

# Formulation and topical delivery of a safflower oil nano-emulsion containing artemether

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The dissertation is presented in an article format, which includes one article for publication in a pharmaceutical journal (Chapter 3) and appendices containing experimental results and discussion (Appendix A – G). The article for publication has a specific Guide for Authors (Appendix H) for publishing.

*“All our dreams can come true if we have the courage to pursue them.”*

*~ Walter E Disney ~*

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*“I can do all things through Him who gives me strength.” ~ Philippians 4: 13*

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#### **Disclaimer**

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# ABSTRACT

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Cutaneous tuberculosis (CTB), one of the forms of extra-pulmonary tuberculosis (TB), occurs 1.5% of the time when the *Mycobacterium tuberculosis* bacterium enters the skin, either through direct contact or through airborne particles (Almaguer-Chávez *et al.*, 2009:562-563; Arora *et al.*, 2006:344; VanderVen *et al.*, 2015:2). Clinical presentations of CTB can vary, but commonly all forms pose as ulcer-like infectious lesions (Bravo & Gotuzzo, 2007:174-177). Current CTB treatment commences through oral TB regimens, which results in unfavourable patient compliance. This is due to an extensive combination of drugs used over a period of months (Van Zyl *et al.*, 2015:634-635). Unfortunately, strains of *M. tuberculosis* are becoming greatly resistant against available active pharmaceutical ingredients (APIs) (Van Zyl *et al.*, 2015:634). This resistance poses another problem during treatment, as patients need to receive therapy they have not had before, which is difficult as only limited APIs are still effective (Almaguer-Chávez *et al.*, 2009:562; Dипiro, 2012:595; Van Zyl *et al.*, 2015:630).

Artemether, a lipophilic derivative of artemisinin, is an existing anti-malarial drug currently being investigated as a potential anti-TB treatment (Haynes, 2016; Miller *et al.*, 2011:2076; Nneji *et al.*, 2013:2619). The effectiveness of artemisinin can be ascribed to the fact that the endoperoxide bridge in their chemical structure leads to the production of free radicals (Nneji *et al.*, 2013:2619; Shrivastava *et al.*, 2010:79). Consequently, artemether can be viewed as an oxidant drug as it can lead to cytotoxic levels of reactive oxygen species (ROS), leading to oxidative stress, proposing an oxidising environment to *M. tuberculosis* and resulting in cell death within the parasitic cell (Ebrahimisadr *et al.*, 2014:1; Haynes, 2015; Haynes, 2016; McIntosh & Olliaro, 2010:2; Nneji *et al.*, 2013:2619; Shahzad *et al.*, 2013:197). Artemisinin combinations have been found to lead to submicromolar activity against *M. tuberculosis* (Shakya *et al.*, 2012:702).

In this study, the aim was to formulate a novel CTB treatment through the topical delivery of artemether. Resistance against existing APIs, together with the lack of topical CTB treatment, presents an opportunity for the investigation thereof (Van Zyl *et al.*, 2015:630). It can be suggested that systemic TB treatment, in combination with topical treatment, could contribute to better treatment of CTB lesions (Van Zyl *et al.*, 2015:636; Wyrzykowska *et al.*, 2012:297).

Firstly, on investigation of the physicochemical properties of artemether, it can be viewed as topically favourable since it is a lipophilic API with an ideal molecular mass and melting point. Secondly, artemether is highly metabolised through the liver when taken orally, hence topical application will be more advantageous (Shahzad *et al.*, 2013:197). Thirdly, as CTB is a cutaneous disorder, it is proposed to be treated through a topical delivery system as direct

contact between the CTB lesions and the API can be achieved. Topical drug delivery of artemether is therefore aimed at keeping the API in the skin, following the direct application thereof to the targeted site, i.e. the epidermis (Williams, 2013:676). The skin being by far the largest and most easily accessible organ represents a great target site and many advantages are proposed by topical drug delivery of which the most important is that it can be viewed as a non-invasive drug delivery system (Williams, 2013:677). This is ascribed to an increase in patient compliance and through the direct application of the API to the target, hence, bypassing the hepatic system (Marrow *et al.*, 2007:37; Naik *et al.*, 2000:319). Although being applied directly to the skin, the API needs to move and permeate through skin layers, but is initially limited by the outer most layer – the stratum corneum (Williams, 2013:682). The lipids within the stratum corneum control and regulate the movement of APIs through the skin and therefore act as a drug flux regulator (Williams, 2003:10). The drug flux is consequently the quantity of an API that can move across the layers of the skin; it is evident that the drug flux is also directly dependent on the API's physicochemical properties (Williams, 2003:28; Williams, 2013:680). Therefore, to result in successful skin permeation, properties such as molecular mass, aqueous solubility, partition coefficient (log P), diffusion coefficient and melting point should be ideal (Allen *et al.*, 2011:42; Williams, 2013:680-682).

Artemether presents with some ideal physicochemical properties for topical delivery, since its molecular mass is less than 500 g/mol (298.37 g/mol) and its melting point is lower than 200 °C (between 86 – 90 °C) (Naik *et al.*, 2000:319; USP, 2013). An ideal octanol-buffer distribution coefficient (log D) for an API to be delivered topically should range between 1 and 3, since this value signifies that the API is soluble in both water and oil (Subedi *et al.*, 2010:339; Williams, 2003:36). Experimental determination of the log D value was calculated to be  $2.35 \pm 0.1170$ , which is ideal for topical drug delivery, whilst the aqueous solubility of artemether was found to be  $0.1053 \pm 0.0022$  mg/ml, which is significantly less than 1 mg/ml, hence, less than optimal for topical delivery (Naik *et al.*, 2000:319). It was proposed that less than optimal properties could be overcome through the formulation of a successful delivery system.

Nano-emulsions can be viewed as a promising topical delivery system due to their small droplets (20 – 200 nm) that can lead to better permeation and drug release, resulting in greater concentration of the API accumulating within the skin (Abolmaali *et al.*, 2011:139; Klang *et al.*, 2015:258; Lai *et al.*, 2008:1; Lu *et al.*, 2014:826). A nano-emulsion is generally constituted by two phases, i.e. a water and an oil phase, dispersed within each other resulting in both hydrophilic and lipophilic characteristics (Gaur *et al.*, 2014:37; Klang *et al.*, 2015: 258). A greater surface area and larger interfacial area, combined with free energy, are contributors to a nano-emulsion being a target site-specific drug delivery system that can result in localised deposition, which is essential for successful CTB treatment (Clares *et al.*, 2014:S91; Lai *et al.*, 2008:1; Lovelyn & Attama, 2011:626).

Many approaches and techniques have been attempted to overcome the stratum corneum properties (Williams, 2013:693). One approach has been the use of a nano-emulsion as a delivery system, since it presents with enhanced penetration (Lovelyn & Attama, 2011:630; Maruno & Da Rocha-Filho, 2010:17). Another successful approach is the incorporation of penetration enhancers in topical formulations (Trommer & Neubert, 2006:108; Williams, 2013:694; Williams & Barry, 2012:129). Penetration enhancers are successful due to the fact that they disrupt, modify and reduce the lipid barrier of the skin, resulting in increased partitioning and absorption of the API (Babu *et al.*, 2006:145; Trommer & Neubert, 2006:108; Wang *et al.*, 2003:1612; Williams, 2013:694). In this study, safflower oil, a natural oil, was employed as chemical enhancer. C<sub>18</sub>-Unsaturated fatty acids, such as linoleic acid and arachidonic acid, have been found to have near optimal enhancement effects (Williams & Barry, 2012:132). Since uncomplicated fatty acids constitute basic components of human skin, the use thereof can be regarded as safe, therefore lowering the possibility of skin irritation (Boelsma *et al.*, 1996:729; Büyüktimkin *et al.*, 1997:433; Gaur *et al.*, 2014:1812; Menon, 2002:S9; Vermaak *et al.*, 2011:922). Safflower oil presents with a high concentration ( $\pm$  75%) of linoleic acid, which plays an important moisturising, healing and anti-inflammatory role when incorporated within topical formulations (Van Wyk & Wink, 2009:81; Vermaak *et al.*, 2011:922; Wolters Kluwer Health, 2009).

Therefore, the aim was to formulate a topical oil-in-water (o/w) nano-emulsion containing 0.8% (w/v) artemether and 5.0% (w/v) safflower oil. Consequently, an optimised nano-emulsion obtained through pre-formulation was formulated within two semi-solid dosage forms, i.e. a nano-emulgel and a conventional emulgel to contain 0.4% (w/v) artemether and 2.5% (w/v) safflower oil. Through characterisation, it could be proposed that the three optimised formulations presented with ideal properties for effective topical drug delivery. Hence, all three formulations presented with small droplets, an ideal surface charge and stability for possible successful permeation.

The effectiveness of each of the three formulations was evaluated by determining whether any API release and/or permeation of the API through the skin had occurred, therefore *in vitro* diffusion studies were conducted on each of the formulations (Wiechers, 2008:23; Williams, 2013:683). Franz cell diffusion studies are based on the employment of a vertical Franz cell method, consisting of a two-chamber diffusion cell, which is separated by a membrane or a piece of skin (Williams, 2013:683). Release of the API from the three different formulations was evaluated through *in vitro* membrane release studies. Following the membrane release studies, skin diffusion studies and tape stripping were done to determine whether any transdermal and/or topical delivery were achieved, respectively.

Experimental flux values of artemether, gained through membrane release studies proved that artemether was released from all three formulations. During the skin studies, only the nano-emulsion resulted in artemether being retained within the stratum corneum-epidermis and although a small amount permeated into the receptor phase, the quantified values were lower than the limit of detection (LOD) as well as the lower limit of quantification (LLOQ). Hence, as a result of this it can be said that artemether was not found within in the systemic circulation and only in the target site, i.e. the outermost layer of the epidermis. The formulated nano-emulgel and conventional emulgel did also not result in any artemether in the skin or through the skin. These non-existing artemether concentration values can possibly be ascribed to the formulation itself and to the pH of the formulations averaging at a pH of  $6.82 \pm 0.03$ ,  $5.14 \pm 0.02$  and  $5.86 \pm 0.02$ , leading to only 0.11%, 5.44% and 1.09% being unionised, respectively. Low unionised species could lead to low or no permeation, whilst high unionised species propose effective permeation of the skin (Li *et al.*, 2012:98; Williams, 2003:38). The low aqueous solubility of artemether ( $0.1053 \pm 0.0022$  mg/ml) (water) and  $0.090 \pm 0.0030$  mg/ml (phosphate buffer solution (PBS) (pH 7.4)) as well as the low initial concentration of artemether within the formulations, ranging between 0.4% and 0.8%, could also influence diffusion results.

To determine the safety of artemether and the optimised topical nano-emulsion on human skin, *in vitro* cytotoxicity studies were conducted on normal immortalised human keratinocytes (HaCaT) cells. Cytotoxicity evaluation on the HaCaT cells commenced through the conduction of a methylthiazol tetrazolium (MTT) assay. Consequently, it was found that the optimised nano-emulsion (with and without artemether) and artemether itself presented as non-cytotoxic as there was less than 20% cell death when the cells were treated with a 0.5% and a 1.0% treatment, respectively. Further *in vivo* experiments or *in vitro* efficacy studies against *M. tuberculosis* would need to be conducted to evaluate the success of the formulation against CTB. It can therefore be proposed that the nano-emulsion would not present toxic when applied to the skin, in low concentrations.

It can be suggested that artemether could be delivered topically and that retention in the epidermis could possibly be achieved. Throughout this study, very low concentrations of artemether were found topically delivered through an optimised o/w nano-emulsion containing artemether and safflower oil. Weak aqueous solubility could possibly explain these low concentrations of artemether quantified. However, further investigations are required as it appears that optimisation of the formula could possibly overcome the challenge of the topical delivery of artemether as a novel CTB treatment.

**Keywords:** Topical drug delivery, cutaneous tuberculosis, artemether, nano-emulsion, safflower oil, Franz cell diffusion.

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# UITTREKSEL

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Kutaneuse tuberkulose (KTB) is 'n vorm van ekstra-pulmonêre tuberkulose (TB), en kom in 1.5% van alle TB gevalle voor wanneer die *Mycobacterium tuberculosis*-bakterieë die vel binnedring; hetsy deur direkte kontak of deur bakterieë in die lug (Almaguer-Chávez *et al.*, 2009:562-563; Arora *et al.*, 2006:344; VanderVen *et al.*, 2015:2). Die kliniese verskynsels van KTB kan wissel, maar in die algemeen word dit gekenmerk deur ulkusagtige, aansteeklike letsels (Bravo & Gotuzzo, 2007:174-177). Huidige KTB behandeling kan lei tot swak pasiëntmeewerkendheid, as gevolg van 'n uitgebreide kombinasie van orale geneesmiddels wat oor 'n behandelingstyd van maande strek (Van Zyl *et al.*, 2015:634-635). Verskeie spesies van *M. tuberculosis* is besig om baie weerstandig te raak teen huidige aktiewe farmaseutiese bestanddele (AFB) (Van Zyl *et al.*, 2015:634). Dus, hierdie weerstandigheid beteken dat 'n pasiënt behandel moet word met geneesmiddels wat hulle nog nie voorheen ontvang het nie; wat problematies is; aangesien beperkte AFBs nog effektief is (Almaguer-Chávez *et al.*, 2009:562; Dipro, 2012:595; Van Zyl *et al.*, 2015:630).

Artemeter, 'n lipofiliese derivaat van artemisinin, is 'n bestaande antimalariamiddel wat tans as 'n moontlike anti-TB behandeling ondersoek word (Haynes, 2016; Miller *et al.*, 2011:2076; Nneji *et al.*, 2013:2619). Die sukses en doeltreffendheid van artemisinin kan toegeskryf word aan die endoperoksiedbrug in hul chemiese struktuur wat kan lei tot die vervaardiging van vry radikale (Nneji *et al.*, 2013:2619; Shrivastava *et al.*, 2010:79). Gevolglik kan artemeter gesien word as 'n oksidant-geneesmiddel, aangesien dit kan lei tot sitotoksiese vlakke van reaktiewe suurstof spesies (RSS), wat aanleiding kan gee tot oksidatiewe stres en vervolgens 'n oksiderende omgewing vir *M. tuberculosis* stel; wat gevolglik kan lei tot die seldood van die parasitiese sel (Ebrahimisadr *et al.*, 2014:1; Haynes, 2015; Haynes, 2016; McIntosh & Olliaro, 2010:2; Nneji *et al.*, 2013:2619; Shahzad *et al.*, 2013:197). Studies het gevind dat artemisinin kombinasies tot sub-mikromolêre aktiwiteit teen *M. tuberculosis* kan lei (Shakya *et al.*, 2012:702).

Die doel tydens hierdie studie was om 'n nuwe behandeling vir KTB te formuleer deur artemeter topikaal af te lewer. Weerstandigheid teen bestaande AFBs, tesame met die gebrek aan topikale KTB behandelings, bied 'n geleentheid om dit te ondersoek (Van Zyl *et al.*, 2015:630). Dit suggereer, om sistemiese en topikale TB behandelings te kombineer, beter behandeling van KTB letsels kan plaasvind (Van Zyl *et al.*, 2015:636; Wyrzykowska *et al.*, 2012:297).

Eerstens, wanneer die fisies-chemiese eienskappe van artemeter ondersoek word, kom dié AFB voor as gunstig vir topikale aflewering; aangesien dit 'n lipofiliese AFB is met 'n ideale molekulêremassa, smeltpunt en oktanol-water-verdelingskoëffisiënt waarde. Tweedens, artemeter word tot 'n groot mate deur die lewer gemetaboliseer wanneer dit oraal geneem word;

dus sal topikale aflewering meer voordelig wees (Shahzad *et al.*, 2013:197). Derdens, word KTB geklassifiseer as 'n velsiekte en daarom word daar voorgestel dat dit topikaal behandel moet word deur gebruik te maak van 'n topikale afleweringstelsel, sodat direkte kontak tussen die KTB letsels en die AFB kan plaasvind. Die doel om artemeter topikaal af te lewer is om die AFB in die vel te behou, nadat dit direk op die geteikende area, bv. die epidermis, toegedien is (Williams, 2013: 676). Die vel kan gesien word as die grootste en mees toeganklike orgaan en verteenwoordig 'n groot teikenarea (Williams, 2013:677). Baie voordele kan toegeskryf word aan 'n topikale afleweringstelsel; waarvan die belangrikste is dat dit gesien kan word as 'n nie-indringende stelsel, dit veroorsaak 'n toename in pasiëntmeewerkendheid en kan ook die hepatiese-stelsel vermy (Marrow *et al.*, 2007:37; Naik *et al.*, 2000:319). Hoewel die AFB direk op die vel aangewend word, is dit nodig vir die AFB om deur die velle te beweeg, maar word aanvanklik deur die buitenste velleag – die stratum korneum, beperk (Williams, 2013:682). Die lipiede binne die stratum korneum beheer en reguleer die beweging van AFBs deur die vel en kan dus as 'n geneesmiddelvloedreguleerder optree (Williams, 2003:10). Die geneesmiddelvloed is gevolglik die AFB konsentrasie wat oor die lae van die vel kan beweeg (Williams, 2003:28). Dit is duidelik dat die geneesmiddelvloed direk afhanklik is van die fisies-chemiese eienskappe van die AFB (Williams, 2013:680). Dus, om tot suksesvolle beweging deur die vel te lei, moet eienskappe, soos die molekulêremassa, wateroplosbaarheid, verdelingskoëffisiënt ( $\log P$ ), diffusiekoëffisiënt en smeltpunt, ideaal wees (Allen *et al.*, 2011:42; Williams, 2013:680-682).

Artemeter het 'n paar ideale fisies-chemiese eienskappe vir topikale aflewering, soos 'n molekulêremassa minder as 500 g/mol (298.37 g/mol) en 'n smeltpunt laer as 200 °C (tussen 86 – 90 °C) (Naik *et al.*, 2000: 319; USP, 2013). Die ideale oktanol-buffer-verdelingskoëffisiënt ( $\log D$ ) om 'n AFB topikaal af te lewer is tussen 1 en 3, aangesien dit aandui dat die AFB in beide water en olie oplosbaar is (Subedi *et al.*, 2010:339; Williams, 2003:36). Die eksperimentele  $\log D$ -waarde was bepaal as  $2.35 \pm 0.1170$  en is ideaal vir topikale aflewering, terwyl die wateroplosbaarheid van artemeter bereken was as  $0.1053 \pm 0.0022$  mg/ml. Die wateroplosbaarheid was minder optimaal vir topikale aflewering, aangesien dit aansienlik laer as die ideale 1 mg/ml is (Naik *et al.*, 2000:319). Nie alle eienskappe van artemeter is ideaal vir topikale aflewering nie en dus word voorgestel dat 'n suksesvolle afleweringstelsel geformuleer moet word om dit te oorkom.

Nano-emulsies kan gesien word as 'n belowende topikale afleweringstelsel, as gevolg van hul klein druppels (20 – 200 nm) wat kan lei tot beter diffusie en geneesmiddelvrystelling; wat gevolglik beter biobeskikbaarheid van die AFB binne die vel kan veroorsaak (Abolmaali *et al.*, 2011:139; Klang *et al.*, 2015:258; Lai *et al.*, 2008:1; Lu *et al.*, 2014:826). 'n Nano-emulsie word oor die algemeen saamgestel deur twee fases, naamlik 'n water- en 'n oliefaas, wat binne mekaar gedispergeer word en vervolgens lei dat 'n nano-emulsie wat beide hidrofiliese en

lipofiliese eienskappe het (Gaur *et al.*, 2014:37; Klang *et al.*, 2015: 258). 'n Groter oppervlakarea en 'n groter tussenvlakarea gekombineer met vrye energie is bydraende faktore wat nano-emulsies in staat stel om as 'n teiken-spesifieke geneesmiddelafleweringsstelsel, op te tree, wat kan lei tot gelokaliseerde aflewering en is gevolglik noodsaaklik vir suksesvolle behandeling van KTB (Clares *et al.*, 2014:591; Lai *et al.*, 2008:1; Lovelyn & Attama, 2011:626).

