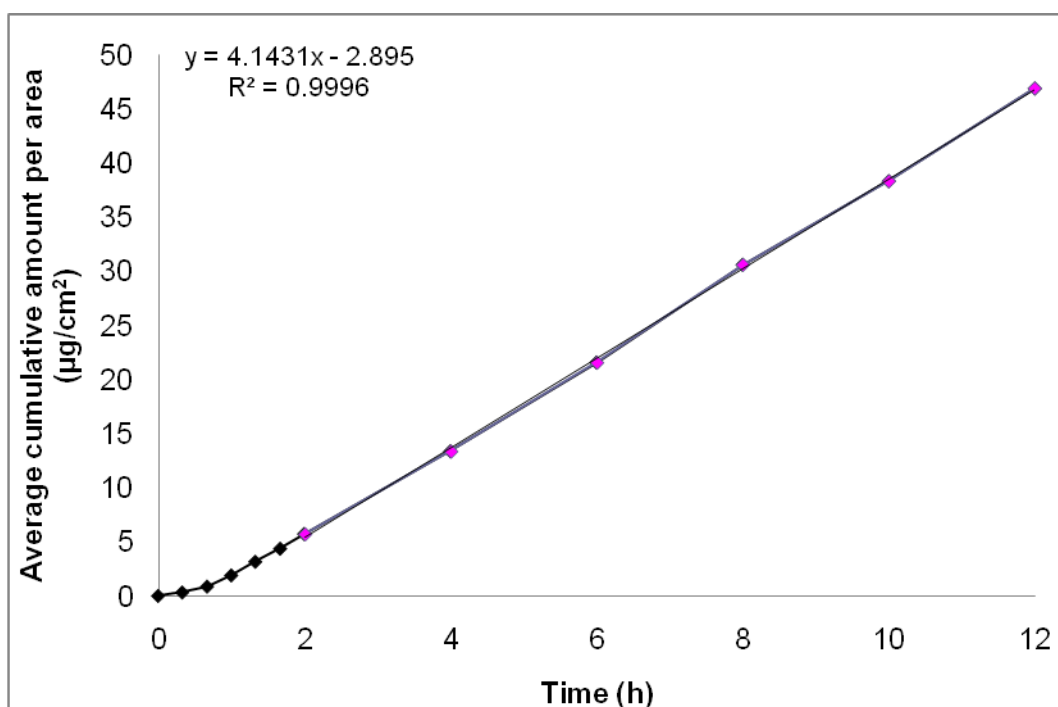


Figure D.23: Average cumulative amount ($\mu\text{g}/\text{cm}^2$) of flurbiprofen that penetrated through the skin as a function of time to illustrate the average flux for (10) from 2 - 12 h.

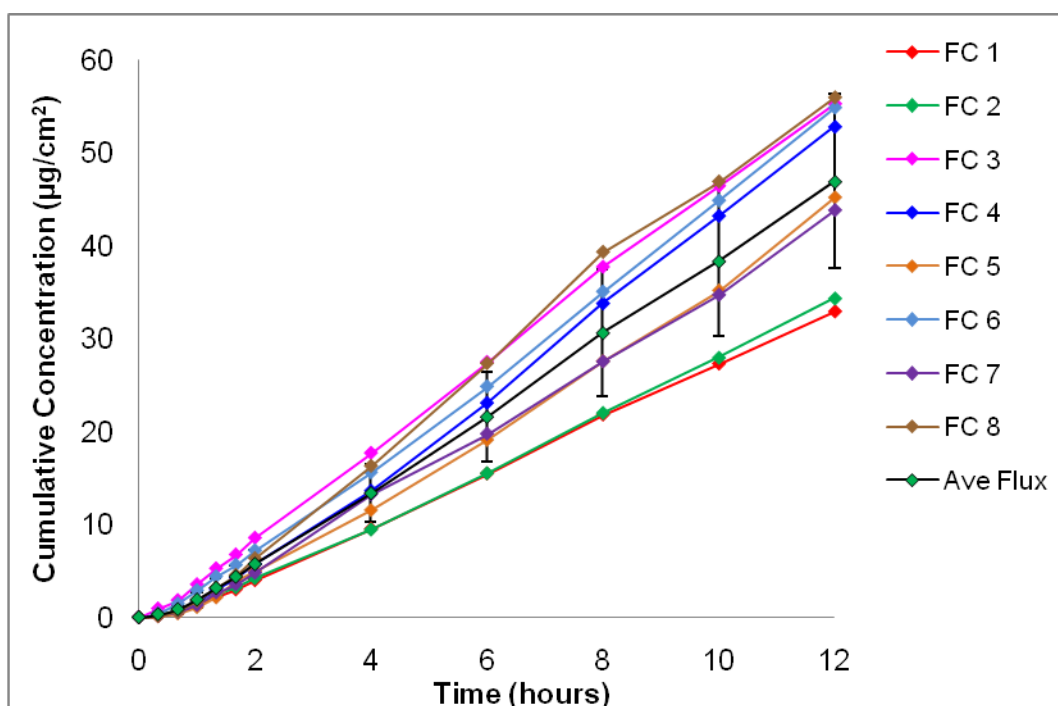


Figure D.24: Cumulative amount flurbiprofen per area ($\mu\text{g}/\text{cm}^2$) for each individual Franz cell of (10) that penetrated through the skin as a function of time.

Comparing the median flux values (Table D.3), the following was observed through the excised human skin after 12 h: Formulation **(1)** ($23.79 \mu\text{g}/\text{cm}^2\cdot\text{h}$) achieved the highest average flux value followed by formulations **(7)** ($17.99 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(2)** ($15.70 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(8)** ($13.16 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(4)** ($11.85 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(3)** ($8.31 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(6)** ($6.68 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(9)** ($5.56 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(5)** ($4.41 \mu\text{g}/\text{cm}^2\cdot\text{h}$) and lastly **(10)** ($4.36 \mu\text{g}/\text{cm}^2\cdot\text{h}$).

Table D.3 Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) and average percentage (%) of diffused flurbiprofen in different emulgels **((1) - (8))** and foams **((9) - (10))** through skin diffusion studies after 12 h. (n = number of Franz cells used)

Formulation	n	Flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)		% Diffused
		Average	Median	Average
Hydrogel emulgel (1)	7	24.12 ± 3.14	23.79	1.47 ± 0.209
Liquid paraffin emulgel (2)	10	16.33 ± 2.83	15.70	0.95 ± 0.160
Avocado oil emulgel (3)	10	8.07 ± 0.80	8.31	0.49 ± 0.575
Grapeseed oil emulgel (4)	9	11.77 ± 2.17	11.85	0.72 ± 0.127
Emu oil emulgel (5)	9	4.82 ± 1.59	4.41	0.28 ± 0.098
Crocodile oil emulgel (6)	10	6.91 ± 1.50	6.68	0.41 ± 0.093
Olive oil emulgel (7)	9	17.80 ± 3.72	17.99	1.09 ± 0.224
Coconut oil emulgel (8)	9	13.60 ± 1.30	13.16	0.85 ± 0.841
Olive oil foam (9)	10	5.22 ± 1.36	5.56	0.30 ± 0.076
Coconut oil foam (10)	8	4.14 ± 0.83	4.36	0.24 ± 0.050

The average flux values did not differ significantly from the median flux values as seen in Table D.3 and Figure D.25. This could have been due to small variations in data points as a result of minimum outliers in the data obtained. The median flux takes all the data into consideration and is therefore unaffected by distortion in the spread of the data and provides a more accurate representation of the true flux value (Gerber *et al.*, 2008:190).