Verskeie benaderings en metodes is al gepoog om die eienskappe van die stratum korneum te oorkom (Williams, 2013:693). Een van hierdie benaderings was die gebruik van 'n nano-emulsie as 'n afleweringsstelsel aangesien dit diffusie deur die vel kan verbeter (Lovelyn & Attama, 2011:630; Maruno & Da Rocha-Filho, 2010:17). Nog 'n suksesvolle benadering is die insluiting van 'n penetrasiebevorderaar in topikale formulerings (Trommer & Neubert, 2006:108; Williams, 2013:694; Williams & Barry, 2012:129). Penetrasiebevorderaars is suksesvol as gevolg van die feit dat hulle die lipiedsamestelling in die stratum korneum kan ontwrig, verander of verminder, wat vervolgens kan lei tot verhoogde diffusie en absorpsie van die AFB (Babu *et al.*, 2006:145; Trommer & Neubert, 2006:108; Wang *et al.*, 2003:1612; Williams, 2013:694). In hierdie studie, word saffloerolie ('n natuurlike olie) gebruik as 'n chemiese penetrasiebevorderaar. C<sub>18</sub>-Onversadigde vetsure, soos linoleïensuur en aragidoonsuur, is bevind om bevorderingseffekte te hê wat naby aan optimaal is (Williams & Barry, 2012:132). Aangesien ongekompliseerde vetsure basiese komponente van die menslike vel uitmaak, kan hierdie vetsure as veilig beskou word en daardeur word die moontlikheid van velirritasie verlaag (Boelsma *et al.*, 1996:729; Büyüktimkin *et al.*, 1997:433; Gaur *et al.*, 2014:1812; Menon, 2002:S9; Vermaak *et al.*, 2011:922). Saffloerolie bevat 'n baie hoë konsentrasie ( $\pm$  75%) linoleïensuur wat 'n belangrike rol speel tydens topikale formulerings aangesien dit hidrerende, genesende en anti-inflammatoriese effekte tot gevolg kan hê (Van Wyk & Wink, 2009:81; Vermaak *et al.*, 2011:922; Wolters Kluwer Health, 2009).

Dus, die doel tydens dié studie was om 'n topikale olie-in-water (o/w) nano-emulsie te formuleer wat 0.8% (m/v) artemeter en 5% (m/v) saffloerolie bevat. Gevolglik was 'n optimale nano-emulsie gedurende pre-formulering geformuleer, waarna dit in twee semi-soliede doseervorme, m.a.w. 'n nano-emuljel en 'n konvensionele emuljel geformuleer was om onderskeidelik 0.4% (w/v) artemeter en 2.5% (w/v) saffloerolie elk te bevat. Deur karakterisering kon daar voorgestel word dat hierdie drie formulerings ideale eienskappe vir effektiewe topikale aflewering, toon. Dus, al drie van die formulerings het met klein druppels, 'n ideale oppervlaklading en stabiliteit vir suksesvolle diffusie voorgekom.

Die effektiwiteit van elk van die drie formulerings is geëvalueer deur vas te stel of enige AFB vrylating en/of diffusie van die AFB deur die vel plaasgevind het. *In vitro* membraan- en veldiffusiestudies was dus op elkeen van die formulerings gedoen (Wiechers, 2008:23; Williams, 2013:683). Franz sel diffusiestudies was uitgevoer deur gebruik te maak van die

vertikale Franz sel metode, wat bestaan uit 'n twee-komponent diffusiesel wat geskei word deur 'n membraan of 'n stuk vel (Williams, 2013:683). Vrylating van die AFB vanuit die drie verskillende formulerings, onderskeidelik, was eerstens geëvalueer deur *in vitro* membraanvrylatingstudies en na afleiding daarvan was veldiffusiétudes gedoen. Dus kon daar vasgestel word of daar enige topikale en/of transdermale aflewering plaasgevind het.

Eksperimentele vloedwaardes van artemeter, verkry deur membraanvrylatingstudies, het bewys dat artemeter wel vanuit al drie van die formulerings vrygestel was. Gedurende die velstudies, het slegs die nano-emulsie behoud van artemeter binne die stratum korneum tot gevolg gehad en alhoewel slegs klein hoeveelhede daarvan die vel deurgedring het tot in die sirkulasie, was hierdie gekwantifiseerde waardes laer as die grens van opsporing (LOD) asook die laagste grens van kwantifisering (LLOQ). Dus kan daar gesê word dat artemeter nie tot in die sistemiese sirkulasie kan beweeg nie, maar wel in die buitenste laag van die epidermis gevind kan word – die teikenarea. Die nano-emuljel en konvensionele emuljel het egter geen artemeter in die vel of deur die vel tot gevolg gehad nie. Hierdie nie-bestaande vloedwaardes kan moontlik toegeskryf word aan die formulering self, sowel as die gemiddelde pH waardes van die formulerings wat by  $6.82 \pm 0.03$ ,  $5.14 \pm 0.02$  en  $5.86 \pm 0.02$ , onderskeidelik net 0.11%, 5.44% en 1.09% ongeïoniseerd is. Lae ongeïoniseerde spesies kan lei tot lae of geen diffusie, waar hoë ongeïoniseerde spesies effektiewe diffusie deur die vel kan veroorsaak (Li *et al.*, 2012:98; Williams, 2003:38). Artemeter se swak wateroplosbaarheid ( $0.1053 \pm 0.0022$  mg/ml) (water) en  $0.090 \pm 0.0030$  mg/ml (fosfaatbufferoplossing (FBO) (pH 7.4)) te same met die aanvanklike lae konsentrasie van artemeter in die formulerings, wat wissel tussen 0.4% en 0.8%, kan ook diffusie resultate beïnvloed.

Om die veiligheid van artemeter en die optimale topikale nano-emulsie op die menslike vel te bepaal, was *in vitro* sitotoksiteitstudies op normale menslike keratinosiete (HaCaT) selle voltooi. Sitotoksiese evaluering op die HaCaT selle het geskied deur die uitvoering van 'n methylthiazol tetrazolium (MTT) toets. Gevolglik was daar gevind dat die optimale nano-emulsie (met en sonder artemeter) en artemeter self as nie-sitotoksies voorgekom het (aangesien daar minder as 20% seldood was) wanneer die selle met 'n 0.5% en 'n 1.0% formulering, onderskeidelik, behandel was. Alhoewel daar voorgestel kan word dat die nano-emulsie en artemeter nie sitotoksies voorkom nie, moet verdere *in vivo* eksperimente of *in vitro* doeltreffendheidstudies voltooi word om die werklike sukses van die formulering teen *M. tuberculosis* in lae konsentrasies te evalueer.

Dit kan voorstel dat artemeter topikaal afgelewer kan word en dat die behoud daarvan in die epidermis moontlik bereik kan word. Gedurende hierdie studie is baie lae konsentrasies van artemeter deur die optimale o/w nano-emulsie (wat artemeter en saffloerolie bevat) topikaal vrygestel. Swak wateroplosbaarheid kan moontlik hierdie lae konsentrasies van artemeter wat

gekwantifiseer is verduidelik. Verdere ondersoek is wel nodig, aangesien dit blyk dat die optimalisering van die formule, die uitdaging is vir die topikale aflewering van artemeter (as nuwe behandeling vir KTB), moontlik kan oorkom.

**Sleutelwoorde:** Topikale geneesmiddelaflewering, Kutaneuse tuberkulose, Artemeter, Nano-emulsie, Saffloerolie, Franz sel diffusie.

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## CHAPTER 1: INTRODUCTION, PROBLEM STATEMENT AND AIMS

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## CHAPTER 2: FORMULATION AND TOPICAL DELIVERY OF A NANO-EMULSION CONTAINING ARTEMETHER AND SAFFLOWER OIL

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# ABBREVIATIONS

---

|                                 |                                                                                    |
|---------------------------------|------------------------------------------------------------------------------------|
| %EE                             | Entrapment efficiency                                                              |
| %RSD                            | Percentage relative standard deviation                                             |
| ACN                             | Acetonitrile                                                                       |
| AFB                             | Aktiewe farmaseutiese bestanddele                                                  |
| AIDS                            | Autoimmune deficiency syndrome                                                     |
| API                             | Active pharmaceutical ingredient                                                   |
| APVMA                           | Australian Pesticides and Veterinary Medicines Authority                           |
| ArtS                            | Artemether stock solution                                                          |
| CEG                             | Conventional emulgel containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil |
| CIR                             | Cosmetic Ingredient Review                                                         |
| CTB                             | Cutaneous tuberculosis                                                             |
| DMEM                            | Dulbecco's Modified Eagle Medium                                                   |
| DMSO                            | Dimethyl sulfoxide                                                                 |
| ED                              | Epidermis-dermis                                                                   |
| FBO                             | Fosfaatbufferoplossing                                                             |
| FBS                             | Foetal Bovine Serum                                                                |
| FDA                             | Food and Drug Administration                                                       |
| H <sup>+</sup>                  | Hydrogen ions                                                                      |
| H <sub>2</sub> O <sub>2</sub>   | Hydrogen peroxide                                                                  |
| HaCaT                           | Human keratinocytes / menslike keratinosiete                                       |
| HCl                             | Hydrochloric acid                                                                  |
| HLB                             | Hydrophilic-lipophilic balance                                                     |
| HPLC                            | High performance liquid chromatographic                                            |
| HRTEM                           | High-resolution transmission electron microscope                                   |
| ICH                             | International Conference of Harmonisation                                          |
| KH <sub>2</sub> PO <sub>4</sub> | Potassium dihydrogen orthophosphate                                                |

|                    |                                                                                |
|--------------------|--------------------------------------------------------------------------------|
| KTB                | Kutaneuse tuberkulose                                                          |
| LDH                | Lactate dehydrogenase                                                          |
| LLOQ               | Lower limit of quantification                                                  |
| LOD                | Limit of detection                                                             |
| Log D              | Octanol-buffer distribution coefficient / oktanol-buffer-verdelingskoëffisiënt |
| Log P              | Octanol-water partition coefficient / oktanol-water-verdelingskoëffisiënt      |
| MDR-TB             | Multi-drug-resistant TB                                                        |
| MEM NEAA           | MEM Non-Essential Amino Acid                                                   |
| MR                 | Mycothiol reductase                                                            |
| MTT                | Methylthiazol tetrazolium                                                      |
| NAD(P)H            | Nicotinamide adenine dinucleotide phosphate                                    |
| NaOH               | Sodium hydroxide                                                               |
| NE1                | O/w nano-emulsion with a Tween® 80:Span® 60 ratio of 4:1                       |
| NE2                | O/w nano-emulsion with a Tween® 80:Span® 60 ratio of 3:2                       |
| NEG                | Nano-emulgel containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil     |
| NH <sub>4</sub> OH | Ammonia                                                                        |
| OH <sup>-</sup>    | Hydroxide ions                                                                 |
| O/w                | Oil-in-water / olie-in-water                                                   |
| OECD               | Organisation for Economic Co-operation and Development                         |
| PBS                | Phosphate buffer solution                                                      |
| PCS                | Photon correlation spectroscopy                                                |
| PdI                | Polydispersity index                                                           |
| Pen/Strep          | Penicillin/Streptomycin                                                        |
| PNE1               | Optimised o/w nano-emulsion placebo (containing no artemether)                 |
| PVDF               | Polyvinylidene fluoride                                                        |
| ROS                | Reactive oxygen species                                                        |
| RSS                | Reaktiewe suurstof spesies                                                     |
| SCE                | Stratum corneum-epidermis                                                      |

|     |                                  |
|-----|----------------------------------|
| SD  | Standard deviation               |
| TB  | Tuberculosis                     |
| TEM | Transmission electron microscopy |
| THF | Tetrahydrofuran                  |
| USP | United States Pharmacopeia       |
| W/o | Water-in-oil                     |
| WHO | World Health Organization        |

# CHAPTER 1:

## INTRODUCTION, PROBLEM STATEMENT AND AIMS

---

### 1.1 Introduction

Tuberculosis (TB) is primarily caused by *Mycobacterium tuberculosis* and annually, accountable for approximately 1.5 – 2.0 million deaths (Almaguer-Chávez *et al.*, 2009:563; Bravo & Gotuzzo, 2007:173). TB can be viewed as a health threat, especially with the growing resistance against existing treatment regimens (Almaguer-Chávez *et al.*, 2009:562; Van Zyl *et al.*, 2015:629). TB can occur as pulmonary or extra-pulmonary presentations; one form of the latter can present itself as cutaneous tuberculosis (CTB), which is responsible for 1.5% of all TB cases and 0.1% – 1.0% of all skin disorders (Almaguer-Chávez *et al.*, 2009:562). Although the presence of CTB is minimal, it should be viewed with more urgency, as patients diagnosed with TB increases on a regular basis (Van Zyl *et al.*, 2015:630). This, together with the fact that there is no existing topical TB treatment, presents an opportunity for the development of novel topical treatment of CTB (Van Zyl *et al.*, 2015:630). It must be proposed that this potential novel topical treatment would be used in combination with existing systemic treatment regimens. Systemic treatment in combination with topical treatment could contribute to better treatment and clearance of CTB lesions (Van Zyl *et al.*, 2015:636; Wyrzykowska *et al.*, 2012:297).

As a result, this study forms part of the MRC flagship programme, MALT-B-Redox, which focuses on the development of alternative TB treatment combinations that can eradicate the TB bacterium. The alveolar macrophage stage is an essential stage in the development of the TB bacterium as it is exposed to large volumes of oxygen (Haynes, 2015; Wyrzykowska *et al.*, 2012:293). Flavoenzyme disulphide reductase, an intracellular enzyme, is responsible for maintaining a suitable reducing environment, which helps the bacterium act against the production of reactive oxygen species (ROS) and other oxidants. An example of such a flavoenzyme disulphide reductase is mycothiol reductase (MR), which produces reduced mycothiol through the transference of electrons from nicotinamide adenine dinucleotide phosphate (NAD(P)H) (Haynes, 2015).

The drug group artemisinin can oxidise these reduced flavin cofactors of flavoenzymes disulphide reductase, thus limiting the production of reduced mycothiol (Haynes, 2015). Artemisinin combinations have been found to lead to submicromolar activity against *M. tuberculosis* and because of this, artemisinin are one of the drug groups that are being investigated as possible treatment of TB (Haynes, 2016; Miller *et al.*, 2011:2076; Shakya *et al.*, 2012:702). Artemisinin, viewed as an oxidant drug, could lead to cytotoxic levels of ROS by

means of affecting MR. This increase of ROS production and oxidative stress is accountable for cell death; and so, therefore the death of the bacterium (Haynes, 2015; Nneji *et al.*, 2013:2619). Consequently, during this study artemether, a semi-synthetic derivative of artemisinin, is investigated as a possible treatment for CTB.

CTB treatments should commence through a topical delivery system. Topical drug delivery aims to keep the active pharmaceutical ingredient (API) in the skin, following direct application to the targeted site (Williams, 2013:676). Although the topical delivery route presents many advantages, the skin is one important limitation that cannot be overlooked (El Maghraby *et al.*, 2008:204). The skin is by far the largest and most intricate organ of the human body, as it covers approximately 2 m<sup>2</sup> of surface area (Williams, 2013:677). The anatomy of the skin, considered as a multi-layered structure, consists of three main layers: the hypodermis, dermis and the epidermis (Geethu *et al.*, 2014:1811). The outer skin layer, the epidermis, consists of the outermost stratum corneum and the underlying viable epidermis (Williams, 2003:2). The latter is constituted by three layers namely the stratum basale, stratum spinosum and the stratum granulosum (Williams, 2003:5). This underlying viable epidermis creates a hydrophilic environment as it is mostly made up of protein and water (Jepps *et al.*, 2013:155; Kute & Saudagar, 2013:372). The epidermis consists largely of keratinocytes, which undergo cell differentiation in order to play an important role in the stratum corneum's composition (Lai-Cheong & McGrath, 2009:223).

Of these four epidermal layers, the outermost stratum corneum acts as the main barrier and is responsible for the protective action of the skin, which is due to the stratum corneum's structure, consisting of 10 to 15 layers of corneocytes (Bouwstra & Ponc, 2006:2081; Venus *et al.*, 2011:471). With the corneocytes being the "bricks" and the lipid bilayers, formed by stratum corneum lipids, representing the "mortar," it can widely be described as a "brick and mortar" model (Menon, 2002:S7). The stratum corneum lipids, which are responsible for the lipophilic nature of this layer, can be divided into three classes, i.e. cholesterol, long chain free fatty acids and ceramides (Menon, 2002:S8). The stratum corneum lipids control and regulate the movement of APIs through the skin and therefore act as a drug flux regulator (Williams, 2003:10). The drug flux value is indicative of the quantity of an API that can move across the layers of the skin (Williams, 2003:28).

An API, presented to the skin through a topical delivery system, undergoes various partitioning and permeation processes (Williams, 2013:679). Drug movement through membranes is used to define the concept of permeation (Williams, 2003:27). APIs can permeate the skin and move across the stratum corneum through three available routes, namely the transcellular-, intercellular- or transappendageal routes (El Maghraby *et al.*, 2008:205; Williams, 2013:679). The skin, especially the stratum corneum, limits the extent of the permeation of an API

(Williams, 2013:682). Consequently, an API's physicochemical properties, such as molecular mass, aqueous solubility, octanol-water partition coefficient (log P) and melting point, require careful consideration when formulating a topical product (Williams, 2013:682).

The United States Pharmacopeia (USP) (2013) states that artemether has a molecular mass of 298.37 g/mol. An ideal molecular mass of less than 500 g/mol can result in successful skin permeation, since larger molecules would permeate through the stratum corneum barrier much slower than smaller molecules (Williams, 2003:37). An ideal API for skin permeation should possess an aqueous solubility of more than 1 mg/ml (Naik *et al.*, 2000:319). Artemether is practically insoluble (USP, 2013) in water and in a study conducted by Haynes *et al.* (2006:2083), it was found to have an aqueous solubility of 117 mg/l, thus less than 1 mg/ml, and therefore does not comply with the solubility properties of an ideal topical API. An API with a melting point of less than 200 °C is ideal for topical delivery, which qualifies artemether as an ideal candidate, as its melting point is between 86 – 90 °C (Drugbank, 2016). The log P is an important property to investigate in what way an API would act within the skin and can be defined as the movement of an API between two phases (Williams, 2003:27). The ideal log P for an API should be between 1 and 3 and APIs that are both lipophilic and hydrophilic would in essence be ideal for topical delivery (Subedi *et al.*, 2010:339). Artemether has a log P value of 3.53 indicating it is highly lipophilic (Drugbank, 2016). Although it does not comply with the log P of an ideal topical API, the lipophilic artemether would probably be retained in the lipophilic stratum corneum and not permeate to the hydrophilic viable epidermis. Formulations with both hydrophilic and lipophilic characteristics such as nano-emulsions can deliver APIs to the stratum corneum, or facilitate percutaneous absorption depending on what is set out to be achieved (Gaur *et al.*, 2014:37).

Henceforth in this study, a nano-emulsion will be used as topical delivery system of artemether. Nano-emulsions can be defined as the composition of emulsified oil and water phases, with droplets ranging from 20 – 200 nm (Chime *et al.*, 2014:77; Solans *et al.*, 2005:102). Depending on the incorporated API, nano-emulsions can be formulated with either water or oil as the core component, forming a water-in-oil (w/o) or oil-in-water (o/w) nano-emulsion (Chime *et al.*, 2014:77; Kela & Kaur, 2013:9203). The rationale behind using a nano-emulsion as a delivery system for topical formulations instead of conventional emulsions is due to the many advantages they possess. Some advantages include the skin being able to tolerate nano-emulsions well, the small droplets enabling easy skin penetration and absorption, together with the advantages of nano-emulsions being used to deliver insoluble APIs, hence improving bioavailability (Chime *et al.*, 2014:79). As a result of these small droplets, stability can also be achieved because there is a lower presence of creaming, coalescence, sedimentation or flocculation (Kela & Kaur, 2013:9206; Lu *et al.*, 2014:826; Tadros *et al.*, 2004:303). The most promising advantages of nano-emulsions is that they can act as penetration enhancers and can

result in targeted or controlled delivery of a drug (Maruno & Da Rocha-Filho, 2010:17). Special techniques applying high-energy such as high-pressure homogenisation or ultrasonication is sometimes needed for the formulation of small droplets in nano-emulsions (Kela & Kaur, 2013:9206; Lovelyn & Attama, 2011:629; Tadros *et al.*, 200:304).