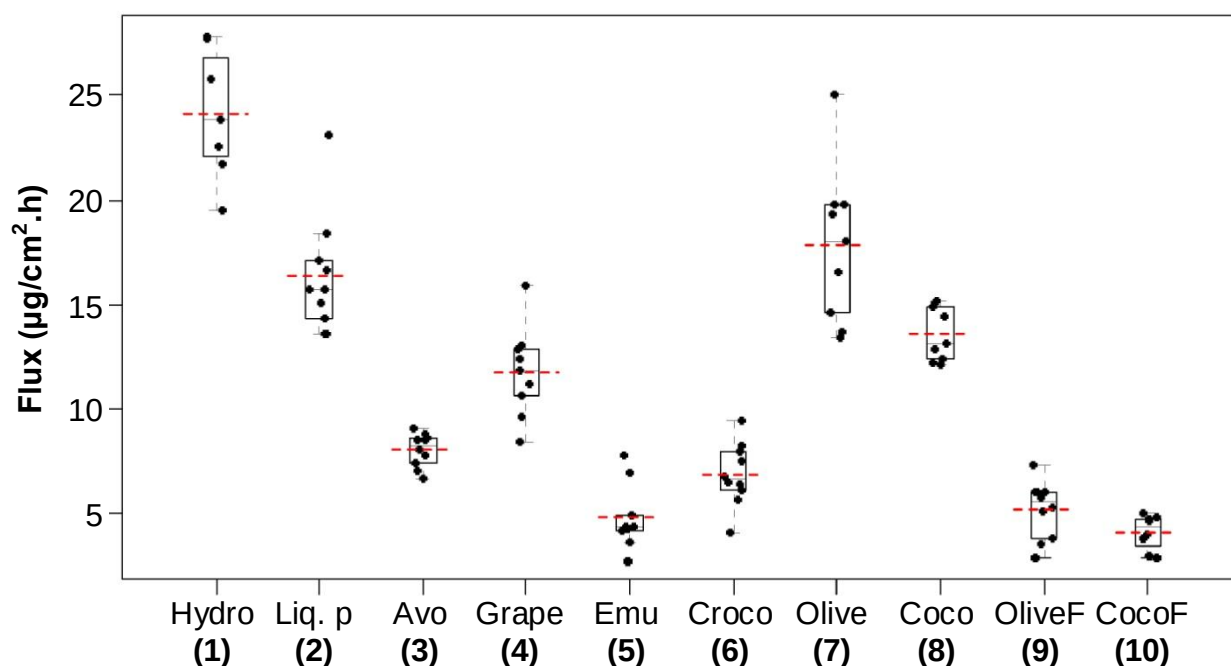


Figure D.25 Box-plot representation of the flux values of flurbiprofen for the different formulations in the skin diffusion studies. The average and median flux values are indicated by the dotted red line and solid line, respectively. The jittered points represent the actual flux values of each cell.

D.3.3.10.1 The effects of hydration on the flux of flurbiprofen

Upon studying the abovementioned box-plot, it is evident that formulations **(1)** performed on average significantly better than all of the other formulations, and this could have been contributed to its high water content. Formulation **(2)** could have caused occlusion of the skin and also caused water accumulation and swelling of the corneocytes, thus also increasing the water content of the intercellular matrix of the stratum corneum (Mitsui, 1997:124; Thomas & Finnin, 2004:699). In a study by Takahashi *et al.* (1991:155-156) it was evident that liquid paraffin contributed to the permeation of another poorly absorbed anti-inflammatory. Various hydrocarbons (i.e. liquid paraffin) have been used as vehicles with a high drug escaping effect and may be used to act as penetration enhancer, but with more of a depletion effect (Leopold & Lippold, 1995:196-197). Water therefore hydrates the skin and cause swelling of the stratum corneum, which opens up the lipid structures within. This could cause an increased flux of the lipophilic flurbiprofen into the skin (Benson, 2005:28; Ranade & Hollinger 2004:213; Williams & Barry, 2004:606).

Contrary, El Maghraby *et al.* (2008:212) stated that an over-hydrated skin could cause too much swelling of the corneocytes and in turn minimise the shunts and negatively affect permeation.

D.3.3.10.2 The general effect of the UFAs on the flux of flurbiprofen

The addition of double bonds (linolenic > linoleic > oleic) within these UFAs are thought to increase the flux of flurbiprofen through the intercellular route; but more so for oleic acid (C18:1), resulting in an increased TEWL by decreasing the barrier function of the stratum corneum. Once these acids are packed within the stratum corneum, they cause a wider channel within and increase the flux of the marker (Fang *et al.*, 2003b:156; Katsuta *et al.*, 2005:1010; Tanojo *et al.*, 1999:102). It is believed, though, that the more double bonds these UFAs consist of, the more difficult it becomes for them to insert themselves within the lipid structure of the skin (Chi *et al.*, 1995:269-270). Formulation **(4)** contained an average amount of oleic- (C18:1) and very high amounts of linoleic acid (C18:2). It also consisted of a small amount of linolenic acid (C18:3), and demonstrated a rather average flux for the marker. Oleic acid (C18:1) present within formulation **(4)** has been observed to be a better enhancer than lauric acid (C12:0). This enhancing ability of the UFA might be due to the *cis* double bond at C₉ in the oleic acid chain. It is believed that the “kink” within the alkyl chain disrupts the well-organised, primarily saturated straight chain skin lipids more so than lauric acid (C12:0) (SFA) (Green *et al.*, 1988:109). Therefore UFAs might increase the flux of flurbiprofen to a greater extent than SFAs with the same chain length (Aungst, 1989:246). UFAs in formulation **(4)** might have caused wider channels within the skin for permeation, due to the packing of the marker within the skin (Chi *et al.*, 1995:270).

D.3.3.10.3 The effects of MUFAs on the flux of flurbiprofen

It is clear that the high oleic acid (MUFA) concentrations present in formulation **(7)** presented with a higher flux when compared to the other oil and foam formulations. Oleic acid modulates the extra-cellular lipid area of the stratum corneum and could therefore enhance the flux of flurbiprofen (Naik *et al.*, 1995:300). This modulation of the lipid domain occurs by fluidisation of the lipid structures within the stratum corneum (Ghafourian *et al.*, 2004:122). Fluidisation might explain the oleic acid induced boost of flurbiprofen within the stratum corneum (Green *et al.*, 1988:103). Oleic acid has a bulky “kink” conformation that disorganise the intercellular lipid layers of the stratum corneum, and may have been the reason for the decreased barrier function. Interesting to know is that UFAs affect the activity of enzymes that regulate the desquamation of the stratum corneum, and as a result causes deterioration of the barrier function around sebaceous glands (Katsuta *et al.*, 2005:1011).

D.3.3.10.4 The effects of PUFAs on the flux of flurbiprofen

Formulation **(3)** contained a significant amount of the PUFA, linolenic acid (C18:3). Linolenic acid (C18:3) consists of three double bonds. It has been noted in literature that an increase in the double bonds causes difficulty for the acid to insert itself within the lipid matrix of the stratum

corneum (Chi *et al.*, 1995:270). The presence of additional double bonds in formulations **(4)**, **(5)** and **(6)** with high linoleic- (C18:2) and lowered linolenic acids (C18:3) decreased the flux of flurbiprofen to some extent, and therefore it is clear that the number of double bonds did not significantly affect the enhancing capacity of these fatty acids when incorporated into the formulations (Santoyo & Ygartua, 2000:247).