Many approaches have been attempted to overcome the stratum corneum barrier properties and to achieve improved drug flux (Williams, 2013:693). The success of penetration enhancers is as a result of the barrier properties of the skin being modified and reduced, resulting in increased partitioning and absorption of the API (Alexander *et al.*, 2012:29). Fatty acids, found in natural oils, are leading chemical penetration enhancers as they have the ability to enhance the movement of APIs across the stratum corneum (Vermaak *et al.*, 2011:922; Wang *et al.*, 2003:1612). Their enhancement is a result of interfering with the lipids in the stratum corneum through the interaction with cellular proteins (Wang *et al.*, 2003:1612). This leads to the disruption of the lipid bilayers and hence an increase in partitioning, especially of lipophilic APIs (Wang *et al.*, 2003:1612; Williams, 2013:694). Two classes of fatty acids are available, namely saturated and unsaturated fatty acids, with the latter having demonstrated more success as penetration enhancers (Wang *et al.*, 2003:1612). Linoleic acid, for example, an 18-carbon fatty acid that is found in natural oils, i.e. safflower oil (as used in this study), is an extremely important unsaturated fatty acid as it is also present in the human skin. Due to the presence in the skin, natural oils are generally safe to use, hence, lowering the possibility of skin irritation (Büyüktimkin *et al.*, 1997:433; Menon, 2002:S9; Vermaak *et al.*, 2011:922). Safflower oil is mostly composed of fatty acids including 75% linoleic, 13% oleic, 6% palmitic and 3% stearic acid (Van Wyk & Wink, 2009:81; Wolters Kluwer Health, 2009).

Due to the lipophilic nature of artemether and not complying with all the ideal characteristics of a topical API, formulating it into an o/w nano-emulsion can help overcome these limitations. Safflower oil, as penetration enhancer, will form the core component of the oil phase of the nano-emulsion together with the lipophilic artemether. This will result in an o/w nano-emulsion containing artemether and safflower with the possibility of leading to better topical delivery. For easier application semi-solid dosage forms of the nano-emulsion will also be investigated. Three formulations, i.e. a nano-emulsion, nano-emulgel as well as a conventional emulgel will be compared to determine the most successful formulation.

## **1.2 Research problem**

Currently there is no existing topical CTB treatment and due to growing resistance, it is necessary to investigate the topical delivery route (Almaguer-Chávez *et al.*, 2009:563; Van Zyl *et al.*, 2015:629). Artemether, a derivative of artemisinin, could possibly provide properties that could be effective against the *M. tuberculosis* bacterium.

As the stratum corneum layer of the skin acts as the main barrier to external influences, such as micro-organisms, due to its complex, impermeable and lipophilic structure it can be viewed as the largest challenge to overcome during topical drug delivery (Subedi *et al.*, 2010:339; Venus *et al.*, 2011:472). Together with this barrier, the physicochemical properties of artemether are not ideal for topical delivery, which poses a grave challenge.

### 1.3 Aims and objectives

The aim of this study is to investigate the topical delivery of artemether by formulating it into an o/w nano-emulsion that contains a natural oil, i.e. safflower oil. The formulated nano-emulsion will be compared to a nano-emulgel as well as a conventional emulgel. This is done to determine which formulation presents more valuable in the topical delivery of artemether. In addition, artemether (the API) and the nano-emulsion will be investigated further, by means of *in vitro* cell culture cytotoxic experiments, to establish the safety towards human skin cells.

The following objectives have been set to achieve the aforementioned aims:

- \* Validate a high performance liquid chromatographic (HPLC) analytical method for the quantitative determination of the artemether concentrations in the formulations.
- \* Determine artemether's aqueous solubility and octanol-buffer distribution coefficient (log D).
- \* Formulate a nano-emulsion, nano-emulgel as well as a conventional emulgel by incorporating artemether, as well as safflower oil in the oil phase.
- \* Characterise the nano-emulsion in terms of droplet size, zeta-potential, morphology, entrapment efficiency, viscosity, pH and visual examination.
- \* Characterise the nano-emulgel and the conventional emulgel in terms of droplet size, zeta-potential, morphology, viscosity, pH and visual examination.
- \* Determine the release of artemether from the nano-emulsion, nano-emulgel and conventional emulgel through membrane diffusion studies.
- \* Determine the transdermal and topical delivery of artemether from the formulated nano-emulsion, nano-emulgel and conventional emulgel through and into the skin, by performing both Franz cell skin diffusion studies and tape stripping, respectively.
- \* Determine the cytotoxic effects of artemether, alone and in the optimised nano-emulsion, by conducting *in vitro* cell culture cytotoxic experiments on human keratinocytes (HaCaT).

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USP **see** United States Pharmacopeia

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# CHAPTER 2:

## FORMULATION AND TOPICAL DELIVERY OF A NANO-EMULSION CONTAINING ARTEMETHER AND SAFFLOWER OIL

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### 2.1 Introduction

TB can be classified as a contagious disease caused by *M. tuberculosis*, which has already infected approximately 2 billion people globally (Dipiro, 2012:584). Approximately 10% – 20% of all TB occurrences do not display pulmonary, but extrapulmonary. CTB, a form of extrapulmonary TB, occurs 1.5% of the time, when the *M. tuberculosis* bacterium enters the skin either through direct contact or through airborne particles (Almaguer-Chávez *et al.*, 2009:562-563; Arora *et al.*, 2006:344; VanderVen *et al.*, 2015:2). CTB can clinically present in a variety of forms, but commonly all forms pose as ulcer-like, infectious lesions (Bravo & Gotuzzo, 2007:174-177).

Although CTB treatment can commence through oral TB regimes, patient compliance is unfavourable. This is due to treatment being based on an extensive combination of drugs used over a period of months (Van Zyl *et al.*, 2015:634-635). Resistant strains of *M. tuberculosis* pose another problem during treatment, as they are becoming untreatable (Van Zyl *et al.*, 2015:634). As a result, the need exists for the development of novel APIs or alternative formulations (VanderVen *et al.*, 2015:2). Existing drugs, such as the anti-malarial drug group artemisinin, is one of the groups being investigated as a possible anti-TB treatment (Haynes, 2016; Miller *et al.*, 2011:2076; Nneji *et al.*, 2013:2619). Artemether, a semi-synthetic derivate of this artemisinin group, will be investigated in this study as a novel topical CTB treatment (McIntosh & Olliaro, 2010:2; Shahzad *et al.*, 2013:197). Used in combination with oral TB treatment, this novel topical treatment hopes to increase patient compliance and decrease the length of treatment as well as side effects.

Although topical and transdermal drug delivery focuses on the delivery of an API to the skin, with topical delivery the API is retained within the skin (Williams, 2013:675). Transdermal delivery, however, is designed for systemic circulation drug delivery (Williams, 2013:676). As artemether can be viewed as a low aqueous soluble and lipophilic API, which is highly metabolised through the liver, the topical delivery thereof presents favourably for investigation (Shahzad *et al.*, 2013:197). The topical drug delivery route, although mostly advantageous, presents with an important limitation, which cannot be overlooked, namely the skin (El Maghraby *et al.*, 2008:204; Wang *et al.*, 2003:1612). The skin is composed of various distinct layers, providing a barrier to the API and consequently influences the topical delivery thereof

(Bouwstra & Ponec, 2006:2081; Ghafourian *et al.*, 2010:612; Williams, 2013:679). This barrier function of the skin is mainly represented by the lipophilic stratum corneum (Bouwstra & Ponec, 2006:2081; Foldvari, 2000:417; Naik *et al.*, 2000:318). In being so lipophilic, the stratum corneum acts as the least permeable layer of all the skin layers, therefore also acting as a drug flux regulator (Marrow *et al.*, 2007:36; Naik *et al.*, 2000:318-319; Williams, 2013:678). A successful delivery system should consequently be selected to contribute to more efficient drug delivery and absorption (Foldvari, 2000:417).

Due to their many advantages, nano-emulsions have been selected as the drug delivery system for the development of a novel CTB treatment. The most positive contribution of nano-emulsions to drug delivery is that they are two-phase dispersions, presenting with both hydrophilic and lipophilic characteristics (Gaur *et al.*, 2014:37). Nano-emulsions can also lead to controlled drug delivery to a targeted site, resulting in depot localisation, which would essentially be needed during the treatment of CTB (Abolmaali *et al.*, 2011:140; Clares *et al.*, 2014:591; Lai *et al.*, 2008:1; Lu *et al.*, 2014:826). Not only can nano-emulsions be used as penetration enhancers, but fatty acids found in natural oils, such as safflower oil, have also been proven to be natural penetration enhancers that can lead to increased API permeation (Boelsma *et al.*, 1996:729).

As a result, the formulation of an o/w nano-emulsion containing safflower oil proposes to deliver artemether topically for the treatment of CTB, used concurrently with systemic treatment.

## **2.2 Tuberculosis**

### **2.2.1 Epidemiology of tuberculosis**

When the epidemiology of TB (pulmonary or extra-pulmonary) is investigated, it is found that TB is primarily caused by the infectious *M. tuberculosis* bacterium, although in some cases, it was reported that *Mycobacterium bovis* was the source of TB (Almaguer-Chávez *et al.*, 2009:563; Bravo & Gotuzzo, 2007:173). TB can most certainly be viewed as a pandemic as it is responsible for approximately 2 – 3 million deaths annually (Almaguer-Chávez *et al.*, 2009:562). It is also ranked number 5 of the causes responsible for deaths worldwide (Almaguer-Chávez *et al.*, 2009:562). According to data gathered by the World Health Organization (WHO), in 2014 approximately 9.6 million people became infected with TB; this large number results in a health threat (WHO, 2016).

For effective API selection and treatment, the *M. tuberculosis* bacterium involved needs to be investigated. *M. tuberculosis* can be viewed as an aerobic, weak gram-positive, acid-fast bacterium and in being aerobic; it is dependent on large volumes of oxygen (Wyrzykowska *et al.*, 2012:293). After the inhalation of the TB mycobacteria, those that are present in airborne

particles can travel to lung alveoli from where pulmonary infections can develop (Almaguer-Chávez *et al.*, 2009:563). A very important development stage for the aerobic TB bacterium is the alveolar macrophage stage, as it exposes the bacterium to great amounts of oxygen, especially when it is present in the lungs (Haynes, 2015; Wyrzykowska *et al.*, 2012:293). As natural defences such as flavoenzyme disulphide reductase (an intracellular enzyme) protects the TB bacterium against ROS, novel treatment must be investigated to overcome this protection (Haynes, 2015).

### **2.2.2 Resistance of tuberculosis**

TB can be viewed as a health threat especially with the growing resistance against existing treatment regimens (Almaguer-Chávez *et al.*, 2009:562; Van Zyl *et al.*, 2015:629). When the *mycobacterium* has built up resistance to one or more of the main treatment drugs, this occurrence is classified as multi-drug-resistant TB (MDR-TB). These cases are complicated, as patients need to receive therapy they have not received before (Dipiro, 2012:595). With an increase in resistant TB strains, effective drugs have become limited (Almaguer-Chávez *et al.*, 2009:562; Van Zyl *et al.*, 2015:630).

Acquired immunodeficiency syndrome (AIDS), low immunity or the use of immunosuppressants are some of the contributors to the increase in resistance against available treatment (Almaguer-Chávez *et al.*, 2009:562). Poverty in developing countries and lengthy treatment periods are also contributors (Wyrzykowska *et al.*, 2012:293). Furthermore, an increase in CTB occurrences can be ascribed to this growing resistance (Van Zyl *et al.*, 2015:629).

### **2.2.3 Cutaneous tuberculosis**

One of the extra-pulmonary cases of TB can appear as CTB, which makes up about 0.1% – 1.0% of all skin disorders (Almaguer-Chávez *et al.*, 2009:562). CTB can be caused through the inhalation of *M. tuberculosis*, which travels to extra-pulmonary sites such as the skin, or through direct contact (Almaguer-Chávez *et al.*, 2009:563; Bravo & Gotuzzo, 2007:174). CTB can be viewed as a manifestation of the skin, presenting in some occurrences with a tuberculous lesion, which can be displayed in a variety of forms (Bravo & Gotuzzo, 2007:174; Van Zyl *et al.*, 2015:629). Although it is known that the skin provides protection against any external factors, when this protection is compromised, it is easier for these mycobacteria to penetrate the skin (Almaguer-Chávez *et al.*, 2009:563).

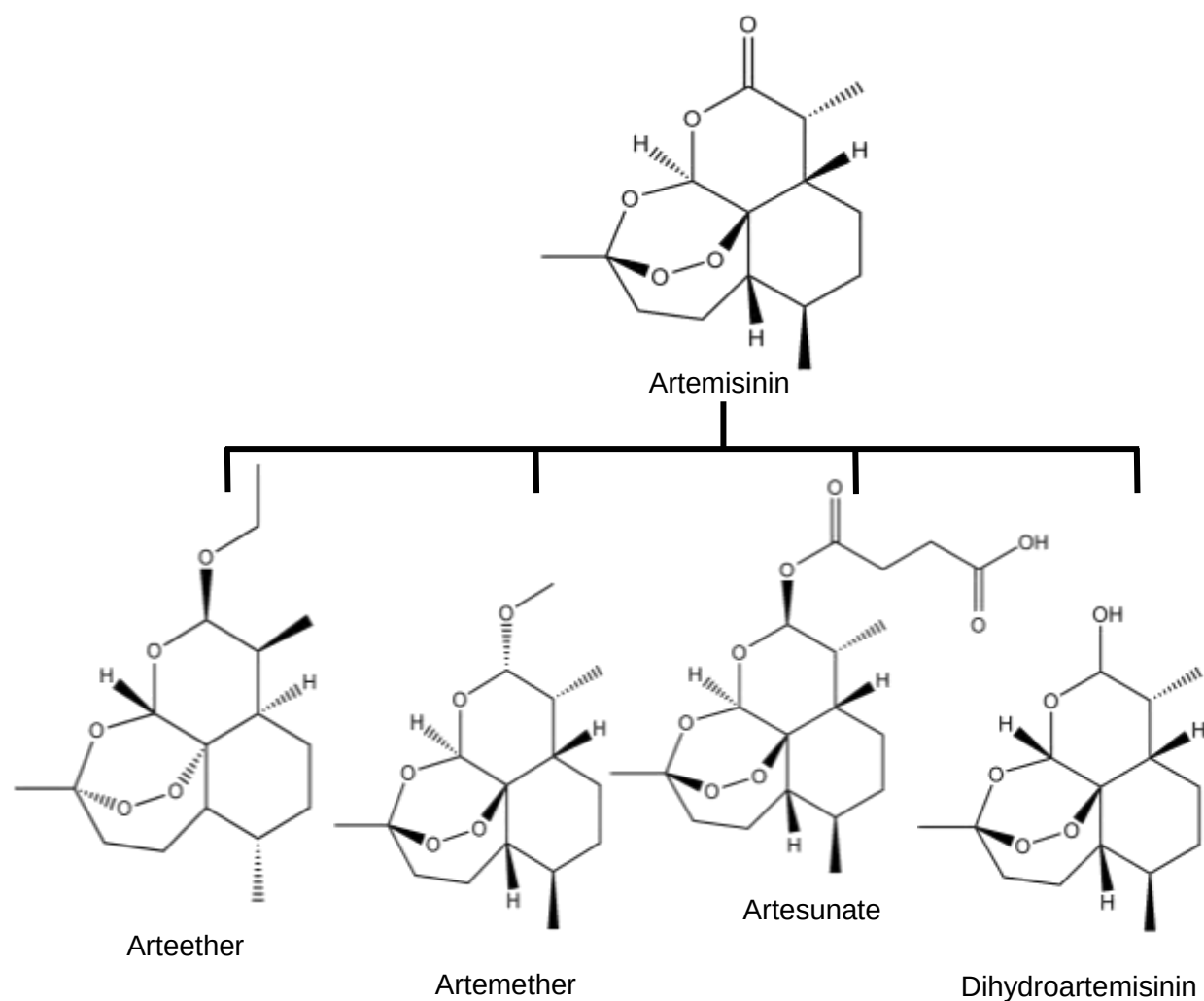
CTB can be characterised into two classes: multibacillary or paucibacillary forms. This characterisation is based on the grounds on which the bacterium multiplies, i.e. through direct inoculations (vaccinations), contiguous infection or by means of haematogenous spreading (spreading through blood) (Bravo & Gotuzzo, 2007:174). Multibacillary forms are primary

inoculated TB, tuberculous chancre, scrofuloderma, TB periorificialis, as well as acute miliary TB (Bravo & Gotuzzo, 2007:174). Paucibacillary forms are more difficult to identify as minimal bacteria are present and consequently it is difficult to isolate them. These forms, for example *lupus vulgaris*, are mainly spread through the blood (Bravo & Gotuzzo, 2007:174). Although the diagnosis of CTB is very difficult, as it presents with a wide clinical range, the most common occurrences of CTB appear as scrofuloderma or as mentioned *lupus vulgaris* (Almaguer-Chávez *et al.*, 2009:563; Bravo & Gotuzzo, 2007:173; Van Zyl *et al.*, 2015:630).

## 2.3 Novel tuberculosis treatment

Growing resistance, together with the fact that there is no existing topical CTB treatment provides an opportunity for the investigation and development thereof (VanderVen *et al.*, 2015:2; Van Zyl *et al.*, 2015:630). The endoperoxide anti-malarial drug group, artemisinin, are being investigated as possible anti-TB treatment (Haynes, 2016; Miller *et al.*, 2011:2076; Nneji *et al.*, 2013:2619).

### 2.3.1 Artemisinin



**Figure 2.1:** Derivatives of artemisinin

Ginghaosu, or better known as artemisinin, is isolated from the leaves or flower clusters of the Chinese plant – *Artemisia annua* (McIntosh & Olliaro, 2010:2; Rogers, 2014; Shrivastava *et al.*, 2010:79). This principal isolate has been used as early as the 1970's, but it was soon found that it possessed weak solubility; resulting in unfavourable bioavailability (McIntosh & Olliaro, 2010:2; Shahzad *et al.*, 2013:197). As a result of this disadvantage, semi-synthetic derivatives were created in order to increase solubility as well as absorption. Chemical modification of artemisinin produced four derivatives namely: arteether, artemether, artesunate and dihydroartemisinin (McIntosh & Olliaro, 2010:2; Shahzad *et al.*, 2013:197) (see Figure 2.1). These four derivatives are normally used as successful treatment against malaria due to their unique structure and effectiveness (Ebrahimisadr *et al.*, 2014:1; McIntosh & Olliaro, 2010:3; Shahzad *et al.*, 2013:197). Consequently, they are being investigated in terms of novel treatment against TB (Haynes *et al.*, 2006:2136; Haynes, 2015; McIntosh & Olliaro, 2010:3; Miller *et al.*, 2011:2076; Van Zyl *et al.*, 2015:630). As TB and CTB are caused through the same *mycobacterium*, *M. tuberculosis*, artemisinin can also be investigated as possible treatment for CTB (Almaguer-Chávez *et al.*, 2009:562-563).