D.3.3.10.5 The effects of medium chain SFAs on the flux of flurbiprofen

It is suggested that UFAs are more effective in absorbing lipophilic drugs than SFAs (Sinha & Kaur, 2000:1135). This was interesting, since formulation **(8)** mostly contained SFAs (of which lauric acid (C12:0) was present in the highest concentration) and depicted a high flux value in comparison with other formulations. Previous research suggested that by adding lauric acid (C12:0) to a mixture it could increase the partition coefficient and diffusivity of an API through the skin (Green *et al.*, 1988:109-110). A possible mechanism of action for lauric acid (C12:0) could have been as a result of a complex formed between flurbiprofen and the fatty acid. The dissociation of this complex into each component at the interface between the stratum corneum and viable epidermis could be the reason for its enhancement of the flux of the marker (Santoyo & Ygartua, 2000:247). Medium chain capric- (C10:0), lauric- (C12:0) and myristic acids (C14:0) were also present within formulation **(8)** and could have increased skin diffusivity for the marker flurbiprofen. Another contributing factor to an increased flux of flurbiprofen obtained with formulation **(8)** could have been due to the shorter chained fatty acids, which might have resulted in a more significant interaction with the skin lipids. Effects of lauric- (C12:0) and myristic acids (C14:0) may have been due to an optimal balance of the partition coefficient and its affinity to the skin in the hydrophobic part of the fatty acid. Hence, this may have been an indication that medium chain fatty acids were more effective in enhancing the permeation of flurbiprofen through the skin (Ogiso & Shintani, 1990:1067).

D.3.3.10.6 Effects of longer chain SFAs on the flux of flurbiprofen

The lowered flux value obtained with formulation **(3)** containing high amounts of oleic acid, could have been as a result of the SFA, palmitic acid's (C16:0) chain length. It is believed that an increase in chain length above C₁₄ does not always result in an enhancing effect (Green *et al.*, 1988:109; Ogiso & Shintani, 1990:1067; Sinha & Kaur, 2000:1135). Although SFAs showed some enhancing activity in this study, Chi *et al.* (1995:270) reported no significant increase for flurbiprofen with SFAs of C₁₆ - C₂₀ in their study.

Interesting to note was the low flux values obtained with formulations **(5)** and **(6)** of animal origin. It was expected that these formulations would have performed better, due to their almost similar fatty acid content compared to human fat (see Table 2.4). Hyaluronic acid, which is a natural constituent in human skin, has been used as a penetration enhancer (Büyüktimkin *et al.*,

1997:373, 382, 434; Strayer *et al.*, 2006:4). However, palmitic- (C16:0) and stearic acids (C18:0) present, have higher melting points and might not have dissolved properly at 37 °C at which these experiments were conducted (Aungst, 1989:245; Ophardt, 2003). The melting points of these SFAs ranged between 62.9 - 69.6°C and are much higher than that of the UFAs, which ranges from -50 to -5 °C. As a result it is likely that linear SFAs with low solubility will also have less potential to disrupt the ordered lipid packing of the stratum corneum to insert themselves within the lipids layers (Chi *et al.*, 1995:270; O'Brien, 2009:266). The stearic acid concentration present within formulation **(5)** was the highest in this formulation of all the oils tested. The low flux of flurbiprofen within the skin could have been as a result of stearic acid's (C18:0) high melting point, and possibly due to the fact that it was a semisolid at the time the skin studies were conducted (Aungst, 1989:245).

It is suggested that the branching of the carbon chain did not increase the disruptive effect of the stratum corneum more significantly than the unbranched chained fatty acids shorter than C₁₈. This indicated that due to stearic acid (C18:0) present in the stratum corneum lipids, it would probably not disrupt the packing of the ordered lipids. However, the branched versions of the same chain length could upset this packing and increase skin penetration (Aungst, 1989:247).

D.3.3.10.7 Effects of the foam formulations high in SFAs and MUFAs on the flux of flurbiprofen

Formulations **(7)** and **(8)** performed better on average, and were therefore formulated into foams **(9)** and **(10)** and compared to the non-foam equivalents. Both foams showed lower flux values compared to their emulgel counterparts **(7)** and **(8)**. This could have been attributed to the air bubbles in the foam causing less contact with the stratum corneum surface, as well as less product present within the donor phase of the Franz diffusion cells, resulting in less flurbiprofen per volume foam when compared to their emulgel counterparts as discussed previously. Furthermore, formulation **(9)** contained more of the MUFA, oleic acid, than formulation **(8)** and this could have been the reason for it showing a slightly higher flux value for flurbiprofen than formulation **(8)**. It is therefore believed that UFAs generally show an increase in the partition coefficient of flurbiprofen (Chi *et al.*, 1995:270). Oleic acid (C18:1) within formulation **(9)** seemed to possess increased enhancing properties compared to lauric acid present within formulation **(10)** (Green *et al.*, 1988:109).

D.3.4 Inferential statistical data analysis

D.3.4.1 Membrane diffusion study

Statistical comparison of the flux values using one-way ANOVA showed that significant differences ($p\text{-value} \approx 0$) existed between the formulations. To investigate which groups differed, Tukey post-hoc tests were performed. Note that there were eight formulations and therefore twenty-eight different comparisons were performed simultaneously. It is also important to note that the results from the Tukey post-hoc comparison revealed a larger number of significant differences than the Dunn's non-parametric multiple comparison tests. For this reason the more conservative Dunn's test results for pharmaceutical interpretation is preferred.

A non-parametric Kruskal-Wallis test was performed next in order to investigate differences amongst the group medians. This test revealed a $p\text{-value}$ of 0.0000, which indicated significant differences amongst the group medians. The Dunn's multiple comparison test results are summarised in an upper matrix form in Table D.4. Since no statistically significant differences ($p > 0.05$) were observed between these formulations, the $p\text{-values}$, coloured in red, indicated significant differences.

Table D.4: Dunn's multiple group comparisons for the membrane diffusion studies (significant differences indicated in red)

(2)	(3)	(4)	(5)	(6)	(7)	(8)	
0.010	1.000	1.000	0.006	1.000	1.000	1.000	(1)
	0.000	0.000	1.000	0.433	1.000	0.235	(2)
		1.000	0.000	0.488	0.075	0.607	(3)
			0.000	0.022	0.002	0.026	(4)
				0.247	1.000	0.134	(5)
					1.000	1.000	(6)
						1.000	(7)

All the red values in Table D.4 have a $p\text{-value}$ smaller than 0.05, which indicated statistically significant differences when two formulations were compared. The aforementioned can be explained as follows: formulation (4) differed significantly from all the formulations except from formulation (1) (1.000) and (3) (1.000). This in turn meant that if (4) was compared to formulations (1) or (3) there would be no statistically significant differences between the two formulations ((4) and (1) or (4) and (3)) due to a $p\text{-value}$ higher than 0.05.

D.3.4.2 Tape stripping

For this experiment the interaction effect between place of measurement (i.e. stratum corneum-epidermis and epidermis-dermis) and formulation were first investigated with a two-way analysis of variance. The p-value was found to be 0.000, which suggested that the interaction was indeed significant. A one-way ANOVA and the Kruskal-Wallis test, depending on the place of measurement were performed. Both the Tukey- and Kruskal-Wallis-tests revealed significant differences between the formulations for the epidermis-dermis experiment. The same results were obtained using only the stratum corneum data. The p-value matrix containing the preferred Dunn's post-hoc tests are provided in Table D.5, for the stratum corneum-epidermis test, and Table D.6 contain the results obtained for the epidermis-dermis tests.

D.3.4.2.1 Stratum corneum-epidermis

Table D.5: Dunn's multiple group comparisons for the stratum corneum-epidermis (significant differences indicated in red)

(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	
1.000	0.858	1.000	0.024	1.000	1.000	1.000	1.000	0.109	(1)
	0.000	1.000	0.000	0.170	1.000	0.021	1.000	0.000	(2)
		0.103	1.000	1.000	0.116	1.000	0.000	1.000	(3)
			0.001	1.000	1.000	0.008	1.000	0.008	(4)
				0.121	0.001	1.000	0.000	1.000	(5)
					1.000	1.000	1.000	0.496	(6)
						1.000	1.000	0.010	(7)
							0.021	1.000	(8)
								0.000	(9)

Of the possible forty-five comparisons, the Tukey-test revealed thirty significant differences amongst the formulations, whilst the Dunn's test revealed only fourteen, with no contradiction to the Tukey test, except removal of significant findings. In Table D.5 it is interesting to see that formulation (6) indicated no statistically significant differences with all of the other formulations, whereas formulation (1) indicated statistically significant differences when compared to formulation (5) ($p = 0.024$), due to a p-value below 0.05 (red).

D.3.4.2.2 Epidermis-dermis

Of the possible forty-five comparisons, the Tukey-test revealed twenty-two significant differences amongst the formulations, whilst the Dunn's test revealed only twelve. The Dunn's test did not reveal any contradictory differences when compared to the Tukey-test (Table D.6), but only revealed less significant differences. The results obtained from the upper matrix form

in the following table indicated no statistically significant differences for formulation **(8)** when compared to any of the formulations as the p-values (black) were higher than 0.05. Comparing formulation **(7)** to the other formulations, it seemed that it had statistically significant differences to formulation **(5)** ($p = 0.005$) with a p-value (red) of less than 0.05.

Table D.6: Dunn's multiple group comparisons for the epidermis-dermis (significant differences indicated in red)

(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	
1.000	1.000	1.000	1.000	0.003	0.980	1.000	0.025	0.001	(1)
	1.000	1.000	1.000	0.004	1.000	1.000	0.040	0.001	(2)
		1.000	0.490	0.047	1.000	1.000	0.336	0.010	(3)
			0.053	0.715	1.000	1.000	1.000	0.187	(4)
				0.000	0.005	0.133	0.000	0.000	(5)
					1.000	0.325	1.000	1.000	(6)
						1.000	1.000	1.000	(7)
							1.000	0.080	(8)
								1.000	(9)

D.3.4.3 Franz cell skin diffusion studies

One-way ANOVA revealed a p-value of 0.000 for the test of significant group differences. A p-value of 0.000 was also observed using the non-parametric Kruskal-Wallis test. Hence, both tests confirmed that there were significant differences amongst the formulations when measuring the flux in the skin diffusion study. In this experiment ten different formulations were compared, which resulted in forty-five different multiple group comparisons, therefore, the Dunn's (following Kruskal-Wallis) post-hoc test was preferred (Table D.7).

Table D.7: Dunn's multiple group comparisons for the skin diffusion studies (significant differences indicated in red)

(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	
1.0000	0.015	0.674	0.000	0.001	1.000	1.000	0.000	0.000	(1)
	0.289	1.000	0.000	0.022	1.000	1.000	0.000	0.000	(2)
		1.000	1.000	1.000	0.166	1.000	1.000	0.933	(3)
			0.094	1.000	1.000	1.000	0.158	0.034	(4)
				1.000	0.000	0.011	1.000	1.000	(5)
					0.012	0.530	1.000	1.000	(6)
						1.000	0.000	0.000	(7)
							0.019	0.004	(8)
								1.000	(9)

As in the case of the membrane flux experiment, we observe that the Tukey post-hoc test revealed a larger number of significant differences (thirty-four out of the forty-five comparisons) when compared to the Dunn's post-hoc test (twenty-two out of the forty-five comparisons). It should be noted that the Dunn's test, reported less significant differences without contradicting the Tukey results. Hence, the more conservative Dunn's test for pharmaceutical interpretation is preferred. In the aforementioned table, formulation **(3)** did not show any statistically significant differences to most of the formulations, it did however indicate statistically significant differences with **(1)** ($p = 0.015$), which had a p-value less than 0.05. When comparing formulations **(4)** with **(10)** ($p = 0.034$) statistically significant differences were observed (red value), p-values greater than 0.05 (indicated in black) yielded no significant differences between formulation **(4)** and the rest of the formulations.

D.4 PREVIOUS STUDIES CONDUCTED ON FLURBIPROFEN UTILISING FATTY ACIDS IN TRANSDERMAL ABSORPTION

In a study by Chi *et al.* (1995:269), UFAs were shown to be potent penetration enhancers for flurbiprofen, and by making use of these acids, the permeation rate of flurbiprofen increased (5.8- to 17.5 fold) through rat skin. While in the same study, no significant increase was observed with their saturated counterparts. The same general trend was observed by Sinha and Kaur (2000:1135), during which a large number of fatty acids were used as penetration enhancers. It also found that UFAs were more effective in enhancing transdermal absorption of an API, than the SFA. This is interesting since saturated, medium chain fatty acids (capric-, lauric- and myristic-), as well as UFAs (oleic-, linoleic- and linolenic-) are reported to be effective penetration enhancers by Babu *et al.* (2006:153). In a study by Chi *et al.* (1995:270), the UFAs showed a large increase in the partition coefficient of flurbiprofen, while with the SFAs, only lauric acid showed penetration-enhancing effects. The penetration effect of the UFAs decreased in order of linolenic acid (C18:3) showing the most potent enhancing effect, followed by oleic- (C18:1), palmitoleic- (C16:1), linoleic- (C18:2) and arachidonic acid (C20:4) (Chi *et al.*, 1995:270).

Also, a study done by Chi *et al.* (1995:270), examined the effect of SFAs on the skin permeation and they incorporated 1% flurbiprofen in a propylene glycol mixture with capric- (C10:0), lauric- (C12:0) and myristic acid (C14:0) in a formulation. It indicated an increase in the permeation rate of flurbiprofen by UFAs (C₁₆ to C₂₀), while no significant increase was detected with their SFA counterparts. This may be attributed to the difference in their lipid solubility and chemical structure differences, plus SFAs have lower solubility, due to their higher melting points (Kanikkannan *et al.*, 2006:19).

Fang *et al.* (2003b:156, 158) tested the UFA, oleic- (C18:1), linoleic- (C18:2) and linolenic acids (C18:3) on the penetration enhancement effect of their marker flurbiprofen. These acids possess the same alkyl chain length but one, two and three double bonds, respectively. The fatty acids increased the drug reservoir within the skin, and increased the flux of flurbiprofen. Linolenic acid (C18:3) (PUFA) increased the flux more so than linoleic acid (C18:2) (PUFA). It detected that the degree of disruption to the stratum corneum (TEWL) did not correlate well with the efficacy of the enhancer, and a relationship between skin disruption and enhancement efficiency can therefore not be assumed.

Fang *et al.* (2003a:313) utilised membranes and human excised skin to determine the influence of chemical penetration enhancers i.e. UFAs on the skin's permeation of flurbiprofen as model drug. The study revealed that oleic- (C18:1), linoleic- (C18:2) and linolenic acid (C18:3) all enhanced the flux of flurbiprofen. Amounts of flurbiprofen trapped within the skin was enhanced more so with oleic acid (C18:1), followed by linolenic- (C18:3) and linoleic acid (C18:2).

The study by Ambade *et al.* (2008:40) evaluated the penetration enhancing effect of microemulsions containing isopropyl myristate and ethyl oleate (oleic acid), which are esters of fatty acids (Martindale, 2012a; Martindale, 2012b) and were used as the oil phase in the topical delivery of flurbiprofen. The release of flurbiprofen was higher from isopropyl myristate- (myristic acid) than from the ethyl oleate (oleic acid) microemulsion. These oily microemulsions had an enhanced flux compared to the gel cream formulations.