### **2.3.2 Artemisinin as tuberculosis treatment**

The effectiveness of artemisinin, as an anti-malarial drug and possible TB treatment, can be ascribed to the fact that the endoperoxide bridge in their chemical structure leads to the production of free radicals (Nneji *et al.*, 2013:2619; Shrivastava *et al.*, 2010:79). These free radicals are due to the interaction between the endoperoxide bridge and the heme molecules found in the parasite's food vacuole (Chadha *et al.*, 2010:190; Shrivastava *et al.*, 2010:79). Peroxides are accountable for the production of free radicals, acting as a source of ROS, causing oxidative stress in the parasitic cells (Ebrahimisadr *et al.*, 2014:1; Nneji *et al.*, 2013:2619). Through increased oxidative stress, an oxidising environment is presented to the *M. tuberculosis* bacterium, therefore leading to cell death (Haynes, 2015; Haynes, 2016; Nneji *et al.*, 2013:2619).

Artemisinin as TB treatment is consequently based on the oxidative effects that artemisinin possesses, which can lead to cytotoxic levels of ROS by means of affecting MR, an intracellular flavoenzyme disulphide reductase enzyme (Haynes, 2015). For this reason, artemisinin can be viewed as an oxidative drug group with the possibility of reducing *M. tuberculosis* (Haynes, 2015). However, natural defences, such as flavoenzyme disulphide reductase, found in the parasite's enzymatic antioxidant system counter the oxidative effects of artemisinin (Haynes, 2015; Nneji *et al.*, 2013:2619). Artemisinin can be irreversibly reduced and as a result, loses its effect and the formation of mycothiol will resume when the reduced flavin is restored by NAD(P)H electrons transferring to the flavoenzyme (Haynes, 2015). To guarantee that this

reactivation of the flavoenzymes, by NAD(P)H cannot occur, a redox drug is needed. This forms the grounds for the MALTB-Redox flagship programme, of which this study is a part of.

### 2.3.3 Artemether as derivative and API

As mentioned, chemical modification of artemisinin resulted in four semi-synthetic derivatives, artemether being one of these derivatives (Da Costa César *et al.*, 2009:737; McIntosh & Olliaro, 2010:2). This study will focus primarily on the topical delivery of artemether delivered through an o/w nano-emulsion containing safflower oil. An optimised o/w nano-emulsion will be formulated into a nano-emulgel, which will be compared to a conventional emulgel.

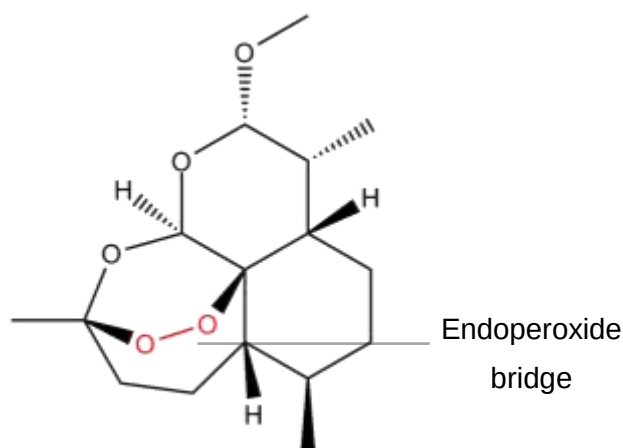
When investigating artemether closer, it can be noted that it undergoes rapid liver metabolism to dihydroartemisinin, the active metabolite (Karbawang *et al.*, 1997:259; Shahzad *et al.*, 2013:197). Liver enzymes accountable for this rapid metabolism are cytochrome P450 enzymes, more specifically cytochrome P450 3A4 and 3A5 (Drugbank, 2016). As a result of this metabolism, artemether is mainly administered as an intramuscular injection (Da Costa César *et al.*, 2009:737). Artemether can also be formulated into a variety of oral dosage forms ranging from tablets, capsules to combination medication (McIntosh & Olliaro, 2010:3). Commercially artemether is implemented in a combination anti-malaria treatment with lumefantrine, better known as Coartem® (Nneji *et al.*, 2013:2620). Although the oral bioavailability of artemether is still very poor, resulting in a half-life of 2 – 3 h, it does possess many advantages such as (Shahzad *et al.*, 2013:197):

- \* low toxicity and irritation (Gao *et al.*, 2013:134),
- \* rapid onset of action (Shahzad *et al.*, 2013:197),
- \* great effectiveness and potency against bacteria (Shahzad *et al.*, 2013:197) and
- \* various routes of administration, with the topical and transdermal route proposing to be ideal (Gao *et al.*, 2013:134; Shahzad *et al.*, 2013:197).

#### 2.3.3.1 Chemical structure of artemether

Artemether's chemical formula is C<sub>16</sub>H<sub>26</sub>O<sub>5</sub>, resulting in a molecular weight of 268.4 g/mol (Ebrahimisadr *et al.*, 2014:1; USP, 2013). Artemether (3R, 5aS, 6R, 8aS, 9R, 10S, 12R, 12aR)-decahydro-10-methoxy-3, 6, 9-trimethyl-3, 12-epoxy-12Hpyrano [4,3-j]-1, 2-benzodioxepin, which acts as an *o*-methyl ether prodrug of dihydroartemisinin, can also be referred to as dihydroartemisinin methyl ether, artemos, β-artemether, etc. (Da Costa César *et al.*, 2009:737; PubChem, 2016; Shrivastava *et al.*, 2010:79). The chemical structure of artemether (see Figure 2.2), consisting of a sesquiterpene lactone, contains an endoperoxide bridge as the core component, which results in the effective mode of action it presents against parasites

(Ebrahimisadr *et al.*, 2014:1; Shahzad *et al.*, 2013:197). A sesquiterpene lactone is a compound consisting of three isoprene units bound to a lactone (a cyclic organic ester ring formed by more than two carbon atoms, a single oxygen atom and a ketone group located at one of the carbons, adjacent to the other oxygen molecule) (Rogers, 2014; Sigma-Aldrich; 2016).



**Figure 2.2:** Chemical structure of artemether

### 2.3.3.2 Physicochemical properties of artemether

A summary of the physicochemical properties of artemether is tabulated in Table 2.1, which includes its molecular mass, melting point, log P, solubility and pKa value.

**Table 2.1:** Summary of the physicochemical properties of artemether

| Physicochemical properties | Value                                                                                                    | Reference                                      |
|----------------------------|----------------------------------------------------------------------------------------------------------|------------------------------------------------|
| <b>Molecular mass</b>      | 298.37 g/mol                                                                                             | Ebrahimisadr <i>et al.</i> , 2014:1; USP, 2013 |
| <b>Melting point</b>       | 86 – 90 °C                                                                                               | Drugbank, 2016                                 |
| <b>Log P</b>               | 3.53                                                                                                     | Drugbank, 2016                                 |
| <b>Solubility</b>          | Very slightly soluble in water; greatly soluble in dichloromethane; soluble in ethanol and ethyl acetate | Sunil <i>et al.</i> , 2010:93; USP, 2013       |
| <b>pKa</b>                 | - 3.9                                                                                                    | Drugbank, 2016                                 |

Artemether is practically insoluble in water (USP, 2013). Its solubility in acetone, as well as dichloromethane, presents very well, but it can be viewed as less soluble in dehydrated ethanol and ethyl acetate (Sunil *et al.*, 2010:93). However, artemether can be viewed as a drug which is not very soluble in water, consisting of a lipophilic log P value of 3.53 (Drugbank, 2016; Karbwang *et al.*, 1997:259; Shahzad *et al.*, 2013:197). Artemether provides a pKa value of - 3.9

(Drugbank, 2016). These properties (summarised in Table 2.1) determine whether artemether would be an ideal API for topical drug delivery and are discussed further in Section 2.7.

## **2.4 Topical and transdermal drug delivery**

For the purpose of keeping the API in the skin, following direct application to the targeted site, topical drug delivery is used (Williams, 2013:676). Transdermal delivery is designed to present the API to the systemic circulation (Williams, 2013:676). During this study, topical drug delivery of artemether will be attempted through an o/w nano-emulsion, nano-emulgel and conventional emulgel containing safflower oil.

### **2.4.1 Advantages of topical drug delivery**

As some orally administered APIs are negatively influenced through a delivery route, topical delivery is a great alternative as it avoids the hepatic and gastrointestinal effects, such as metabolism, gastric pH and food influences (Marrow *et al.*, 2007:37; Naik *et al.*, 2000:319). An increase in patient compliance is achieved as the formulation is applied directly onto the skin and can also be removed without any trouble if necessary (Marrow *et al.*, 2007:37; Naik *et al.*, 2000:319). As a result of this, topical delivery can also be seen as a non-invasive drug delivery route (Naik *et al.*, 2000:319). It is possible to achieve controlled release of the API over extended periods, which can result in steady-state conditions (Jepps *et al.*, 2013:153; Marrow *et al.*, 2007:37). Importantly, improved bioavailability and effectiveness of the API can be achieved (Jepps *et al.*, 2013:153).

### **2.4.2 Disadvantages of topical drug delivery**

Although advantageous, the topical drug delivery route presents one important limitation i.e. the skin that cannot be overlooked (El Maghraby *et al.*, 2008:204; Wang *et al.*, 2003:1612). Effective delivery of APIs is consequently challenged by this skin barrier (Wang *et al.*, 2003:1612). As a result of this disadvantage, the APIs with appropriate physicochemical properties for topical drug delivery are very limited (Marrow *et al.*, 2007:38). The possibility of skin irritation may also exist (Marrow *et al.*, 2007:38).

## **2.5 The skin**

### **2.5.1 Structure and function of the skin**

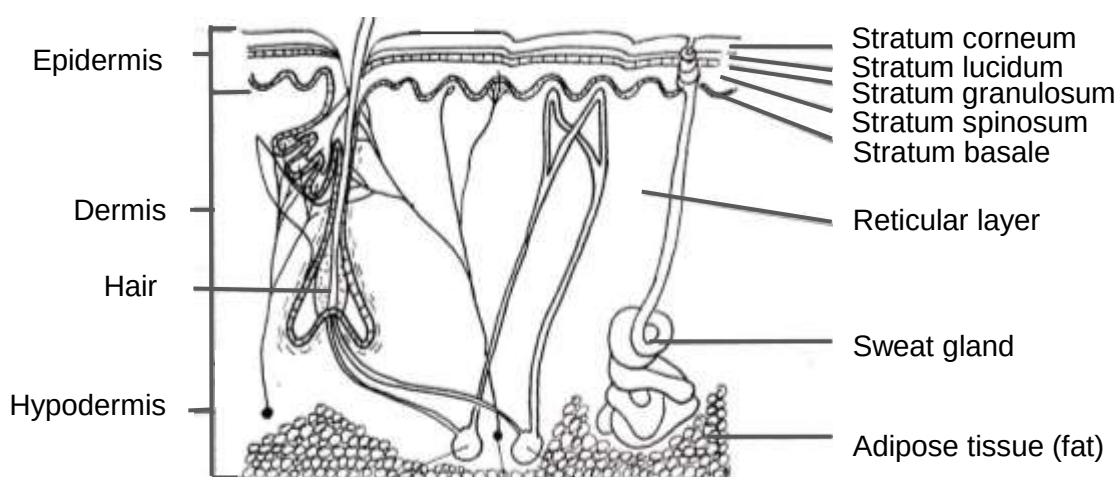
The largest and most easily accessible organ of the human body is the skin (Subedi *et al.*, 2010:339). The skin is the most intricate human organ as it covers approximately 2 m<sup>2</sup> surface area of the body – making up 15% of the total mass of an average adult (Lai-Cheong & McGrath, 2009:223; Wickett & Visscher, 2006:S98; Williams, 2013:677). Viewed as the largest

organ, the skin provides a surface which is greatly accessible for the effortless absorption of APIs (Naik *et al.*, 2000:319). The skin's intricate structure is due to the composition of various distinctive layers (Bouwstra & Ponc, 2006:2081; Menon, 2002:S3). This complex and multi-layered structure is mainly designed to form a protective barrier against the infiltration of external factors such as micro-organisms (Venus *et al.*, 2011:472; Williams, 2013:677).

Besides acting as a protective layer, the skin possesses many other functions:

- \* with an acidic pH, the skin can also act as a chemical barrier resulting in antimicrobial effects (Ng & Lau, 2015:8),
- \* restricts the loss of water (Wickett & Visscher, 2006:S98; Williams, 2013:677),
- \* plays important immunological and sensory functions (Wickett & Visscher, 2006:S98),
- \* plays a role during the production of vitamin D (Wickett & Visscher, 2006:S98) and
- \* manages thermoregulation (Wickett & Visscher, 2006:S98).

When examined closely the anatomy of the skin consists of three main layers each made up of different types of cells, contributing to each layer's function and role; these three layers are the epidermis, dermis and hypodermis (Foldvari, 2000:417; Ng & Lau, 2015:4). These layers have the ability to potentially limit the permeation process of APIs as they provide a sequence of barriers to the API (Williams, 2013:677). For this reason, investigating these layers is very important, as they play a vital role in the permeation process during the administration of topical APIs (Jepps *et al.*, 2013:154). Figure 2.3 illustrates a diagrammatic representation of the main layers that are characteristically of the skin's structure.



**Figure 2.3:** A diagrammatic representation of the human skin structure (adapted from Burton, 1997).

### **2.5.1.1 The epidermis**

The epidermis forms the outermost layer of the skin, as it is composed of different epidermal cell types such as keratinocytes, melanocytes and Langerhans cells (Williams, 2013:678). The epidermis, the layer found on top of the dermis can be viewed as a structure composed of four layers: the stratum basale, stratum spinosum, stratum granulosum and the outermost stratum corneum (Ng & Lau, 2015:4; Venus *et al.*, 2011:471; Williams, 2013:678) (see Figure 2.3). The epidermis can further be divided into the viable and non-viable epidermis (Kute & Saudagar, 2013:272).

#### **2.5.1.1.1 Viable epidermis**

The epidermis without the stratum corneum is referred to as the lower epidermal layer or the viable epidermis (Jepps *et al.*, 2013:154; Ng & Lau, 2015:7). The viable epidermis is the layer located between the stratum corneum and the dermis and is made up mostly of protein and water (Jepps *et al.*, 2013:155; Kute & Saudagar, 2013:372). Consequently, it can be viewed as a hydrophilic environment, as lipids represent only a small portion of this layer (Jepps *et al.*, 2013:155; Kute & Saudagar, 2013:372). Approximately 95% of the viable epidermis consists of keratinocytes, where the remainder of the cells are composed of other epidermal cells (Ng & Lau, 2015:4).

#### **2.5.1.1.2 Non-viable epidermis as skin barrier**

The stratum corneum, being the most outer layer of the epidermis, is referred to as the non-viable epidermis (Kute & Saudagar, 2013:368) (see Figure 2.3). The stratum corneum acts as the main physical barrier and is predominantly responsible for the protective action of the skin (Bouwstra & Ponc, 2006:2081; Kute & Saudagar, 2013:368; Naik *et al.*, 2000:318; Venus *et al.*, 2011:471). This protective action can be ascribed to the morphology of the stratum corneum, as it can be viewed as a unique and very thin layer (Naik *et al.*, 2000:318). Its morphology, consisting of 10 to 15 layers of corneocytes (dead keratinocytes), forms by far the least permeable layer of all the skin layers (Marrow *et al.*, 2007:36; Naik *et al.*, 2000:318; Williams, 2013:678).

The impermeable stratum corneum is due to corneocytes that are arranged in a scaffold-like lattice, closely surrounded by highly dense, intercellular lipid bilayers (El Maghraby *et al.*, 2008:2004; Jepps *et al.*, 2013:154; Venus *et al.*, 2011:471; Williams, 2013:677). For this reason it can be described as a “brick and mortar” model - the flattened corneocytes, being the “brick” and the lipid bilayers, formed by distinctive stratum corneum lipids, representing the “mortar” (Menon *et al.*, 2012:4; Ng & Lau, 2015:4; Williams, 2013:677). The stratum corneum lipids can be divided into three classes: cholesterol, long chain free fatty acids and ceramides

(Menon *et al.*, 2012:4; Williams, 2013:677). Of all these groups, the ceramides are the largest as they make up 50% of the stratum corneum lipids and are therefore viewed as the most important component in the organisation of the stratum corneum (Marrow, 2007:37; Ng & Lau, 2015:4). More than three hundred different types of ceramides have been identified in human stratum corneum (Ng & Lau, 2015:5). The importance of these ceramides are based on their structure as they consist of a sphingoid moiety, which comprises a polar head group, a hydrocarbon chain and a fatty acid moiety (another hydrocarbon chain of fatty acid origin) (Ng & Lau, 2015:5). Long chain unsaturated fatty acids play an important role in the organisation and barrier properties of the skin, as well as during the formulation of topical products (Gaur *et al.*, 2014:1812).

The stratum corneum lipids are also responsible for the control and regulation of the API movement through the skin, thus acting as a drug flux regulator (Williams, 2003:10). The drug flux indicates the quantity of API that can move across the layers of the skin (Williams, 2003:28). Drug flux is greatly influenced by the stratum corneum barrier (Marrow, 2007:36). For the most part APIs tend to encounter the stratum corneum first during this topical permeation process – it can consequently be viewed as a rate-limiting barrier (El Maghraby *et al.*, 2008:204; Jepps *et al.*, 2013:153). Therefore, this brick and mortar structure, consisting of hydrophilic corneocytes and lipophilic bilayers, presents a challenge to both hydrophilic and lipophilic molecule absorption (Baibhav *et al.*, 2011:66).

#### **2.5.1.2 Dermis**

On top of the hypodermis and beneath the viable epidermis, the dermis is positioned with a thickness of approximately 3 – 5 mm (Jepps *et al.*, 2013:677; Kute & Saudagar, 2013:372). The principal component of the skin is the dermis, from where it provides strength and elasticity to the skin as it consists mainly of collagen and elastin (Ng & Lau, 2015:4). A unique hydrophilic environment is created by a mucopolysaccharide gel which surrounds the collagen and elastin (Jepps *et al.*, 2013:677; Williams, 2013:677). Although this layer does not contain any dividing cells, it is rich in blood vessels, vessels that carry lymph, as well as nerve endings (Foldvari, 2000:418).

#### **2.5.1.3 Hypodermis**

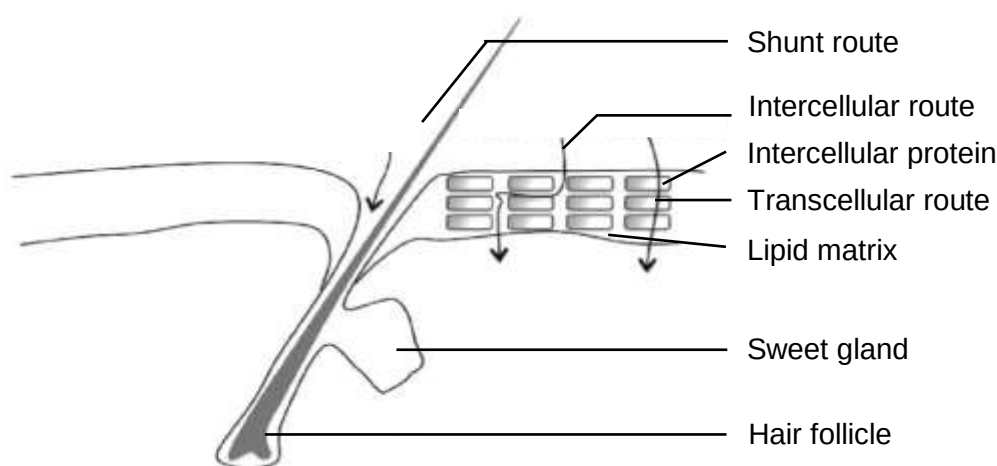
The hypodermis, also known as the subcutaneous layer, consists of adipose tissue (fat), which forms the most inner layer below the dermis (Geethu *et al.*, 2014:1811; Ng & Lau, 2015:7; Williams; 2013:677). This layer's main function is to provide protection against shock of physical nature, insults the body may encounter, it provides insulation against temperature changes and can also act as storage of high-energy molecules (Williams, 2013:677).