D.5 CONCLUSION

Natural oils have similar properties to those of ideal penetration enhancers and do not show any pharmacological activities. These oils are relatively inexpensive and spread well over the skin. During the skin diffusion studies, the marker flurbiprofen had been released from all of the formulations and some degree of penetration enhancement activity between the different emulgel formulations was indicated. Differences noted between oils containing UFAs and the same amount of carbon on the chain seemed to have had different penetration enhancement activities (Büyüktimkin, 1997:361-362,434). These types of enhancers (fatty acids) with polar (hydrophilic) head groups and long hydrophobic chains penetrate into the intercellular lipids of the stratum corneum and disrupt the endogenous ceramides, thereby increasing diffusion of an API through the skin (Hadgraft & Finnin, 2006:367-368).

Throughout this study, the flux of the marker and the concentrations within the stratum corneum-epidermis seemed to be enhanced more significantly by oils containing UFAs, but predominantly oleic acid (MUFA). Oleic acid (MUFA) was most effective in the enhancement of the flux of the marker, but not more so than formulation **(8)**, which predominantly contained medium chain SFAs. Structural alteration in the stratum corneum lipids with oils containing high

lauric acid (SFA) values, seemed of a lesser extend. This explained why oils, high in oleic acid portrayed increased penetration enhancing effects compared to oils containing average amounts of MUFAs and PUFAs. The reason for this may have been due to the conformational “kinks” found within the chains (the *cis*-double bond isomers) of these UFAs, which disrupt the normal packing of the stratum corneum (Santoyo & Ygartua, 2000:246).

Solubility and diffusion of an API increase with increasing temperature; and although it was strived to maintain temperatures during these experiments at normal skin temperature (32°C), variations ± 1 °C could have caused significant differences in the rates of absorption (Smith, 1990:28). However, a previous study denoted that even though oleic acid increased skin permeation of lipophilic and hydrophilic APIs, the combined effect with applied heat did not illustrate a significant effect on the enhancement of the API (Ohara *et al.*, 1995:283).

Data from the membrane studies indicated that the highest release of flurbiprofen was from formulations **(5)**, **(2)** and **(7)**, respectively. This was followed by formulations **(6)**, **(8)**, **(1)** and **(3)**; with **(4)** depicting the lowest flux. However, when comparing the results of the membrane studies to the skin diffusion studies, it could have been that the skin itself played an important part in the process of transdermal absorption, due to the partitioning behaviour between the skin and the emulgel formulation (Fang *et al.*, 2003a:316).

Fatty acid effects on drug delivery can vary through human skin. The fact that there was a significant interaction between the formulations and the place of measurement (i.e. stratum corneum-epidermis and epidermis-dermis), was a normal phenomenon and could have occurred due to the differences in biological materials. These discrepancies might have been due to the variation of skin samples used per study (Williams & Barry, 2004:610).

Results obtained from diffusion studies performed on the stratum corneum-epidermis indicated rather high flurbiprofen concentrations for most of the formulations, as observed for formulations **(2)**, **(9)**, **(7)**, **(4)**, **(1)** and **(6)**, respectively. If the site of action in transdermal delivery is the stratum corneum and the molecule is lipophilic (i.e. flurbiprofen) it will automatically partition into and accumulate in the stratum corneum. A drawback of dermal delivery is the uptake of the API into the bloodstream (Wiechers, 2008a:11-12). When an API is applied to the skin and intended to serve a local action to the immediate underlying tissue, it is suggested that flurbiprofen would provide a prolonged, localised effect, with minimal systemic absorption (Panwar *et al.*, 2011:333), due to the lowered concentration flurbiprofen found within the epidermis-dermis region, as was observed during this study for some of these formulations. Generally, topically applied APIs are preferred to penetrate into, and confine to the skin layers in order to produce a localised effect, hence, the permeation of flurbiprofen through the skin is not necessarily desired (Ramchandani & Toddywala, 1997:559). In many cases, enhancement encourages a localised

effect by increasing drug concentration within the skin, whereas transdermal enhancement requires transport through the dermal layers into systemic circulation. Only a few enhancers are powerful enough to deliver therapeutic levels and macromolecules to the blood (Foldvari, 2000:419).

Formulations **(8)**, **(3)**, **(10)** and **(5)** did not reveal significantly high flurbiprofen concentrations within the stratum corneum-epidermis. However, it could have been due to the fact that the marker inside the emulgels penetrated the epidermis-dermis very slowly through the layers of the cell membranes in the stratum corneum (Martini, 1998:153).

With regards to the flurbiprofen concentration depicted within the epidermis-dermis for formulation **(1)**, the marker had to first diffuse out of the aqueous surrounding of the hydro-formulation, before it could permeate through the skin. The partitioning time of the marker between the skin and the hydrogel might have caused a reduction in the drug reservoir within the skin when compared to the flurbiprofen concentrations in the stratum corneum-epidermis (Fang *et al.*, 2003a:316).

By hydrating the stratum corneum with formulation **(1)**, its pliability was increased, thus inhibiting TEWL. Hydration causes swelling of the stratum corneum, an increase in diameter of the sweat ducts and an increase in the degree of folding of the stratum corneum. The introduction of moisture into the stratum corneum might have increased the transdermal absorption of flurbiprofen, which, together with artificial holes that might have formed during skin preparation, could have modified the apparent permeability of the tissue to the marker. Skin tissue softens when saturated with water, thereby dramatically increasing its permeability and thus, facilitating the penetration of most (not all) materials through the skin (Barry, 1983:28, 107,145). It is therefore clear that even the safest and effective penetration enhancer (water) can change the nature of the stratum corneum (Barry, 2001a:106; Ranade & Hollinger, 2004:211).

Franz cell diffusion studies indicated that formulation **(1)** achieved the highest median flux compared to the rest of the formulations. Besides the hydration aspect as discussed, the increase in the median flux could have been due to the marker's low affinity for the vehicle and its eagerness to diffuse out of the formulation into the skin. The more soluble an API within a formulation is; the slower the permeation of the API would be into the skin (Takahashi *et al.*, 1991:155). A formulation may alter the properties of the skin, and enhance, or retard the permeation of the API within the formulation by increasing its diffusivity and/or solubility within the stratum corneum (Walters & Brain, 2009:482). The results of the skin diffusion studies obtained very similar flux values for formulations **(2)** and **(7)**. Takahashi *et al.* (1991:156) tested both liquid paraffin, as found in formulation **(2)** and the oleic acid found within formulation **(7)**. They concluded that both portrayed some enhancing effects as seen in this study as well. The

liquid paraffin in formulation **(2)** depicted a slightly higher enhancing effect compared to the oleic acid included in formulation **(7)**, which suggested the importance of selecting a vehicle that has a low affinity for the tested API. However, a study by Babu *et al.* (2006:140) concluded that the flurbiprofen enhancement factor increased predominantly by oleic- (C18:1) and linolenic acid (C18:3) (UFA), regardless of the formulation.

The stratum corneum contains predominantly C₁₆ or longer chains, therefore, UFAs or branched chains can disrupt the packing of these chains to a higher effect within the stratum corneum. The formulation of **(7)** contained branched chains (i.e. oleic acid), which might therefore have disrupted the stratum corneum packing and led to enhanced penetration of the marker. Naturally occurring fatty acids generally contain a *cis*-configuration. UFAs are more disruptive to the stratum corneum's lipid packing than SFAs of the same chain length in skin permeation (Aungst, 1989:247; Zamora, 2005). Comparing the formulations containing natural oils in the skin diffusion studies, formulation **(7)** depicted the highest median flux. Penetration enhancers cause a disordered packing of the lipids in the stratum corneum, by interacting with the polar lipid head groups or with the hydrophobic lipid tails (Foldvari, 2000:420). Altering the barrier function of the intercellular lipid pathway of the stratum corneum causes conformational "kinks" of the hydrocarbon chains within this pathway and causes small openings in the intercellular pathway of the stratum corneum that increases its fluidity. Disruptions of the polar head groups within the intercellular domain alters the packing of its lipid alkyl chains and possibly disrupt its lamellar packing, resulting in the marker entering through these openings and traversing across the skin (Marjukka Suhonen *et al.*, 1999:152-153).