## 2.5.2 Drug transport through the skin

As mentioned earlier the structure of the skin can influence the drug flux and consequently the transport of API molecules through the skin (Williams, 2013:679). Drug delivery and transport through the skin is complicated, not only due to the stratum corneum barrier, but also as a result of various processes that are needed (Baibhav *et al.*, 2011:67).

### 2.5.2.1 Skin delivery

Skin delivery can be viewed as a complicated process as it consists of several steps (Wiechers, 2008:7). Initially the API molecules within a drug delivery system, i.e. in this study a nano-emulsion, nano-emulgel or conventional emulgel needs to diffuse to the surface of the skin from the dispersion or semi-solid dosage form. Various partitioning and permeation steps follow in order for the API to reach the stratum corneum and/or the systemic circulation facilitated by moving through the stratum corneum (Wiechers, 2008:7). These partitioning and permeation steps can be achieved through three possible penetration pathways that are presented by the skin (Wiechers, 2008:7). These pathways include the shunt/follicular route, transcellular route and intercellular route and are illustrated in Figure 2.4.



**Figure 2.4:** Penetration pathways through the skin (adapted from Schroeter *et al.*, 2013).

#### 2.5.2.1.1 Shunt route

The shunt route, where API molecules move along and through hair follicles, apocrine glands, eccrine glands and sweat glands, can also be referred to as the follicular route or as the transappendageal pathway (Gaur *et al.*, 2014:1813; Williams, 2003:678). Continuous pathways or channels are formed moving across and bypassing the barrier of the stratum corneum (Marrow *et al.*, 2007:38; Williams, 2003:31). Making up about 0.1% of the surface of the skin, this route is seldom used, mostly by lipophilic APIs, as sebum is present in these glands yielding easy diffusion (Gaur *et al.*, 2014:1813; Jepps *et al.*, 2013:160; Marrow *et al.*, 2007:38).

### 2.5.2.1.2 Transcellular route

The transcellular route demands that the API should cross the alternating corneocyte layers found within the lipid matrix (Ng & Lau, 2015:9). This route consists of the API molecule moving through the hydrophilic corneocytes of the stratum corneum cells, and then through the lipid matrix (Gaur *et al.*, 2014:1813; Marrow *et al.*, 2007:38). As a result, this route requires a variety of partitioning and diffusion steps as the hydrophilic corneocytes are surrounded by a lipid matrix (Marrow *et al.*, 2007:38). As a hydrophilic environment is created by the corneocytes, this can be viewed as a popular route for hydrophilic APIs.

### 2.5.2.1.3 Intercellular route

The intercellular route makes use of the lipid matrix existing within the stratum corneum (Marrow *et al.*, 2007:38; Williams, 2003:38). This lipid matrix presents a challenge to diffuse and partition through as it presents a complicated pathway for the API (Marrow *et al.*, 2007:38; Ng & Lau, 2015:9). This route is thus more suitable for non-polar APIs, as these molecules can dissolve and diffuse through the non-polar lipid matrix (Geethu *et al.*, 2014:1813).

## 2.6 Mathematical model

Mathematical principals and equations can be used to give a better understanding in terms of skin permeation. It can therefore be used as an aid during the design and formulation of a topical drug delivery system (Williams, 2013:680). Fick's first law of diffusion is one of these mathematical aids.

### 2.6.1 Fick's first law of diffusion

Passive diffusion is the movement of an API molecule from a higher concentration to a lower concentration. Steady-state and passive diffusion can best be described by making use of Fick's first law of diffusion (Williams, 2013:681). Hence, this mathematical model can be used to describe the transport and skin penetration of the API during *in vitro* studies (Dancik *et al.*, 2008:182).

$$J = DK\Delta c / h$$

**Equation 2.1**

In Equation 2.1,  $J$  can be viewed as the diffusive flux,  $D$  represents the API diffusion coefficient,  $K$  is the skin-vehicle partition coefficient,  $\Delta c$  can be viewed as the concentration difference and  $h$  as the diffusional path length (the stratum corneum thickness) (Dancik *et al.*, 2008:182; Hadgraft, 2001:2).

Equation 2.1 can be simplified to Equation 2.2 especially when taken into consideration that the applied concentration ( $C_{app}$ ) is normally larger than the concentration present in the skin.

$$J = k_p \cdot C_{app}$$

**Equation 2.2**

This mathematical model can supply further information in regards to diffusivity, as it is also representative of the drug flux (Naik *et al.*, 2000:320). Thus, it proposes that an increased flux can be achieved when the API diffusivity is increased (Naik *et al.*, 2000:320). The physicochemical characteristics and properties of both the skin and the API can consequently influence the diffusion and delivery of APIs (Naik *et al.*, 2000:320; Wiechers & Watkinson, 2008:63; Williams, 2013:677).

## **2.7 Properties influencing topical drug delivery**

### **2.7.1 Ideal physicochemical properties**

Being a complex and multi-layered structure, APIs with ideal physicochemical properties are required in order to achieve skin permeation and absorption, since the skin can restrict the extent of permeation of APIs (Williams, 2013:680). It is evident that the drug flux is directly dependent on the permeant's physicochemical properties (Williams, 2013:680). The APIs available and suitable for topical drug delivery are therefore limited (Williams, 2013:675).

An API's molecular mass, melting point, solubility, log P, diffusion coefficient and pKa are some of the properties which require careful consideration when formulating a topical product (Allen *et al.*, 2011a:42; Williams, 2013:682).

#### **2.7.1.1 Molecular mass**

An important property of an API to take into consideration is the size and shape of the API molecule (Williams, 2003:36). The molecular mass and size can influence diffusivity (Williams, 2003:36). Therefore, it has been suggested that there is a relationship between the molecular weight and transdermal flux (Barry, 2002:513; Williams 2003:36). Large molecules are prevented from penetrating the skin due to its barrier properties and so few APIs, ideal for topical delivery, are available (Williams, 2013:680). Polar molecules also show weak penetration through the stratum corneum (Steele, 2009:224). Ideal APIs for topical drug delivery should possess a molecular mass smaller than 500 g/mol (Naik *et al.*, 2000:319; Williams, 2013:675). Artemether is an API which falls within this narrow range as it presents with a molecular mass of 298.37 g/mol (USP, 2013).

### **2.7.1.2 Melting point**

Studies have shown there is a direct correlation between an API's melting point and its aqueous solubility (Williams, 2003:37). Consequently, APIs possessing a low melting point will present good aqueous solubility and vice versa (Williams, 2003:37). The melting point of an API can also be indicative of its permeation through the skin. An API ideal for transdermal and topical delivery should present with a melting point of lower than 200 °C (Naik *et al.*, 2000:319). Artemether has a melting point between 86 and 90 °C, posing as ideal for topical delivery (USP, 2013).

### **2.7.1.3 Aqueous solubility**

Aqueous solubility is another important property to investigate. A solubility value is indicative of how well an API will dissolve within a medium and consequently be absorbed through the skin (Steele & Austin, 2009:24). Solubility of an API can determine the bioavailability of an API. It is found that an API with an aqueous solubility of greater than 1 mg/ml is ideal for topical drug delivery (Naik *et al.*, 2000:319). Encyclopaedia Britannica (2016) states there is a correlation between solubility and permeation, hence, good solubility leads to better permeation. The solubility of an API can also be influenced by its lipophilic nature (Williams, 2003:37). Artemether is practically insoluble in water (USP, 2013) and in a study conducted by Haynes *et al.* (2006:2136); it was found that artemether had an aqueous solubility of 0.117 mg/ml, so less than 1 mg/ml (therefore not being ideal for topical drug delivery).

### **2.7.1.4 Partition coefficient**

When the distribution of API molecules between two phases, i.e. a lipophilic and a hydrophilic phase, is investigated it can be referred to as the log P of an API (Williams, 2013:676). The lipophilic nature of an API can be ascribed to its partition coefficient. This is an indication of how the API will be distributed throughout the hydrophilic and lipophilic parts of the stratum corneum (Williams, 2013:676). It is thus also indicative of the drug flux of the API (Barry, 2002:512). It has been found that faster permeation through the skin can be achieved with lipophilic molecules (Williams, 2003:37). Ideally an API for topical drug delivery should possess both lipophilic and hydrophilic characteristics in order to permeate through the brick and mortar-like stratum corneum (Naik *et al.*, 2000:319; Williams. 2003:37).

Due to this, the ideal log P for an API should be between 1 and 3, as this value means the API is soluble in water and oil (Subedi *et al.*, 2010:339; Williams, 2003:36). Artemether can further be viewed as a drug which is not very soluble in water, as it consists of a lipophilic log P value of 3.53 (Drugbank, 2016; Karbwang *et al.*, 1997:259; Shahzad *et al.*, 2013:197). This could mean that artemether would not permeate further than the stratum corneum.

#### **2.7.1.5 Diffusion coefficient**

Diffusion can be defined as the movement of API molecules from a higher to a lower concentration (Williams, 2003:27). The diffusion coefficient (D) of an API describes the movement of the API through an area; especially with what ease the API will move through tissue (Williams, 2003:27; Williams, 2013:676). It is expressed by area (cm<sup>2</sup>) per time (h or s) (Williams, 2003:27). Passive diffusion, which is defined by Fick's diffusion laws, can be used to describe API movement through and across the skin (Williams, 2013:675) (this is discussed in Section 2.6.1). The complicated brick and mortar structure of the stratum corneum results in limited diffusivity (Barry, 2002:512). The state of matter of the formulation can determine the speed at which diffusion can occur (Barry, 2002:512). The diffusivity, as well as the bioavailability, can also be impacted by the number of hydrogen bonding groups in the API's structure, which is a result of interactions which can occur between the lipid polar head groups and the hydrogen bonds within the API's structure (Thomas & Finnin, 2004:699; Williams, 2013:680). It has been proposed that a molecule should ideally not possess more than five hydrogen bond donors and no more than ten hydrogen bond acceptors (Ashford, 2013:324). As artemether presents with only five hydrogen bond acceptors and no donors it can be proposed that it will display with favourable absorption outcomes (PubChem, 2016).

#### **2.7.1.6 pH, pKa and ionisation**

Although the skin exhibits with an acidic nature due to its low pH averaging at 5, pH values ranging between 4 and 7 are also used to describe the skin's pH (Ng & Lau, 2015:8; Williams, 2013:678). A pH value measuring lower than 3 or higher than 9 can cause harm to the skin and consequently influence permeability (Naik *et al.*, 2000:319). The pH can therefore influence dissociation rates of weak acids and bases; this depends on the API's pKa or pKb values (Barry, 2002:511). An effective membrane gradient can be influenced by both the pH, as well as the ionisation of the API (Barry, 2002:511).

Ionisation is important when taking into consideration that the barrier produced by the skin is mainly of a lipophilic nature (Williams, 2003:38). It is noted that APIs that are unionised are ideal for transdermal and topical drug delivery as they can result in effective permeation and diffusion (Li *et al.*, 2012:98; Williams, 2003:38). Based on the pH-partition hypothesis, it is concluded that unionised molecules present with easy permeability across these lipid layers, whereas ionised molecules show little to no degree of penetration into the stratum corneum (Barry, 2002:511; Williams, 2003:38). It has been postulated that the concentration of a greatly ionised API, which moves through the skin, can be achieved at an appropriate pH level (Li *et al.*, 2012:985). Williams (2003:38-39) states that a unionised API can present with a high

permeability coefficient and low aqueous solubility, whereas an ionised API can present with low permeability and high aqueous solubility.

By using an altered form of the Henderson-Hasselbalch equation (Equations 2.3 and 2.4) the ionisation of an API or formulation could be determined:

$$\% \text{ionised} = 100 / 1 + \text{anti-log} (\text{pKa} - \text{pH}) \quad \text{Equation 2.3}$$

$$\% \text{unionised} = 100 - \% \text{ionised} \quad \text{Equation 2.4}$$

According to Drugbank (2016), artemether provides a pKa of - 3.9. Due to this negative pKa value, it was calculated (with abovementioned Equations 2.3 and 2.4) that artemether would possess little unionised species. At a pH of 7 and 5, only 0.0794% and 7.3500% of species were unionised respectively, resulting in artemether being greatly ionised. This could conclude that diffusivity through the skin could be limited, but with the help of penetration enhancers and through being incorporated within an o/w nano-emulsion, this problem hopes to be overcome.

#### 2.7.1.7 Investigating artemether in terms of ideal topical delivery properties

**Table 2.2:** API investigated in terms of ideal topical physicochemical properties

| Physicochemical property  | Ideal topical delivery values | Artemether   |
|---------------------------|-------------------------------|--------------|
| <b>Molecular mass</b>     | < 500 g/mol                   | 298.37 g/mol |
| <b>Melting point</b>      | < 200 °C                      | 86 – 90 °C   |
| <b>Log P</b>              | 1 – 3                         | 3.53         |
| <b>Aqueous Solubility</b> | > 1 mg/ml                     | 0.117 mg/ml  |

Table 2.2 is used as a guide to determine whether artemether would be an ideal candidate for topical drug delivery. The ideal physicochemical properties of a topical and transdermal API is compared to the properties of artemether. In terms of molecular mass and melting point, it can be seen that artemether adheres to these ideal properties, as artemether presents with a molecular mass of 289.37 g/mol (less than 500 g/mol) and a melting point of 86 – 90 °C (less than 200 °C) (Naik *et al.*, 2000:319; USP,2013; Williams, 2003:37). With a low solubility and a log P value of above 3, it is indicative that artemether is a very lipophilic API. This could propose that although it does not comply with the log P and aqueous solubility of an ideal topical API, the lipophilic nature of artemether could ensure it would probably be retained in the stratum corneum and not permeate to the hydrophilic viable epidermis (Subedi *et al.*, 2010:339). For this reason, an o/w nano-emulsion, nano-emulgel and conventional emulgel will be formulated in an attempt to deliver artemether into the stratum corneum.

## **2.8 Overcoming the skin barrier**

Topical drug delivery is challenging due to the barrier that is presented by the skin and so an opportunity exists to investigate approaches to overcome it (Williams & Barry, 2012:128). Most APIs lack the ideal physicochemical properties needed to cross the stratum corneum and for this reason, some modification of the skin's properties is needed (Trommer & Neubert, 2006:106). Many approaches and techniques have been attempted to overcome the stratum corneum properties and to achieve improved drug flux (Shahzad *et al.*, 2012:1686; Williams, 2013:693). A successful approach is the incorporation of penetration enhancers in topical formulations (Trommer & Neubert, 2006:108; Williams, 2013:694; Williams & Barry, 2012:129).

### **2.8.1 Penetration enhancers**

Penetration enhancers are better known as permeation enhancers, accelerants or absorption promoters and their function is accurately described by their name (Büyüktimkin *et al.*, 1997: 358). Penetration enhancers can be viewed as a group of chemicals that can interact and interfere with intercellular proteins, as well as with the lipids in the stratum corneum. The success of penetration enhancers is due to the fact that they disrupt, modify and reduce the barrier properties of the skin, resulting in increased partitioning and absorption of the API (Trommer & Neubert, 2006:108; Wang *et al.*, 2003:1612; Williams, 2013:694). Ideal enhancers should pose no toxicity, no irritation, are pharmacologically inactive and could be used together with other excipients (Williams, 2013:694).

#### **2.8.1.1 Types of penetration enhancers**

Penetration enhancers can be subdivided into two approaches, namely chemical or physical, which can be utilised to assist with API penetration (Wang *et al.*, 2003:1612). Physical enhancers include: electro-osmosis, electroporation, iontophoresis, sonophoresis and temperature (Asbill & Michniak, 2000:37). A variety of chemical enhancers are available which includes: alcohols, amines, esters, fatty acids, surfactants, etc. (Naik *et al.*, 2002:321). Of the two, chemical enhancers present as most successful. The success of these chemical enhancers on penetration is because they temporarily compromise the properties of the skin barrier, which leads to the movement and entry of molecules through the skin (Naik *et al.*, 2000:321). In this study, chemical enhancers, more specifically fatty acids, were used to enhance penetration of artemether through the skin.

#### **2.8.1.2 Fatty acids in natural oils**

Since as early as the 1960's, fatty acids have been investigated in terms of their effect on skin permeation (Wang *et al.*, 2003:1612). Fatty acids, found in natural oils, are leading chemical penetration enhancers, as they have the ability to successfully enhance the movement of APIs

across the stratum corneum (Boelsma *et al.*, 1996:729; Vermaak *et al.*, 2011:922; Wang *et al.*, 2003:1612). This forms the main rationale behind using fatty acids in topical formulations (Boelsma *et al.*, 1996:729). Enhanced penetration can be achieved either through lipid interaction, protein interaction, or creating changes in the partitioning through the stratum corneum (Büyüktimkin *et al.*, 1997:360-361). A disruption of the lipid bilayers is created, providing diffusion and consequently increased API permeation and partitioning (Babu *et al.*, 2006:138; Wang *et al.*, 2003:1612; Williams, 2013:694).

Oils sourced from natural seeds are triglyceride esters composed of a variety of fatty acids, contributing and supporting the effective use thereof in cosmetic and topical products (Sharma & Kundu, 2006:984; Vermaak *et al.*, 2011:922). When fatty acids are viewed in terms of their chemical structure, it can be seen they consist of an aliphatic hydrocarbon chain and a carboxyl group on the end of the chain (Babu *et al.*, 2006:144). They consist of between 14 to 22 carbons in their chain, which contains 1 to 3 double bonds (Sharma & Kundu, 2006:984). Fatty acids therefore differ only in the length of the hydrocarbon chains or in terms of the amount, position and configuration of the *cis*-double bond (Babu *et al.*, 2006:144). It has been found that the length of the hydrocarbon chain can have an effect on the permeation enhancement of APIs across the skin (Babu *et al.*, 2006:145).

Fatty acids are available as two types, namely saturated and unsaturated fatty acids, with the latter having demonstrated the most enhancement effects (Wang *et al.*, 2003:1612). Effective unsaturated fatty acids consist of more double bonds as well as *cis*-configurations, disturbing intercellular lipids to a great extent (Trommer & Neubert, 2006:108-114; Williams & Barry, 2012:132). Fatty acids, consisting of longer hydrocarbon chains, have a high affinity for the lipids located in the stratum corneum and as a result of this have a greater effect on permeation (Babu *et al.*, 2006:145). C<sub>18</sub>-Unsaturated fatty acids have been found to have near optimal enhancement effects (Williams & Barry, 2012:132). Büyüktimkin *et al.* (1997:433) states that the use of natural oils as penetration enhancers can be viewed as safe as they are widely used in the food and cosmetic industry. They can also be regarded as safe seeing as uncomplicated fatty acids, such as linoleic acid and arachidonic acid, form basic components of human skin (Boelsma *et al.*, 1996:729; Gaur *et al.*, 2014:1812).

### **2.8.1.3 Safflower oil as natural oil and penetration enhancer**

As safflower, or *Carthamus tinctorius*, can be described as an herb with toothed, prickly leaves and a striking yellow to deep red flower, it forms part of the *Asteraceae* family (Domagalska, 2010:6-8; Toma *et al.*, 2014:539; Wolters Kluwer Health, 2009). Names such as American saffron, Zafran and Bastard saffron can also be used to refer to safflower (Wolters Kluwer Health, 2009). Safflower fruits are small, one-seeded nut shaped and white of colour. The

flowers, seeds, as well as the oil extracted from the seeds can all be utilised (Van Wyk & Wink, 2009:81).

The oil extracted from the safflower seeds is mainly used and is composed of fatty acids including: 75% linoleic, 13% oleic, 6% palmitic and 3% stearic acid (Van Wyk & Wink, 2009:81; Wolters Kluwer Health, 2009). Safflower oil contains the highest concentration of linoleic acid of all the plant oils (Van Wyk & Wink, 2009:81). As mentioned, this linoleic acid (a C<sub>18</sub>-unsaturated fatty acid), found in natural oils such as safflower oil, is also present in the human skin (Vermaak *et al.*, 2011:922). Consequently, the incorporation of linoleic acid within a topical formulation can lead to skin moisturising, play a role during the skin healing process of dermatosis and can have possible anti-inflammatory effects (Vermaak *et al.*, 2011:922). Due to the presence in the skin, it is believed these natural oils are generally safe to use, hence lowering the possibility of skin irritation (Büyüktimkin *et al.*, 1997:433; Menon, 2002:S9; Vermaak *et al.*, 2011:922). The Cosmetic Ingredient Review (CIR) Expert Panel found safflower oil not to cause any skin irritations, photosensitisation, or sensitisation (Cosmeticsinfo, 2015).

As a result of this little known irritation, safflower oil is implemented in various sectors, from cosmetics to the food industry. At present, there are various products available, which contain safflower oil, such as a topical 3% safflower oil lotion (Cable, 2009:611; Domagalska, 2010:6). It has also been incorporated in cosmetics during the formulation of soaps, lotions, creams and even hair-care products, mostly as an emollient (Cable, 2009:611). Studies have shown that safflower oil possess wound healing and moisturising properties (Athar & Nasir, 2005:39). It has been stated that safflower oil is mainly used as delivery system for topical or oral preparations (Cable, 2009:611).

## **2.9 Topical delivery system selection**

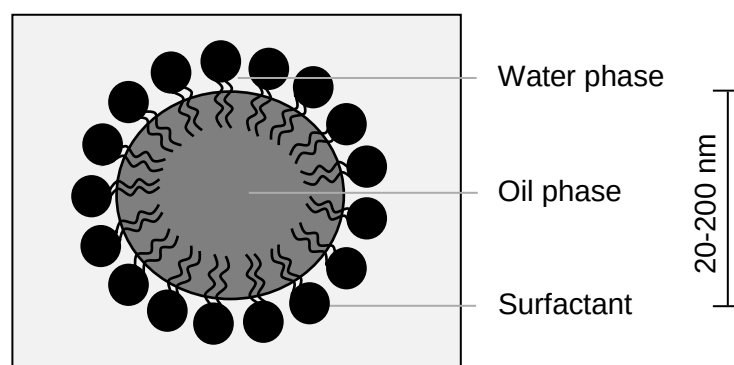
The role of the delivery system is to deliver the API to the site where an effect is needed; during the treatment of CTB, the epidermis is the main target site (Hyma *et al.*, 2014:4). It is important that the selected delivery system is successful in the delivery and release of the API (Hyma *et al.*, 2014:4; Weiss, 2014:472). It should also be noted that the characteristics of the selected delivery system and API can influence the API delivery and response (Hyma *et al.*, 2014:4).

### **2.9.1 Nano-emulsions as delivery system**

Nano-emulsions have been formulated as early as the 1940's (Bhatt & Madhav, 2011:2482). Nano-emulsions, also referred to as mini-emulsions, fine-disperse emulsions or submicron emulsions are characterised according to their small nano-sized droplets averaging between 20 – 200 nm (Abolmaali *et al.*, 2011:139; Forgiarini *et al.*, 2001:2076; Lovelyn & Attama,

2011:626). A nano-emulsion can be defined as a heterogeneous, two-phase dispersion with the main components consisting of oil and water, which is stabilised by surfactants (Abolmaali *et al.*, 2011:140; Chime *et al.*, 2014:77; Gaur *et al.*, 2014:37; Kela & Kaur, 2013:9203; Lovelyn & Attama, 2011:626; Reddy *et al.*, 2013:86). This two-phase dispersed nano-emulsion creates both hydrophilic and lipophilic characteristics, which can be used to deliver APIs to the stratum corneum (Gaur *et al.*, 2014:37).

Depending on the incorporated API, a nano-emulsion can be formulated with either water or oil as the core component, hence forming a w/o or o/w nano-emulsion (Chime *et al.*, 2014:77; Kela & Kaur, 2013:9203). The last mentioned will be formulated in this study (see Figure 2.5). Lipophilic APIs, such as artemether, are mainly delivered through o/w nano-emulsions (Kela & Kaur, 2013:9203). When investigating an o/w nano-emulsion the oil, ranging from natural or synthetic lipids, fatty acids or triglycerides, are dispersed as nanometric sized droplets, in the water phase (Abolmaali *et al.*, 2011:140). These small droplets of nano-emulsions lead to a reduction in gravitational force, which results in appropriate Brownian motion, forming a stable nano-emulsion (Kela & Kaur, 2013:9203). Characteristically, nano-emulsions are unique due to their long-term physical stability (Bouchemal *et al.*, 2004:242; Forgiarini *et al.*, 2001:2076; Reddy *et al.*, 2013:86; Tadros *et al.*, 2004:303; Wu *et al.*, 2001:64).



**Figure 2.5:** Oil-in-water nano-emulsion droplet

## 2.9.2 Applications of nano-emulsions in topical drug delivery

Nano-emulsions can be viewed as a promising delivery system due to the many advantages they present (Lai *et al.*, 2008:1; Solans *et al.*, 2005:102). Consequently, it is especially valuable during the topical delivery of APIs (Klang *et al.*, 2015: 258). Nano-emulsions have been greatly implemented in a variety of topical and transdermal fields ranging from cosmetics to gene delivery (Kela & Kaur, 2013:9206).

### 2.9.2.1 Advantages of nano-emulsions

Nano-emulsions are kinetically stable due to their droplet size, which is in the nanometric range (Abolmaali *et al.*, 2011:140; Lu *et al.*, 2014:826; Tadros *et al.*, 2004:303). As a result, stability can be achieved because there is a lower presence of creaming, coalescence, sedimentation or flocculation (Lu *et al.*, 2014:826; Tadros *et al.*, 2004:303). This can also be attributed to the fact that there is a reduction in forces created by gravity (Kela & Kaur, 2013:9206; Lu *et al.*, 2014:826; Tadros *et al.*, 2004:303).

For this reason, increased permeation effects and drug release through the stratum corneum can also be obtained by using nano-emulsions as the delivery system of APIs; this, along with the presence of the large surface area created by the nano-emulsion, can lead to larger concentrations of the API accumulating in the skin (Kela & Kaur, 2013:9206; Klang *et al.*, 2015:258; Lu *et al.*, 2014:826; Tadros *et al.*, 2004:304). Favourable delivery of APIs can also be achieved due to the fact that nano-emulsions can act as penetration enhancers leading to greater bioavailability (Lovelyn & Attama, 2011:630; Maruno & Da Rocha-Filho, 2010:17). Gaur *et al.* (2014:37) stated that another advantage of nano-emulsions are that they possess both hydrophilic and lipophilic characteristics, which can therefore be used to deliver APIs either to the lipophilic stratum corneum or help facilitate movement through the hydrophilic layers of the skin.

Successful application in topical products and cosmetics is a result of the small droplets causing the product to accumulate evenly on the skin and enhance API delivery through the skin (Bouchemal *et al.*, 2004:242; Tadros *et al.*, 2004:304). Low surface tension contributes to uniform depositing of the API (Kela & Kaur, 2013:9206; Lovelyn & Attama, 2011:629). Greater surface area, larger interfacial area combined with free energy also contributes to nano-emulsions being target site-specific drug delivery systems of APIs (Bhatt & Madhav, 2011:2293; Lovelyn & Attama, 2011:626). Hence, nano-emulsions can also lead to controlled drug delivery to a targeted site, resulting in depot localisation (Abolmaali *et al.*, 2011:140; Clares *et al.*, 2014:591; Lai *et al.*, 2008:1; Lu *et al.*, 2014:826). This localised depot could aid successful treatment of CTB.

Nano-emulsions can make self-administration possible, fewer applications are needed and the treatment can be eliminated at any time (Kela & Kaur, 2013:9206; Lovelyn & Attama, 2011:630). Patient compliance can also be achieved due to the pleasant and aesthetic feel on the skin (Bouchemal *et al.*, 2004:242; Kela & Kaur, 2013:9206; Maruno & Da Rocha-Filho, 2010:17; Tadros *et al.*, 2004:304). Nano-emulsions can be incorporated into a variety of formulations ranging from creams and emulgels to liquids and sprays (Bhatt & Madhav, 2011:2483). They display with a low presence of toxic or irritant effects on the skin and can generally be regarded

as safe, as they do not cause any harm to cells of human or animal nature (Shah *et al.*, 2010:25; Thakur *et al.*, 2012:224).

### **2.9.2.2 Disadvantages of nano-emulsions**

Special methods and expensive equipment, such as high-pressure homogenisers, used during the high-energy emulsification method are sometimes needed for the formulation of nano-emulsions especially when small droplets are needed (Kela & Kaur, 2013:9206; Lovelyn & Attama, 2011:629; Tadros *et al.*, 2004:304). Special knowledge of Ostwald ripening effects, which is a major instability problem present with nano-emulsions, interfacial chemistry and the role of surfactants, etc. is required (Kela & Kaur, 2013:9206; Tadros *et al.*, 2004:304).

### **2.9.3 Methods of nano-emulsion formulation**

Although kinetically stable, nano-emulsions are not very thermodynamically stable and therefore energy is needed during the formulation process. Nano-emulsions can be formulated by using either a high or low-energy emulsification method (Abolmaali *et al.*, 2011:141; Tadros *et al.*, 2004:307).

#### **2.9.3.1 High-energy emulsification methods**

This method is based on the application of mechanical devices, such as ultrasonicators, microfluidisers or high-pressure homogenisers, which creates high-energy forces (of about 500 to 5000 psi) to break up the oil and water phase (Bhatt & Madhav, 2011:2294; Lovelyn & Attama, 2011:627; Tadros *et al.*, 2004:308). This creates an intense turbulence within the mixture, breaking the droplets into nano-sized droplets (Bhatt & Madhav, 2011:2294). Although this method is successful in producing nano-sized droplets, its biggest disadvantage is the fact that the high-energy force creates an increase in heat production limiting the APIs that can be used during this method (Bhatt & Madhav, 2011:2294; Kela & Kaur, 2013:9203; Lovelyn & Attama, 2011:627).

#### **2.9.3.2 Low-energy emulsification methods**

This method is based on utilising energy stored within the system, thus very little additional energy is used to produce nano-emulsions (Chime *et al.*, 2014:92; Kela & Kaur, 2013:9203; Lovelyn & Attama, 2011:627). Phase behaviour, as well as the properties of the materials used, contributes to the formation of small droplets (Kela & Kaur, 2013:9203). Low-energy methods can be used to achieve droplets which are ultra-small (Lovelyn & Attama, 2011:627). Low-energy methods can be divided into three methods: self-nano-emulsification, solvent displacement method, as well as phase inversion temperature method (Chime *et al.*, 2014:92).

In this study, a coarse emulsion will be formulated based on self-nano-emulsification where after, a high-energy emulsification method, i.e. ultrasonication, will be applied in order to reduce droplets size, resulting in a nano-emulsion (Thakur *et al.*, 2012:223).

## **2.10 Semi-solid formulation: Gels**

Since nano-emulsions present in a liquid form, it needs to be formulated within a semi-solid formulation in order to increase the ease of application to the skin. Most topical and transdermal conditions can be treated with semi-solid applications (Mahalingam *et al.*, 2008:267; Williams, 2013:689). Semi-solid dosage forms aid the delivery of APIs and formulations as they function as carriers (Gupta & Garg, 2002:144). A variety of semi-solid dosage forms are available ranging from ointments and creams to gels (Allen *et al.*, 2011b:272; Gupta & Garg, 2011:144). For the topical delivery of artemether, as used in this study, a gel will be formulated as a semi-solid dosage form.

A gel can be defined as a jellylike semi-solid created through the mixture of a dispersion within an aqueous medium and a thickening agent (Allen *et al.*, 2011b:278). A gel is very advantageous as it presents as greaseless, easy spreadable and easy to remove, together with emollient properties and compatibility with many excipients (Gaur *et al.*, 2014:37; Weiss, 2014:475). Direct contact between the API and the skin or site of absorption can thus be achieved through gels (Mahalingam *et al.*, 2008:288). During the formulation of gels, the incorporation of lipophilic APIs is difficult, which presents as a disadvantage of this semi-solid dosage form (Hyma *et al.*, 2014:2).

### **2.10.1 Emulgel**

An emulgel therefore exists so that lipophilic APIs, such as artemether, can be delivered through a gel-like semi-solid (Gaur *et al.*, 2014:38; Hyma *et al.*, 2014:2; Kute & Saudagar, 2013:368). An emulgel can be defined as the combination of a coarse emulsion and a gel, hence a w/o or an o/w emulsion is mixed with a gelling agent creating an emulgel (Khullar *et al.*, 2011:117; Kute & Saudagar, 2013:368). Furthermore, emulgels can offer the many advantages of gels such as:

- \* avoidance of the hepatic system (Baibhav *et al.*, 2011:66),
- \* easy administration and thus increased patient compliance (Baibhav *et al.*, 2011:66),
- \* APIs with a short half-life can be delivered (Baibhav *et al.*, 2011:66) and
- \* APIs can be delivered to a specific target (Baibhav *et al.*, 2011:66).

One of the shortcomings of emulgels is that they present with weak permeability as their large droplets struggle to permeate through the skin (Baibhav *et al.*, 2011:66). To overcome this, nano-emulgels are formulated proposing a better permeability response (Baibhav *et al.*, 2013:369).

### **2.10.2 Nano-emulgel**

Individually nano-emulsions propose many advantages, yet the formulation of a nano-emulgel can further increase these advantages and so increase the use thereof in topical applications as well (Baser *et al.*, 2015:1872; Eid *et al.*, 2014:1). Nano-emulgels are formulated just like emulgels, except a nano-emulsion (instead of a coarse emulsion) is combined with a gelling agent (Eid *et al.*, 2014:1; Hyma *et al.*, 2014:4). Through this combination, advances can be made in better stability, increased viscosity, easy spreadability and removal, patient acceptability and most importantly, increased penetration of the skin can be achieved (Eid *et al.*, 2014:1). Controlled release of APIs, especially those with a short half-life, can also be achieved (Panwar *et al.*, 2011:337).

Consequently, in this study, a nano-emulgel, as a semi-solid dosage form, presents as the most appropriate option for the topical delivery of artemether. The formulated o/w nano-emulsion containing artemether and safflower oil will be formulated into a nano-emulgel, which will be compared to a conventional emulgel.

### **2.11 Conclusion**

TB and CTB are infectious diseases caused by *M. tuberculosis*. Growing resistance of *M. tuberculosis* against available treatment poses a great threat. This results in an increase in CTB occurrences. To treat CTB, a target specific delivery system is needed for a direct and local effect. This could be achieved through topical drug delivery. It must be noted that when novel topical treatment of CTB can be achieved and formulated, it should be used concurrently with systemic TB treatment; this could possibly shorten the period of treatment. Although topical delivery offers many advantages, the main limitation created by the skin makes API delivery challenging.

With its hydrophilic and lipophilic characteristics, the skin, especially the stratum corneum, presents as a barrier and rate limiting step during topical drug delivery. The choice of API and the drug delivery system is just as important to investigate for effective drug delivery. Much thought needs to be given to overcoming this barrier and how drug flux through the skin can be increased. Penetration enhancers are one of the methods used to overcome the barrier properties, by interfering with the cellular proteins and consequently disrupting the structure of the stratum corneum.

Hence, it is aimed to formulate an o/w nano-emulsion containing artemether and safflower oil as a novel treatment of CTB. The lipophilic artemether is therefore formulated into the oil phase of an o/w nano-emulsion. Safflower oil, a natural oil containing an unsaturated fatty acid, i.e. linoleic acid, is used as the oil phase within the nano-emulsions, also acting as penetration enhancer. This is predicted to overcome the stratum corneum's barrier, resulting in the topical delivery of artemether. The o/w nano-emulsion containing artemether and safflower oil will also be incorporated within a hydrogel in order to produce a semi-solid state, hence a nano-emulgel. To determine the value of the nano-emulgel, it will be compared to a conventional emulgel.

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# CHAPTER 3:

ARTICLE FOR THE PUBLICATION IN INTERNATIONAL JOURNAL OF PHARMACEUTICS

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Chapter 3 is written in article format for the purpose of publication in the International Journal of Pharmaceutics. The complete guide for authors is stipulated in Appendix H. Only the instructions and formatting, as specified by the Guide for Authors, was used during the writing of this article, except that the text in the manuscript was justified for the ease of reading.

# **Formulation and topical delivery of artemether as a novel cutaneous tuberculosis treatment**

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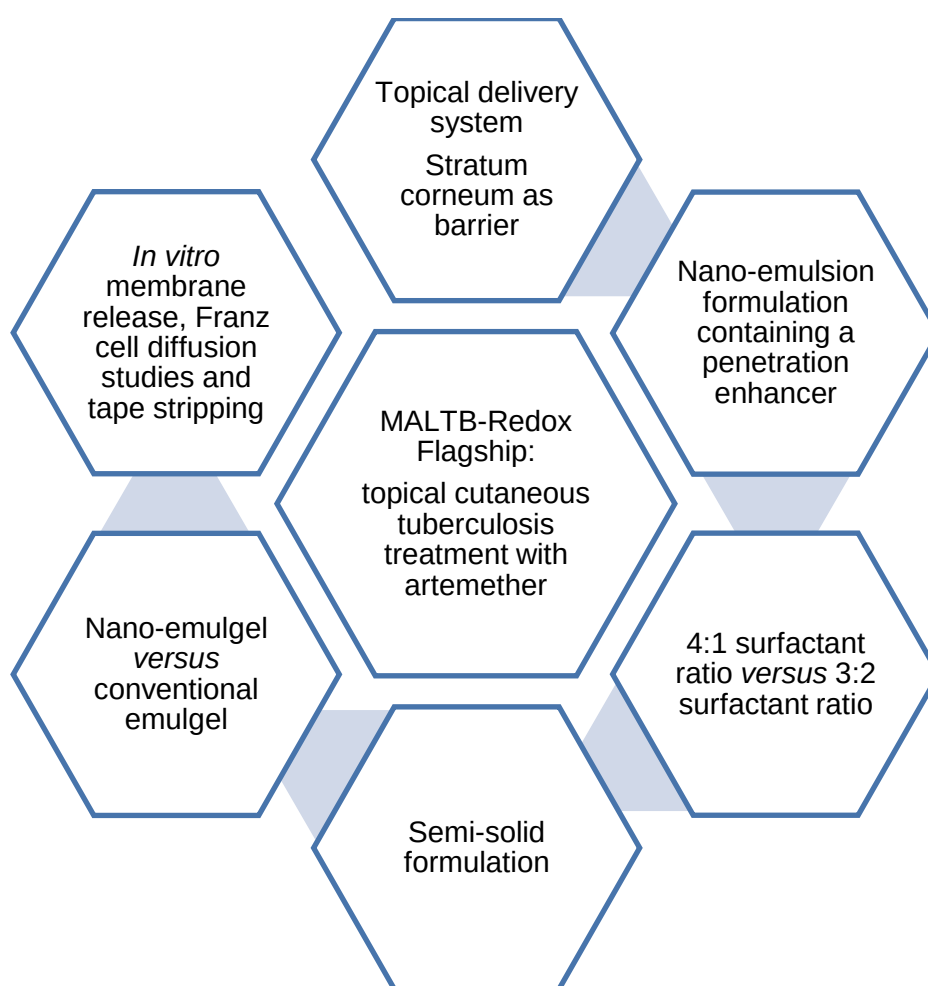
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## Abstract

Currently there is no topical treatment available for cutaneous tuberculosis (CTB). During this study, the aim was to formulate a novel topical CTB treatment. With artemether's lipophilic nature and the barrier presented by the stratum corneum, an oil-in-water (o/w) nano-emulsion with droplets between 20–200 nm was chosen as the delivery system. This study was conducted to evaluate the formulation and topical delivery of artemether within an optimised o/w nano-emulsion containing safflower oil as penetration enhancer. Due to the low viscosity, the optimised nano-emulsion was incorporated within a nano-emulgel as a semi-solid dosage form that was compared to a conventional emulgel; formulations with spherical, nano-sized droplets with a highly negative surface charge ( $>40$  mV) were formulated. Membrane release studies over 6 h were conducted to determine if the drug was released from the vehicle and the optimised nano-emulsion had the highest average flux. Thereafter, diffusion studies and tape stripping was performed to evaluate transdermal and topical delivery. Through *in vitro* cytotoxicity studies on immortalised human keratinocytes (HaCaT), it was proposed that artemether and the optimised nano-emulsion were non-cytotoxic in low concentrations. During this study, it was found that the optimised nano-emulsion presented as advantageous for the topical delivery of artemether.