Although formulation **(8)** did not yield the highest flux, compared to the other oil formulations, it did however perform relatively well compared to formulations, **(3)**, **(4)**, **(5)** and **(6)** that primarily contained more UFAs than the predominantly SFAs found within formulation **(8)**. The fatty acids found in formulation **(8)** were mostly of the short and medium chain SFAs. Caproic acid (C6:0) was a minor component, whereas caprylic- (C8:0) and capric acid (C10:0) dominated. Lauric acid (C12:0), a medium chain SFA, was present in high concentrations in formulation **(8)** (Mitsui, 1997:125; Ralston, 1948:25, 29). It is believed that if the ordered regions of the lipid layer of the stratum corneum consist of C₆ to C₈ hydrocarbons and are disordered it might ensure enhanced permeation of the marker. Thus the fluidity of the intercellular lipids of the stratum corneum as well as an effective free volume may have been increased, by increasing partitioning and diffusivity during these tests. The fatty acids's alkyl chain might have penetrated into the lipid layers of the stratum corneum to the same depth in order to induce fluidisation (Warner *et al.*, 2001:1152) and could have enhanced the flux of flurbiprofen through this region.

The foam formulations did little to improve the general release of the marker and the drawback of these foam formulations could be that the propellant technology might become rather

complex and expensive to manufacture. This may explain why pharmaceutical manufacturers tend to shy away from these types of skin delivery systems. There was thus no significant clinical advantage gained by using foam, compared to the emulgel formulations (Purdon *et al.*, 2003:75). However, a study conducted by Deng *et al.* (2004:80) delivered an API from the foam at an increased rate, compared to the semisolid cream, solution and ointment they used in their study.

Compounds with a log P between 1 and 3 will show good absorption within the skin. The marker flurbiprofen has a log P value of 4.16, and will thus, partition easily into the stratum corneum due to its apparent lipophilicity. If the partition coefficient is low, there will be little partitioning into the skin lipids. However, a too high partitioning coefficient can cause low partitioning, because of its inability to partition out of the stratum corneum (Craig, 1990:541; Fang *et al.*, 2003a:319; Thomas & Finnin, 2004:699; Wiechers & Watkinson, 2008:63). It therefore seemed as if a parabolic relationship exists between log P values and the transport of an API through the skin. Highly lipophilic APIs (i.e. flurbiprofen) will preferably reside within the lipophilic regions of the stratum corneum as observed with results obtained for some of the formulations from the tape stripping data. The partition coefficient refers to the lipophilicity of an API in the context of equilibrium of the unionised species between the aqueous and organic phases (skin); and the distribution of the species will depend on the pH thereof. The concentration of the ionised species in the aqueous phase will have an effect on the overall partition coefficient that affects the diffusion coefficient between the layers of the skin (Steele & Austin, 2009:22-23).

Ideally, the aqueous solubility of an API considered for skin permeation, should be higher than 1 mg/ml (Naik *et al.*, 2000:319). The API should possess both lipid- and aqueous-solubility characteristics, but if it is too hydrophilic, the active would be unable to diffuse into the stratum corneum, whereas if too lipophilic, it would remain within the stratum corneum-epidermis. Therefore, this may have been the reason for the marker penetrating into the viable epidermis-dermis (Barry, 1983:109-113; Hadgraft & Finnin, 2006:365). The solubility of flurbiprofen in phosphate buffer solution at 25 °C from literature is 2.94 ± 0.18 mg/ml, which may indicate that it might permeate well through the skin (Ambade *et al.*, 2008:37). The main pathway for permeation of most drugs is the intercellular lipid domain of the stratum corneum (Moghimi *et al.*, 1999:515), and it is suggested that flurbiprofen will partition through this route (Fang *et al.*, 2003a:319). The diffusion of lipid- and aqueous soluble, topically applied APIs, are subject to the structure of the intercellular compartment of the stratum corneum in that it is made up of lamellar double bilayers. These bilayers comprise of lipid components (i.e. ceramides, cholesterol and fatty acids), which have polar heads and water associated with them. Hence, for an API to cross the bilayers of the stratum corneum, perpendicular to the axis, it needs to cross a lipid and aqueous phase alternately (Sloan & Wasdo, 2006:53). These physicochemical

properties seemed to have correlated well with the results found. Flurbiprofen showed good release properties from most of the natural oil emulgel formulations during the membrane studies. It permeated well into the dermatomed excised skin, by penetrating the stratum corneum, with subsequent accumulation thereof in the stratum corneum for most of the formulations, showing less partitioning into the viable hydrophilic epidermis-dermis.

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APPENDIX E

JOURNAL OF NATURAL MEDICINES: INSTRUCTIONS FOR AUTHORS

“Journal of Natural Medicines” is pleased to announce the launch of its new online manuscript submission and tracking system. This new system makes it possible for authors to submit manuscripts via the Internet, provides online peer review services, and tracks manuscripts through the review process. Key features include automatic conversion of authors’ submissions into PDF format as well as supporting submissions in various file formats and special characters. Authors are requested to use Editorial Manager for all submissions of new manuscripts.

E.1 EDITORIAL POLICY

The Journal of Natural Medicines invites papers that make a significant contribution to the knowledge and understanding of naturally occurring medicines and their related foods and cosmetics, including identification and structure elucidation of natural products, biosynthesis, biotechnology, and pharmacology of herbs, natural products, and Kampo formulas. Papers are also published concerning chemical and botanical identification of herbs or their products where such information contributes to the overall safety of plant-based medicines currently and/or formerly in use. Submission of a paper implies that it has been approved by all the named authors, that it has not been published before, and that it is not under consideration for publication elsewhere.

Acceptance of papers will be decided by the Editorial Board after examination by at least two referees. Manuscripts that require revision and/or correction and/or condensation will be returned to the authors with comments specifying alterations from the referees and/or the Editorial Board.

The following types of papers may be submitted. (i) Original Papers: Original Papers are the usual form of publication of this journal describing original experimental results. (ii) Notes (up to 4 printed pages): Notes are brief articles reporting new facts and/or valuable data. (iii) Rapid Communications (up to 3 printed pages): These are short preliminary reports of novel and significant findings that merit urgent publication. Full details of the Rapid Communication may

be published later as a comprehensive original paper, in this or another journal. An explanatory statement is required for urgent publication. (iv) Reviews (up to 12 printed pages): Reviews covering recent discoveries or topics of current interest are generally invited. (v) Mini-Reviews (up to 6 printed pages): These provide a short overview of a particular subject and are also generally invited. In the case of a non-invited Review or Mini-Review, a short summary describing the outline should be sent to the Chief Editor for approval before submitting a manuscript. (vi) Natural Resource Letters (up to 6 printed pages): Natural Resource Letters are reports on biological screening data, field observations, market surveys, quality evaluation or quantitative methods of crude drugs, etc., which may contribute to the scientific knowledge of the readers.

➤Chief Editor: Hiromitsu Takayama

E-mail: takayama@p.chiba-u.ac.jp

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E.3 LEGAL REQUIREMENTS

The author(s) guarantee(s) that the manuscript will not be published elsewhere in any language without the consent of The Japanese Society of Pharmacognosy and Springer, that the rights of third parties will not be violated, and that the publisher will not be held legally responsible should there be any claims for compensation.