**Keywords:** Artemether, Topical delivery, Nano-emulsion, Semi-solids, Cutaneous tuberculosis, Franz cell

## Graphical Abstract



## 1 Introduction

Tuberculosis (TB), primarily caused by *Mycobacterium tuberculosis*, can occur as pulmonary or extra-pulmonary presentations (Almaguer-Chávez et al., 2009). One form of the latter, which makes up approximately 1.5% of all TB cases, can present itself as cutaneous tuberculosis (CTB). Of all the skin disorders, 0.1% to 1.0% is caused through CTB (Almaguer-Chávez et al., 2009; Bravo & Gotuzzo, 2007). As the number of patients diagnosed with TB increases regularly, although the presence of CTB is minimal, it should be viewed with more urgency (Van Zyl et al., 2015). An opportunity for the development of a novel topical treatment of CTB is presented due to this increase, together with the fact that there is no existing CTB treatment (Van Zyl et al., 2015). It must be noted that this potential novel topical treatment should be used in combination with existing systemic treatment regimens, as this could contribute to better treatment and clearance of CTB lesions (Van Zyl et al., 2015; Wyrzykowska et al., 2012).

As a result, this study forms part of the MRC flagship programme, MALT-Redox, which focuses on the development of alternative TB treatment combinations that can overpower the TB bacterium. An essential stage in the development of the TB bacterium is the alveolar macrophage stage, as it is exposed to large volumes of oxygen (Haynes, 2015; Wyrzykowska et al., 2012). The bacterium can act against the production of reactive oxygen species (ROS) and other oxidants through flavoenzyme disulphide reductase (i.e. mycothiol reductase (MR)), an intracellular enzyme, as it is responsible for maintaining a suitable reducing environment. Consequently, MR produces reduced mycothiol through the transference of electrons from nicotinamide adenine dinucleotide phosphate (NAD(P)H) (Haynes, 2015).

The drug group artemisinin can oxidise these reduced flavin cofactors of flavoenzymes disulphide reductase, thus limiting the production of reduced mycothiol. Artemisinin combinations have been found to lead to submicromolar activity against *M. tuberculosis* and because of this it is one of the drug groups currently being investigated as a possible treatment of TB (Haynes, 2016; Miller et al., 2011; Shakya et al., 2012). Artemisinin can be

viewed as an oxidant drug, as it could lead to cytotoxic levels of ROS by means of affecting  
MR. This increase in ROS production and oxidative stress is accountable for cell death and  
therefore, the death of the bacterium (Haynes, 2015; Nneji et al., 2013). Consequently,  
during this study artemether, a semi-synthetic derivative of artemisinin, was investigated as  
a possible treatment for CTB.

Hence, to treat CTB, a topical delivery system was needed as this delivery route is aimed at  
keeping the active pharmaceutical ingredient (API) in the skin, following the direct application  
thereof to the targeted site (Williams, 2013). Although the topical delivery route presents  
many advantages, there is one important limitation that cannot be overlooked – the skin (El  
Maghraby et al., 2008). The skin is by far the largest and most intricate organ of the human  
body, covering approximately 2 m<sup>2</sup> of surface area (Williams, 2013). When examined  
closely, the anatomy of the skin, known as a multi-layered structure, is found to be extremely  
complex, consisting of three main layers, i.e. the hypodermis, dermis and the epidermis  
(Geethu et al., 2014). The outer skin layer, the epidermis, is divided into the outermost  
stratum corneum and the underlying viable epidermis (Williams, 2003). Consequently, the  
outermost stratum corneum acts as the main barrier and is responsible for the protective  
action of the skin, which can be ascribed to the stratum corneum's structure (Bouwstra &  
Ponec, 2006; Venus et al., 2011). Structurally it can be described as a "brick and mortar"  
model, since the corneocytes represents the "bricks" and the lipid bilayers, formed by  
stratum corneum lipids, represent the "mortar" (Menon, 2002). The stratum corneum lipids,  
which are responsible for the lipophilic nature of this layer, can be divided into three classes,  
i.e. cholesterol, long chain free fatty acids and ceramides (Menon, 2002). Thus, the stratum  
corneum lipids control and regulate the movement of APIs through the skin and therefore act  
as a drug flux regulator (Williams, 2003). The drug flux value is consequently indicative of  
the quantity of an API that can move across the layers of the skin (Williams, 2003).

Usually an API is presented to the skin by means of a delivery system from where it must  
undergo various partitioning and permeation processes (Williams, 2013). Permeation can  
be described as drug movement through membranes (Williams, 2003). The skin, especially

the stratum corneum, limits the extent of the permeation of an API (Williams, 2013). Consequently, an API's molecular mass, aqueous solubility, partition coefficient (log P) and melting point are some of the physicochemical properties that requires consideration during  
60 topical pre-formulation (Williams, 2013).

Ideally for topical drug delivery, an API should possess an aqueous solubility of more than 1 mg/ml and a log P of between 1 and 3 (Naik et al., 2000). Artemether does not comply with the solubility and log P of an ideal topical API, as it presents with an aqueous solubility of less than 1 mg/ml (practically insoluble) and a lipophilic log P value of 3.53 (Drugbank, 2016;  
65 Karbwang et al., 1997; USP, 2013), however, in spite of this, its lipophilic nature could ensure it would probably be retained within the stratum corneum and not permeate to the hydrophilic viable epidermis. Formulations such as nano-emulsions possess hydrophilic and lipophilic properties, which can be used to deliver APIs to the stratum corneum (Gaur et al., 2014). Henceforth in this study, a nano-emulsion was used as the topical delivery system of  
70 artemether.

Nano-emulsions can be defined as the composition of emulsified oil and water phases, with droplets ranging from 20 – 200 nm (Chime et al., 2014; Solans et al., 2005). Depending on the incorporated API, nano-emulsions can be formulated with either water or oil as the core component, forming a water-in-oil (w/o) or an oil-in-water (o/w) nano-emulsion (Chime et al.,  
75 2014; Kela & Kaur, 2013). The rationale behind using a nano-emulsion as a delivery system for topical formulations, instead of conventional emulsions, is due to the many advantages they possess. Some advantages include the skin being able to tolerate nano-emulsions well and the small droplets enabling easy skin penetration and absorption, as well as being able to deliver insoluble APIs; all advantages improving bioavailability (Chime et al., 2014). Due  
80 to the small droplets, stability can also be achieved as there is a lower presence of creaming, coalescence, flocculation or sedimentation (Kela & Kaur, 2013; Lu et al., 2014; Tadros et al., 2004). The most promising advantage of nano-emulsions is that they can act as penetration enhancers and can result in targeted or controlled delivery of a drug (Maruno & Da Rocha-Filho, 2010).

85 As such, many approaches have been attempted to overcome the stratum corneum barrier properties and to achieve improved drug flux (Williams, 2013). The success of penetration enhancers is due to the fact that they modify and reduce the barrier properties of the skin, resulting in increased partitioning and absorption of the API (Alexander et al., 2012). Fatty acids, found in natural oils, are leading chemical penetration enhancers as they have the

90 ability to enhance the movement of APIs across the stratum corneum (Vermaak et al., 2011; Wang et al., 2003). Their enhancement is a result of interfering with the lipids in the stratum corneum through the interaction with cellular proteins (Wang et al., 2003). This leads to the disruption of the lipid bilayers and hence, an increase in partitioning, especially of lipophilic APIs (Wang et al., 2003; Williams, 2013). Fatty acids exist as saturated or unsaturated fatty

95 acids, of which the unsaturated fatty acids have demonstrated more success as penetration enhancers (Wang et al., 2003). Linoleic acid, for example, an 18-carbon fatty acid found in natural oils, i.e. safflower oil (as used in this study), is an extremely important unsaturated fatty acid as it is also present in human skin (Vermaak et al., 2011). Due to their presence in the skin, it is believed natural oils are generally safe to use, thus lowering the possibility of

100 skin irritation (Büyüktimkin et al., 1997; Menon, 2002; Vermaak et al., 2011). Safflower oil is mostly composed of fatty acids, including 75% linoleic, 13% oleic, 6% palmitic and 3% stearic acid (Van Wyk & Wink, 2009; Wolters Kluwer Health, 2009).

In this study, the topical delivery of a novel CTB treatment aimed at retaining the lipophilic artemether within the epidermis, since CTB, with its various superficial presentations, served

105 as the target site (Frankel et al., 2009). Due to the lipophilic nature of artemether and not complying with all the ideal properties of a topical API, the aim was to formulate artemether into an o/w nano-emulsion to help overcome these limitations and possibly lead to the better topical delivery thereof. Safflower oil, as a penetration enhancer, formed the core component of the oil phase of the nano-emulsion together with the lipophilic artemether. To

110 improve the application of the nano-emulsion to the skin, semi-solid dosage forms, i.e. a nano-emulgel and a conventional emulgel, were formulated.

## 2 Materials and Methods

### 2.1 Materials

Artemether was acquired from DB fine chemicals (Johannesburg, RSA); the natural oil, safflower oil, was obtained from Sharon Bolel chemical marketing (Edenvale, RSA); the lipophilic surfactant sorbitan monostearate (Span® 60) and liquid paraffin were obtained from Fluka (Aston Manor, RSA); the hydrophilic surfactant polyoxyethylene 20 sorbitan fatty acid ester (Tween® 80) was obtained from Merck Chemicals (Midrand, RSA); Xanthan gum was purchased from Warren Chem Specialities (Cape Town, RSA). A phosphate buffer solution (PBS) (pH 7.4) was prepared using UnivAR® sodium hydroxide (NaOH) and UnivAR® potassium dihydrogen orthophosphate dihydrogen (KH<sub>2</sub>PO<sub>4</sub>), both of which were supplied by Merck (Wadeville, RSA).

For the mobile phase preparation, HPLC chromatographic grade LiChrosolv® acetonitrile (ACN), supplied by Merck Millipore (Halfway House, RSA), was used together with deionised water obtained through using a Millipore® Milli-Q water system (Millipore Corporation, Bedford, MA). All other chemicals were of chromatographic grade and no further purification was needed.

### 2.2 Methods

#### 2.2.1 Formulation of artemether topical products

Three formulations, i.e. an optimised o/w nano-emulsion (**NE**) (containing 0.8% w/v artemether and 5% w/v safflower oil), a nano-emulgel (**NEG**) and a conventional emulgel (**CEG**) (each containing 0.4% w/v artemether and 2.5% w/v safflower oil) were formulated during this study. The **NE**, as obtained through investigating different surfactant ratios, was formulated within semi-solid form, i.e. a **NEG** and a **CEG**. Figure 1 is a diagrammatical representation of the formulation of the three artemether topical products.

Figure 1: Diagrammatic representation of the preparation of a nano-emulsion, nano-emulgel or a conventional emulgel

### 2.2.1.1 Formulation of nano-emulsion

Table 1: Ingredients used to formulate the nano-emulsions

In being lipophilic, artemether was formulated within the oil phase of an o/w nano-emulsion. During pre-formulation studies, a low-energy emulsification method, i.e. solvent displacement method was employed, but a large amount of the organic solvent remained in the dispersion, which could possibly influence the skin's integrity. Hence, a high-energy emulsification method, i.e. ultrasonication was employed instead to result in nano-sized droplets. Before the surfactant ratios were explored, various ultrasonication times, using an ultrasonicator (Model UP200St, Hielscher Ultrasonics, Teltow, DE) and ultrasonication bath (Elma Electronic GmbH, Pforzheim, DE), were investigated. Through investigations, it was found that the ideal ultrasonication time was three 1 min intervals, with an ultrasonic probe, followed by 15 min in the ultrasonication bath; these times resulted in the most stable dispersions. Following this, two surfactant ratios were investigated: a surfactant ratio of Tween<sup>®</sup> 80:Span<sup>®</sup> 60 (4:1) and a Tween<sup>®</sup> 80:Span<sup>®</sup> 60 (3:2). The ingredients mentioned in Table 1 were used respectively for the two ratios. Both dispersions presented as a white, semi-translucent liquid with no phase separation, oil droplets or sedimentation visible.

### 2.2.1.1 Formulation of a nano-emulgel and a conventional emulgel

Table 2: Ingredients used to formulate the semi-solid dosage forms

The **NEG** and **CEG** consisted of two phases. For each of the semi-solids the same process and ingredients (found in Table 2) were used, the only difference was a nano-emulsion was incorporated within the **NEG**, whilst a coarse emulsion was incorporated within the **CEG**. Xanthan gum was slowly dispersed in heated water, while homogenising at  $\pm 777$  rpm. Once a clear hydrogel base was formed, the optimised **NE** or coarse emulsion was added, respectively, to create a **NEG** or **CEG**. The oil phase consisted of liquid paraffin, Span<sup>®</sup> 60 and Tween<sup>®</sup> 80. The ingredients were mixed through continuous stirring and heat. Once all the excipients were dissolved, the oil phase was added to the hydrogel while still homogenising at  $\pm 777$  rpm. With the addition of the oil phase, the mixture became white in

colour. The hot plate was then turned off and homogenised until a temperature of around 40°C was reached. The mixture was left to cool at room temperature.

### **2.2.2 Quantitative analysis of artemether**

170 An artemether high pressure liquid chromatography (HPLC) analytical method was successfully validated and found to be sensitive, responsive and reliable for the quantification of the artemether at the Analytical Technology Laboratory (ATL), North-West University (NWU), Potchefstroom, RSA. An Agilent® 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1100 gradient pump was used. The  
175 system was also fitted with a UV detector, set at a wavelength of 216 nm, as well as an autosampler injection mechanism. Chromatograms were analysed by making use of ChemStation Rev. A.10.02 acquisition and analysis software. A laboratory environment with a controlled temperature of 25°C was maintained. A Venusil® XBP C<sub>18</sub> (2) reverse phase column (100 Å pores, 150 x 4.6 mm) with a particle size of 5 µm was used (Agela  
180 Technologies, Newark, DE). The mobile phase, consisting of 70% of acetonitrile (ACN) and 30% HPLC water, was injected at 50 µl with a flow rate of 1 ml/min. A calibration curve was determined with each analysis by injecting freshly prepared artemether standard solutions.

### **2.2.3 Standard preparation**

A standard solution was prepared by accurately weighing 5 mg of artemether and dissolving  
185 it in a 25 ml volumetric flask made up to volume with mobile phase (ACN:water (70:30)). The standard solution was injected into the HPLC at different injection volumes of 2.5, 5.0, 7.5, 10.0, 12.5, 25.0 and 50.0 µl.

### **2.2.4 Physicochemical properties**

#### **2.2.4.1 Aqueous solubility**

190 Prior to sample preparation, the water bath was preheated to 32°C (conditions used during diffusion studies) as this temperature simulated the external temperature of the skin (Williams, 2013). Six clean test tubes were taken, three were filled with 3 ml HPLC water using a micropipette and the other three were filled with 3 ml of PBS (pH 7.4). An excess amount of artemether was added to each of the six test tubes resulting in saturated

195 solutions. These test tubes were placed into the preheated water shaker bath (32°C) for 24 h. After 24 h the test tubes were removed and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into an HPLC vial. The samples were analysed in duplicate by HPLC.

#### 2.2.4.2 Octanol-buffer distribution coefficient (log D)

200 PBS (pH 7.4) and *n*-octanol, in equal volumes, were equilibrated for 24 h to ensure co-saturation of the two phases occurred. The two co-saturated solutions were separated within a separating funnel; the top layer in the separating funnel represented the octanol phase, whilst the bottom layer was the PBS (pH 7.4). After separation, 1 g of artemether was weighed and placed in a beaker containing 20 ml of the pre-saturated octanol.

205 Thereafter, 3 ml of the pre-saturated octanol/artemether solution was placed in three different test tubes and to this, an equal amount of pre-saturated PBS (pH 7.4) was added, respectively. The test tubes were placed into a shaker water bath overnight at 32°C. The test tubes were then removed and 1 ml of the octanol phase (top layer) was taken by making use of a micropipette and placed into a clean 10 ml volumetric flask. The volumetric flask

210 was made up to volume with methanol. This was done for all three test tubes containing the octanol phase in order to dilute the octanol (to be suitable for HPLC analysis). A volume was taken from this solution and placed into an HPLC vial; the second phase (PBS (pH 7.4)) of each tube was also placed into an HPLC vial, without dilution. All the vials were then analysed and the concentration of artemether in each phase determined by means of HPLC.

215 This experiment was done in duplicate.

The octanol-buffer distribution coefficient (log D) was determined in terms of the ratio of the API concentration in the *n*-octanol (oily) phase to the API concentration in the buffer (PBS) phase. Calculations to determine log D were done by applying Equation 1.

Log D = concentration in *n*-octanol/concentration in PBS

**Equation 1**

## 220    **2.3 Characterisation of artemether topical formulations**

The three formulations, the **NE**, the **NEG** and the **CEG**, were characterised in terms of pH, viscosity, droplet size and zeta-potential. Transmission electron microscopy (TEM) was completed on the **NE** and its entrapment efficacy (%EE) was also determined.

### **2.3.1 pH**

225    The pH of each formulation (the **NE**, the **NEG** and the **CEG**) was measured using a Mettler Toledo® pH meter (Mettler Toledo, CU), equipped with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU).

### **2.3.2 Viscosity**

A Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA), connected to a  
230    thermostatic water bath (controlled at  $\pm 25^{\circ}\text{C}$ ), was used to determine the viscosity of the three formulations. The Viscometer was equipped with a T-bar 18 and 25 spindle to measure the **NE** and the semi-solids' (the **NEG** and the **CEG**) viscosity, respectively. Formulations were placed into the thermostatic water bath an hour prior to the conduction of the experiment in order to acclimatise to  $\pm 25^{\circ}\text{C}$ . An amount of each formulation was  
235    transferred to the sample chamber and fitted to the small sample adapter of the viscometer. The small sample adapter was rotated at a specified speed of 200 rpm to measure the viscosity. A percentage torque was achieved and multipoint data was collected on Rheocalc T 1.2.19 software at 10 sec intervals for a minute. Viscosity readings were described in terms of centipoise (cP) at room temperature ( $\pm 25^{\circ}\text{C}$ ); the average viscosity values were  
240    calculated thereafter.

### **2.3.3 Droplet size**

A Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK) was used to determine the droplet size of each formulation. Diluted samples of the formulations were used during this characterisation by placing 10 ml of HPLC water into a polytop. Two drops  
245    of the **NE** and one drop of the semi-solids (**NEG** and **CEG**), respectively, were added to a polytop and mixed thoroughly. These diluted samples were used to ensure improved

readings, since dilution decreases the amount of interaction between the droplets (Gaumet et al., 2008). Each sample was measured in triplicate.

#### **2.3.4 Zeta-potential**

250 A Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK) was used to determine the zeta-potential of each of the three formulations. Diluted samples of the three formulations were prepared: two drops of the **NE** and one drop of the semi-solids (**NEG** and **CEG**), respectively, were added to a polytop containing the 10 ml HPLC water and mixed thoroughly. Each sample was measured in triplicate and the average determined.

#### **2.3.5 Transmission electron microscopy**

The morphological characteristics of the **NE** were studied at the Electron Microscopy Laboratory of the North-West University (NWU), Potchefstroom, RSA through a FEI Tecnai G2 20S-Twin 200 kV high-resolution transmission electron microscope (HRTEM) (Czech Republic, EU) with an Oxford INCA X-Sight EDS System, equipped with a Gatan bottom  
260 mount camera. Samples of a placebo **NE** (containing no artemether) (**PNE**), to avoid harm to the HRTEM, were diluted and a small amount of the dilution was placed on a microscopic carbon-coated 300 mesh copper grid using a micropipette. This was stained using osmium tetroxide, resulting in images of high-contrast quality as it is able to preserve any unsaturated fatty-acid present (Nomaki et al., 2015). It was allowed to dry for 10 – 15 min,  
265 where after the osmium stained carbon-coated grid was investigated at the adequate voltage under the TEM.