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E.4 MANUSCRIPT PREPARATION

Manuscripts must be written in English using Times font and typed in double-line spacing with 3 cm margins; pages should be numbered. Authors whose first language is not English are urged to have their manuscript read by a colleague who is a native English speaker and is familiar with their field of work before submitting the paper.

Manuscripts with extensive English language deficiencies will be returned to the author without the full reviewing process.

➤ Documentation of experimental biological materials.

Use the correct scientific name and indicate who identified the biological materials. The herbarium deposit site and voucher number should be recorded. Authors who purchase herbal materials from companies must make provision for their deposit in a herbarium.

➤ Pharmacological investigations of extracts require detailed extract characterization.

This includes botanical characterization of plant material(s), solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). A chromatographic (e.g., HPLC profile recorded at different wavelengths) or chemical characterization (quantities of typical constituents) must be presented. The investigation must clearly indicate a dose/activity dependence in comparison with a reference compound (positive control) together with the appropriate statistics.

The manuscript should be arranged as follows:

➤ Title page (including an article title, name(s) of author(s), affiliation(s) and address(es) of the author(s), e-mail address, telephone and fax numbers of the corresponding author.).

➤ Abstract (200 - 250 words).

➤ Four to six keywords.

Text of the paper: Introduction; main text (divided into sections, if appropriate); Acknowledgments; References; tables; figure legends

➤ Abbreviations. Abbreviations must be spelled out in full at their initial appearance in the abstract and main text, followed by the abbreviation in parentheses. Thereafter, the abbreviation may be employed.

However, the following need not be defined: AIDS (acquired immunodeficiency syndrome), ATP (adenosine 5'-triphosphate), cAMP (adenosine 3',5'-cyclic monophosphate), CD (circular dichroism), cDNA (complementary DNA), CoA (coenzyme A), COSY (correlated spectroscopy), DNA (deoxyribonucleic acid), ED₅₀ (50% effective dose), EI-MS (electron ionization mass spectrometry), FAB-MS (fast atom bombardment mass spectrometry), FAD (flavin adenine dinucleotide), GC-MS (gas chromatography-mass spectrometry), HMBC (heteronuclear multiple bond connectivity), HMQC (heteronuclear multiple quantum coherence), HPLC (high-pressure liquid chromatography, high-performance liquid chromatography), HSQC (heteronuclear single quantum coherence), IC₅₀ (inhibitory concentration, 50%), IR (infrared), LD₅₀ (50% lethal

dose), mRNA (messenger RNA), MS (mass spectrum), NMR (nuclear magnetic resonance, as ^{13}C -NMR, ^1H -NMR), NOE (nuclear Overhauser effect), NOESY (nuclear Overhauser and exchange spectroscopy), ORD (optical rotatory dispersion), P450 (as in cytochrome P450), RNA (ribonucleic acid), rRNA (ribosomal RNA), TLC (thin-layer chromatography), tRNA (transfer RNA), UV (ultraviolet).

- Units. The following units should be used: length (m, cm, mm, μm , nm, Å), mass (kg, g, mg, μg , ng, pg, mol, mmol), volume (l, ml, μl), time (s, min, h, d), temperature ($^{\circ}\text{C}$, K), radiation (Bq, Ci, dpm, Gy, rad), concentration (M, mM, mol/l, mmol/l, mg/ml, $\mu\text{g}/\text{ml}$, %, $\%(\text{v}/\text{v})$, $\%(\text{w}/\text{v})$, ppm, ppb).
- Spectral and elemental analysis data. Please report spectral and elemental analysis data in the following format, ^1H -NMR (CDCl_3) δ : 1.25 (3H, d, $J=7.0$ Hz), 3.55 (1H, q, $J=7.0$ Hz), 6.70 (1H, m). ^{13}C -NMR (CDCl_3) δ : 20.9 (q), 71.5 (d), 169.9 (s). IR (KBr) cm^{-1} : 1720, 1050, 910. UV $\lambda_{\text{max}}(\text{H}_2\text{O})$ nm ($\log \epsilon$): 280 (3.25). MS m/z : 332 (M^+), 180, 168. HRFAB-MS m/z : 332.1258 (Calcd for $\text{C}_{18}\text{H}_{20}\text{O}_6$: 332.1259). $[\alpha]_{\text{D}}^{23}$ -74.5° (c 1.0, MeOH). Anal. Calcd for $\text{C}_{19}\text{H}_{21}\text{NO}_3$: C, 73.29; H, 6.80; N, 4.50. Found: C, 73.30; H, 6.88; N, 4.65.
- Nomenclature. The nomenclature of chemical compounds should be in accordance with the nomenclature rules formulated by IUPAC. Alternatively, naming may conform to the nomenclature in the index of Chemical Abstracts or the Ring Index.
- X-ray crystal structure analyses. When structure determination by X-ray crystallographic analysis is a central theme of the paper, the data required for registration with the Cambridge Crystallographic Data Base (CCDB) should be attached as supplementary material. Although this does not apply in cases where crystallographic analysis plays only a supplementary role, crystal data (unit cell parameters, space group, Z density) and R-factor should still be noted. Atomic coordinates, bond lengths and angles, thermal parameters, and torsion angles should be presented when they are important for the issues addressed in the paper. When papers have been accepted, the authors are advised to register the data with CCDB.

E.5 REFERENCES

The list of References should include only works that are cited in the text and that have been published or accepted for publication. Personal communications should be mentioned only in the text. If available, the DOI can be added at the end of the reference in question.

References should be numbered in the order in which they appear in the text and be listed in numerical order. Journal titles should be abbreviated according to Chemical Abstracts. References should be styled as follows, with correct punctuation:

1. Kuroyanagi M, Umehara K, Hayashi T, Hirayama Y (2002) Cell differentiation inducers and antiandrogenic active compounds from zingiberis rhizoma. *Nat Med* 56:47–502
2. Chen W, Punja Z (2002) Agrobacterium-mediated transformation of American ginseng with a rice chitinase gene. *Plant Cell Rep* 20:1039–1045 DOI 10.1007/s00299-002-0446-z3
3. Tsukaya H, Iokawa Y, Kondo M, Ohba H (2005) Large-scale general collection of wild-plant DNA in Mustang, Nepal. *J Plant Res* DOI 10.1007/s10265-005-0196-44
4. Capasso F, Gaginella TS, Grandolini G., Izzo AA (2003) *Phytotherapy*. Springer, Berlin Heidelberg New York
5. Bauer BA (2003) Herbal therapy and the elderly. In: Cherniack P, Cherniack N (eds) *Alternative medicine for the elderly*. Springer, Berlin Heidelberg New York, pp 111–132

E.6 TABLES AND FIGURES

All figures (photographs, graphs or diagrams) and tables should be cited in the text, and each should be numbered consecutively throughout with arabic numerals. All artwork that is provided must be of a size that will fit within the width of single or double columns (single-column width is 8.4 cm; double-column width is 17.4 cm). Single-column artwork is strongly recommended. The publisher reserves the right to reduce or enlarge illustrations.

Tables must be numbered consecutively with arabic numerals. They should have a title explaining any abbreviation used in that table. Footnotes to tables should be indicated by superscript lowercase letters (or asterisks for significance values and other statistical data).

Illustrations must be restricted to the minimum needed to clarify the text. Figure parts should be identified by lowercase roman letters (a, b, etc.). If illustrations are supplied with uppercase labeling, lowercase letters will still be used in the figure legends and citations.

Figure legends must be brief, self-sufficient explanations of the illustrations. The legends should be placed at the end of the text.

All figures should be submitted as separate files and should not be integrated within the text. The preferred figure formats are EPS (Encapsulated Postscript) for vector graphics exported from a drawing program and TIFF for halftone illustrations. EPS files must always contain a preview in TIFF of the figure. Microsoft PowerPoint files and illustrations embedded in Microsoft Word documents are not acceptable. The file name should include the figure number.

- Line drawings. Inscriptions should be legible, with initial capital letters and appropriately scaled to the size of the drawing. Scanned line drawings should be digitized with a

resolution of 800 dpi relative to the final figure size.

- Halftone illustrations (black and white and color). Magnification should be indicated by scale bars. For scanned halftone illustrations, a resolution of 300 dpi is usually sufficient.
- Color illustrations should be saved as RGB (8 bits per channel) in TIFF format.

E.6.1 Artwork Guidelines

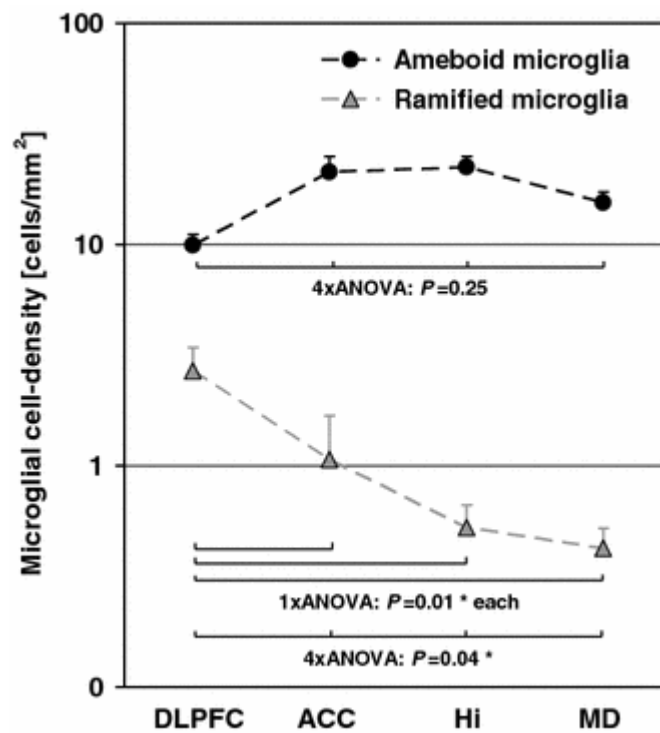
For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

E.6.1.1 Electronic figure submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

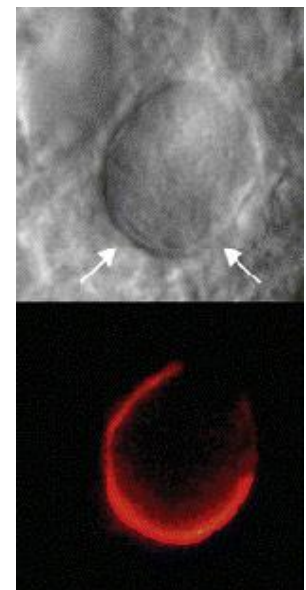
E.6.1.2 Line art

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.



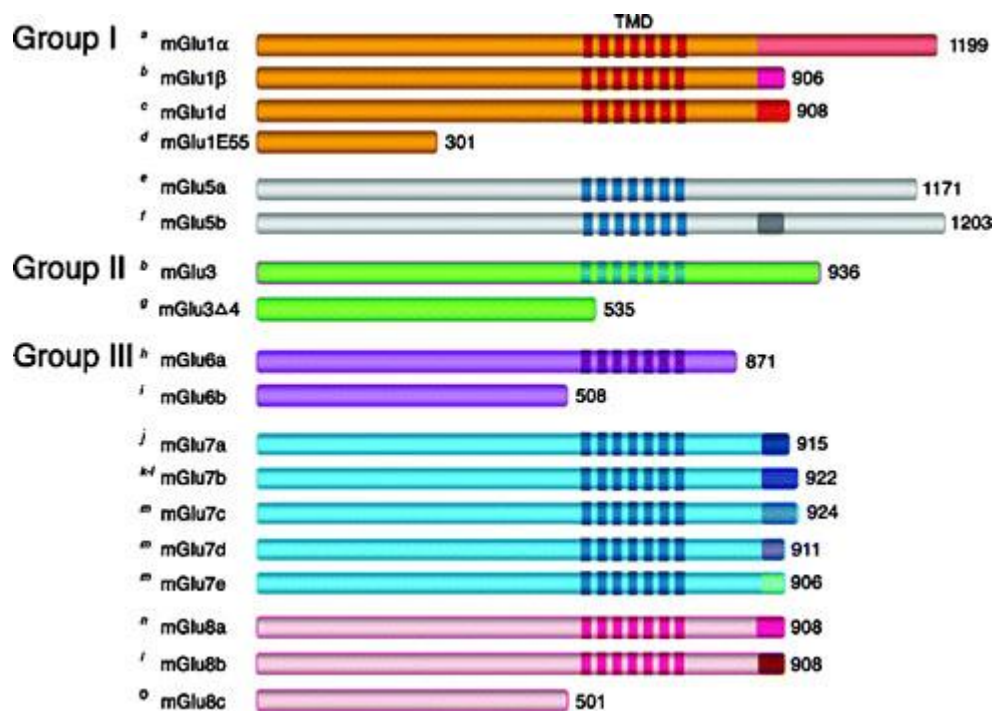
E.6.1.3 Halftone art

- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.



E.6.1.4 Combination art

- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.



E.6.1.5 Color art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

E.6.1.6 Figure lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2 - 3 mm (8 - 12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8 pt type on an axis and 20 pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

E.6.1.7 Figure numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
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- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

E.6.1.8 Figure captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
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- When preparing your figures, size figures to fit in the column width.
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- Any figure lettering has a contrast ratio of at least 4.5:1

E.7 CONFLICT-OF-INTEREST POLICY

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E.9 ELECTRONIC SUPPLEMENTARY MATERIAL

Electronic Supplementary Material (ESM) for a paper will be published in the electronic edition of this journal provided the material is:

- submitted in electronic form together with the manuscript;
- accepted after peer review.

ESM may consist of:

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After acceptance for publication, ESM will be published as received from the author in the online version only. Reference will be given in the printed version.

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- (i) Handling charges: JPY 4,000 per printed page.
- (ii) Charges for excess pages in Notes, Rapid Communications and Natural Resource Letters: JPY 8,000 per printed page in addition to the handling charges.

E.12 DATE OF ISSUE

The journal will be issued on 10th of January, April, July, and October each year.

(Revised on 7 October 2011)

APPENDIX F

ETHICAL APPROVAL



NORTH-WEST UNIVERSITY
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NOORDWES-UNIVERSITEIT
INSTITUTIONAL OFFICE

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 018 299-4852
Web: <http://www.nwu.ac.za>

ETHICS APPROVAL OF PROJECT

This is to certify that the next project was approved by the NWU Ethics Committee:

Project title :

APPLICATION FOR THE USE OF BIOLOGICAL MATERIAL OBTAINED FROM HUMAN SUBJECTS IN EXPERIMENTS

Student/Projectleader : Prof. J Du Plessis

Ethics number: NWU-00114-11-A5

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Issue Date: 2011-08-25

Expiry date: 2016/08/24

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project.

Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

The formal ethics approval certificate will follow shortly.

Yours sincerely

A handwritten signature in black ink, appearing to be 'H. Halgryn'.

HM Halgryn
NWU Research Ethics Secretariate