#### **2.3.6 Entrapment efficiency**

The %EE of artemether in the **NE** was quantified by means of HPLC. Samples of the **NE** were centrifuged at the Laboratory for Applied Molecular Biology (LAMB) of the North-West  
270 University (NWU), Potchefstroom Campus, RSA, using an Optima L-100XP Ultra-centrifuge (Beckman Coulter, RSA) at 25000 rpm for 30 min, at a temperature of 23°C, presenting a supernatant. Of the supernatant, ±1 g was withdrawn by means of a needle and syringe, where after it was transferred to a 50 ml volumetric flask and made up to volume with methanol. The amount of artemether in the supernatant was quantified by means of HPLC.

Equation 2, adapted from Kurakula et al. (2012), was used to calculate the %EE of the **NE**, where  $C_t$  is the total API concentration and  $C_f$  is the concentration of free untrapped drug.

$$\%EE = [(C_t - C_f) / C_t] \times 100 \quad \text{Equation 2}$$

## 2.4 Diffusion experiments

### 2.4.1 Membrane release studies

Twelve vertical Franz cells were used during each of the membrane release studies. Two of the twelve vertical Franz cells served as a control as they contained a dispersion or semi-solid without artemether, i.e. a **PNE**. Each vertical Franz diffusion cell consisted of a donor and receptor compartment, to which Dow Corning® high vacuum grease was applied. A magnetic stirrer was placed into the receptor compartment before placing the membrane on top. A polyvinylidene fluoride (PVDF) synthetic membrane with a pore size of 0.45 µm was placed between the two vacuum greased compartments. The two compartments were placed together and the sides were sealed with more vacuum grease to prevent leakages. The receptor compartment, with a diffusion area of 1.075 cm<sup>2</sup>, has a capacity of 2.0 ml and represents the blood flow of the body and was therefore filled with 2.0 ml (37°C) preheated absolute ethanol (receptor phase). When the receptor compartment was filled, it was visually examined to make sure there were no air bubbles present. Each individual donor compartment was filled with 1.0 ml of the respective preheated formulation (32°C). The donor compartment was then covered with two layers of Parafilm® and a plastic cap to prevent any loss of the formulation during the experiment. A horseshoe clamp was used to fasten the two compartments (donor compartment on top of the receptor compartment) securely together. This was done for all twelve Franz cells. The Franz cells were then placed in a water bath, at a constant temperature of 37°C, on a Variomag® (Variomag, USA) magnetic stirring plate, which was placed in a Grant® water bath (Grant Instruments, UK). The entire receptor phase was extracted through the sampling port and refilled with fresh absolute ethanol (37°C) hourly for 6 h. The content of the receptor compartment was then placed into HPLC vials and analysed by HPLC analysis.

#### 2.4.2 Skin preparation

Caucasian, female skin, obtained from abdominoplasty surgery, was used during skin diffusion studies. Ethical approval for the use of biological material, i.e. human skin, was  
305 obtained from the Research Ethics Committee of the North-West University (reference number NWU-00114-11-A5). The patient, who donated the skin, was required to complete an informed consent form and remained anonymous at all times. The skin was placed in the freezer at -20°C after collection until used. To prepare the dermatomed skin samples, the skin was visually examined for any defects or stretch marks, where after a dermatome™  
310 (Zimmer TDS, UK) was used to cut pieces of skin, with a thickness of approximately 400 µm, by pressing on the skin with the dermatome at a 30 – 45° angle and with constant pressure. The dermatomed skin was placed on Whatman® filter paper to dry and covered with aluminium foil. It was placed in the freezer at -20°C until needed for skin diffusion studies, when required circular shapes were cut out of the dermatomed skin and used between the  
315 two compartments of the vertical Franz cells.

#### 2.4.3 Skin diffusion studies

During the *in vitro* skin permeation studies, the same technique as for the membrane release studies (as described in Section 2.4.1) was employed; the differences were the PVDF synthetic membranes, used in the membrane studies, were replaced with circular  
320 dermatomed skin samples (with the stratum corneum facing upwards) and the receptor compartment was filled with 2.0 ml PBS (pH 7.4), instead of absolute ethanol, which was preheated to 37°C.

#### 2.4.4 Tape stripping

Once the skin had been exposed to the formulation for 12 h, the diffusion studies were  
325 completed, the Franz cells' compartments were disconnected and the skin samples carefully removed. The skin samples were pinned to Parafilm®, which was stapled to a wooden board, and lightly dabbed with tissue paper to dry them in order to remove any excess formulation (Walters & Brain, 2008). Next, the stratum corneum-epidermis (SCE) was removed by making use of adhesive tape (3M Scotch® Magic™ Tape), which was cut into an

adequate size to just cover the diffusional area, which was approximately 1.075 cm<sup>2</sup> (indicated by the imprint made on the skin by the Franz cells). The layers of the stratum corneum were removed using sixteen tape strips. The first strip was discarded as it was part of the procedure to remove any excess formulation and API; the other fifteen strips were used to remove the SCE until the area glistened, then they were placed inside a polytop, which contained 5 ml absolute ethanol. The remaining skin, i.e. the epidermis-dermis (ED), was cut into pieces and placed inside another polytop, also containing 5 ml absolute ethanol. Both polytops, containing the tape strips and the ED respectively, were left overnight ( $\pm 8$  h) in the fridge at  $\pm 4^{\circ}\text{C}$ ; thereafter, the samples were filtered and analysed by means of HPLC.

## 2.5 Data analysis

For each diffusion experiment, samples were analysed by means of HPLC to provide a linear line. This linear line was used to determine the concentration of artemether, i.e. drug flux in each of the Franz cells (Ng et al., 2010). For analysis purposes, the cumulative concentration ( $\mu\text{g}/\text{cm}^2$ ) of artemether that diffused through the membrane was plotted against time and the flux could be determined according to the linear line formed. The average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of artemether that diffused through the synthetic membrane was determined by the linear regression at hourly intervals for 6 h.

To determine the outcome of the skin diffusion studies (topical and/or transdermal delivery), the average concentration that diffused into the skin ( $\mu\text{g}/\text{ml}$ ) was calculated for the SCE and the ED; the cumulative amount of artemether per diffusion area ( $\mu\text{g}/\text{cm}^2$ ) that had diffused through the skin after 12 h was also calculated.

## 2.6 Cytotoxicity studies

Toxicity of the **NE** needed to be evaluated on humans or cell cultures of human nature. *In vitro* cytotoxicity studies were therefore conducted on HaCaT (immortalised human keratinocytes) cells by employing a methylthiazol tetrazolium (MTT) assay. It is known that the dehydrogenase enzymes in the mitochondria of metabolically active cells would change the water soluble MTT into an insoluble, purple formazan. This purple formazan is therefore

indicative of cell viability since it is unable to cross cell membranes, therefore remaining in the healthy cells (Fotakis & Timbrell, 2006).

The *in vitro* cytotoxicity testing was performed on the HaCaT cells to establish any possible change in the amount of viable cells. The HaCaT cells were seeded in a 96-well plate at a density of 20000 per well and incubated at 37°C (5% CO<sub>2</sub>, 95% humidity) for 24 h to ensure the cells attached to the well plate surface. The HaCaT cells were maintained in a cell culture flask (75 cm<sup>2</sup>) containing Dulbecco's Modified Eagle Medium (DMEM), or high glucose adequate growth medium, supplemented with 10% Foetal Bovine Serum (FBS), 1% non-essential amino-acids (NEAA), 4 mM L-glutamine and 1% of Penicillin/Streptomycin (Pen/Strep). It was then cultured in a CO<sub>2</sub> incubator at standard culturing conditions (37°C, 95% humidity and 5% CO<sub>2</sub>).

The MTT-reagent (2 mg/ml) was added to the HaCaT cell lines 24 h after treatment with the **PNE**, the **NE** and the artemether stock solution (**ArtS**), and the 96-well plates were incubated for a further 3 h until a purple precipitate was visible. Once visible, the growth medium was removed, the excess MTT reagent was also carefully removed and the purple formazan was dissolved by adding 200 µl dimethyl sulfoxide (DMSO) to each well plate (Lee et al., 2012:17). This was left at room temperature for 2 h. The amount of formed purple formazan was then spectrophotometrically measured with the SpectraMax<sup>®</sup> Paradigm<sup>®</sup> Multi-Mode Microplate reader (Molecular Devices, California, USA) at 570 nm (630 nm is normally used as the reference wavelength), using the SoftMax<sup>®</sup> Pro 6.2.1 Software (Riss et al., 2013:2).

### 3 Results and Discussion

#### 3.1 Formulation of the optimised nano-emulsion

An optimised **NE**, containing 0.8% (w/v) artemether and 5% (w/v) safflower oil, with a surfactant ratio of Tween<sup>®</sup> 80:Span<sup>®</sup> 60 (4:1), was obtained through pre-formulation. The optimised **NE** was formulated by preparing two phases: an oil phase (phase A) and a water phase (phase B). After the ingredients of phases A and B were added together, each phase was heated and stirred on a heated magnetic stirring plate. Once all the phases were

adequately mixed, phase A was added to phase B by means of drop wise addition with a syringe. It was then left to mix on the stirring plate – this is referred to as the coarse emulsion. In order to obtain nano-sized droplets, ultrasonication, a high-energy emulsification method, was applied to the coarse emulsion.

### 3.2 Formulation of a nano-emulgel and a conventional emulgel

Two semi-solids, i.e. the **NEG** and the **CEG** of the optimised **NE** and coarse emulsion were formulated. The semi-solids each contained 0.4% (w/v) artemether and 2.5% (w/v) safflower oil. Both semi-solids presented as a homogenous, white, smooth, gel-like semi-solid. No oil droplets, phase separation, sedimentation or undissolved excipients were visible.

### 3.3 Physicochemical properties

#### 3.3.1 Aqueous solubility

During this study it was found that artemether had an experimental aqueous solubility of  $0.1053 \pm 0.0022$  mg/ml in water and  $0.090 \pm 0.0030$  mg/ml in PBS (pH 7.4). It is known that an ideal topical API should possess an aqueous solubility larger than 1 mg/ml (Naik et al., 2000). With an aqueous solubility greatly lower than 1 mg/ml, it can be expected that artemether would have difficulty permeating through the skin.

#### 3.3.2 Log D

Experimentally it was found that artemether presented with a log D value of  $2.35 \pm 0.1170$  at pH 7.4; literature indicates that an API ideal for topical drug delivery presents with a log P value between 1 and 3 (Williams, 2013). As this value falls within acceptable ranges, it could suggest that artemether might present with effective topical drug delivery.

### 3.4 Characterisation of the formulations

The characteristics, such as pH, viscosity, droplet size and zeta-potential values, as obtained for each formulation, are summarised in Table 3.

Table 3: Characterisation results of the formulations containing artemether and safflower oil.

A pH value measuring lower than 3 or higher than 9 can cause harm to the skin and consequently influence permeability (Naik et al., 2000:319). Although all three of the

formulations (the **NE**, the **NEG** and the **CEG**) presented with an acceptable pH; it proposed very low unionised species. As based on the pH-partition hypothesis, unionised molecules present with easy permeability across these lipid layers; whereas ionised molecules show little to no degree of penetration into the stratum corneum (Barry, 2002; Williams, 2003). Viscosity readings indicated that the **NE** was not very viscous, whilst the semi-solids were rather viscous. Literature states that a zeta-potential, higher than 30 mV or lower than -30 mV, especially for a dispersion with small droplets can present with great stability (Eid et al., 2014; Silva et al., 2012). The average measured zeta-potential for all three formulations were highly negative and therefore ideal for topical delivery and could result in successful permeation through the skin (Duangjit et al., 2011). The %EE of artemether within the **NE** was determined to be 99.578%, suggesting almost full incorporation of artemether within the **NE**. A **PNE** with droplets within the range of 20 –200 nm was formulated and measured through TEM, as seen from Figure 2.

Figure 2: TEM micrographs of the **PNE** sonicated for 3 min and placed in an ultrasonication bath for 15 min

### 3.5 Diffusion experiment results

#### 3.5.1 Membrane release experiments

Membrane release studies affirmed artemether was released from all three formulations. The optimised **NE** presented with the highest percentage release ( $32.22 \pm 1.76\%$ ) compared to the semi-solids, **NEG** ( $11.36 \pm 0.60\%$ ) and **CEG** ( $9.47 \pm 0.57\%$ ) after 6 h. When the average flux values were compared, the **NE** presented with an average flux value of  $1011.4 \pm 66.968 \mu\text{g}/\text{cm}^2\cdot\text{h}$ , followed by the **NEG** at  $127.57 \pm 12.178 \mu\text{g}/\text{cm}^2\cdot\text{h}$  and lastly, the **CEG** at  $100.39 \pm 9.294 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ; a higher release was thus obtained for the **NE** when compared to the semi-solids. When the **NEG** and the **CEG** were compared, the **NEG** presented with a higher percentage release, which could be correlated with its droplet size, as one of the disadvantages of emulgels is they are constituted through large droplets, which limit permeation (Baibhav et al., 2011).

The large difference between the average flux values could be ascribed to the viscosity of the formulations. It has been proposed that lower viscosity could lead to an increase in API release from the dispersion (Chime et al., 2014). Generally, nano-emulsions present with very low viscosity compared to a nano-emulgel or conventional emulgel, which in turn are greatly viscous. When the two semi-solids, i.e. the **NEG** and the **CEG**, were compared, the **NEG** presented with a better average flux than the **CEG**. This permeation difference between the **NEG** and the **CEG** can be ascribed to the large droplets within the **CEG**, which limit permeation (Baibhav et al., 2011).

### 3.6 Skin diffusion results

No transdermal delivery of artemether was found for any of the three formulations (the **NE**, the **NEG** and the **CEG**). The lack of artemether found transdermally could possibly be ascribed to the API (artemether) itself or to the delivery system used (the **NE**, the **NEG** or the **CEG**). The hydrophilic systemic circulation, presents unfavourable for the lipophilic and weak aqueous soluble artemether, therefore limiting the quantification thereof. Lipophilic APIs could also be retained within the lipophilic layers, such as the SCE, therefore decreasing permeation into the circulation (Yourick et al., 2008). Since nano-emulsions could aid targeted delivery of an API by formulating a lipophilic API within the oil droplets of the oil phase, slow release could be a result, as well as targeting in the SCE (Hörmann & Zimmer, 2016). It can also be proposed that the initial concentration within the three formulations were too low to result in systemic delivery. The pH of the **NE**, the **NEG** and the **CEG** measured at 6.85, 5.14 and 5.86, respectively, and low unionised species of 0.11%, 5.44% and 1.09% were calculated for each of the three formulations. As a result, the lack of unionised species of artemether within each of the formulations (the **NE**, the **NEG** and the **CEG**) could be used as explanation for no quantification thereof in the systemic circulation. Although, no quantifiable amounts of artemether were found within the systemic circulation, it should be kept in mind that the aim of the study was to deliver artemether topically and not transdermally.

### 3.7 Tape stripping

The tape stripping technique produced data about whether topical delivery (within the SCE or within the ED) of artemether was achieved (OECD, 2004). In this study, the topical delivery of a novel CTB treatment was aimed at retaining the lipophilic artemether within the epidermis. The physicochemical properties of artemether suggested it would remain within the lipophilic stratum corneum and not permeate further. Concentrations of artemether were only detected in the SCE for the **NE**, but not for the two semi-solids. Yourick et al. (2008) suggested that a lipophilic API would result in a higher concentration of the API in the SCE (lipophilic layers) as opposed to the ED (hydrophilic layers). The average concentration of artemether in the SCE was quantified as  $21.173 \pm 9.842$   $\mu\text{g/ml}$ . When the samples of the semi-solids, i.e. the **NEG** and **CEG**, were analysed no artemether was quantified within the SCE; no concentration of artemether, for all three formulations (the **NE**, the **NEG** and the **CEG**) was quantified in the ED. It can be proposed that the lipophilic artemether was retained within the SCE and therefore was unable to diffuse to further layers; this could possibly explain why artemether was only found in the SCE and not in the ED for the **NE**. The fact the ED provided a hydrophilic environment and not a favourable lipophilic environment, could also aid these results. Very low unionised species were measured, respectively, for the three formulations (the **NE**, the **NEG** and the **CEG**), which could propose weak permeability as the formulations were largely ionised. Consequently, the lack of unionised species of artemether within each of the formulations could be used as explanation for no quantification thereof in the ED.

### 3.8 Cytotoxicity studies

Determination of viable cells was conducted using the Trypan Blue exclusion test by employing a haemocytometer. The live cells (purple formazan colour) were counted under a microscope using the counting chamber of the haemocytometer. López-García et al. (2014) stated that a treatment or compound can be determined as cytotoxic according to the following percentage of cell viability (it must be noted that these guidelines are normally assay and cell line specific): non-cytotoxic: >80%; weak cytotoxicity: 80% – 60%; moderate

cytotoxicity: 60% – 40%; and strong cytotoxicity: <40%. For the HaCaT cell line, the MTT %cell viability was assessed after 12 h of treatment with the **PNE**, the **NE** and the **ArtS**, respectively, in a 0.5%, a 1.0% and a 2.0% concentration. A set of cells were left untreated and served as a control group.

As seen from Table 4, a concentration dependent decrease and trend was viewed for all three of the treatments (**PNE**, **NE** and **ArtS**). No noticeable difference in %cell viability was observed for the different treatments in the 0.5% and the 1.0% concentrations, however, for the 2% concentration, a greater cell death was noted for all three treatments. Since the %cell viability of the different treatments were relatively high for the 0.5% and the 1.0% concentrations, these yielded percentages above 80%, therefore both were considered to be non-cytotoxic (López-García et al., 2014). Conversely, 2.0% concentrations presented with a drastic decrease in the %viable cells (for all three treatments). The %cell viability results for the 2% concentrations of **PNE** and **ArtS**, ranged between 60% and 40% and can subsequently be classified as having a moderate cytotoxicity, whilst the 2.0% concentration of the **NE** is considered as weak cytotoxic, with a %cell viability of 63.178%. Consequently, the **NE** presented as the least cytotoxic treatment, as observed in the 0.5% and the 1.0% concentrations, whilst the **ArtS** resulted in the highest %cell death. It can therefore be proposed that the **NE**, when compared to the **PNE** and the **ArtS**, had the same concentration dependent effect on the HaCaT cells – all three treatments were therefore relatively safe to use on the skin in low concentrations of 0.5% and 1.0%. Further investigation of the cytotoxicity and efficacy of artemether is needed, especially *in vivo* experiments, as it could provide the most accurate results.

Table 4: Percentage cell viability of the HaCaT cells after treatment

#### 4 Conclusion

This study aimed to investigate topical delivery, more so, the topical delivery of artemether as novel CTB treatment to the epidermis. The first aim was to formulate an optimised nano-emulsion containing artemether and a natural oil, i.e. safflower oil. Secondly, since the

optimised nano-emulsion was not very viscous, it was aimed to formulate it within a semi-solid dosage form; a nano-emulgel that was compared to a conventional emulgel for easier application. These formulations were characterised and evaluated for their topical delivery effects.

Following characterisation, the API release from the formulations was investigated. It can be said that artemether had been released from all three formulations (the **NE**, the **NEG** and the **CEG**), as found during the membrane release studies. The optimised **NE** presented with the highest average flux, followed by the **NEG** and lastly the **CEG**, after 6 h during the membrane release studies. Thereafter, the three formulations were investigated during *in vitro* skin diffusion studies, where artemether was only quantified within the SCE for the optimised **NE**, but not for the semi-solids. No concentrations of artemether were quantified for any of the three formulations in the ED, hence only the optimised **NE** resulted in topical delivery of artemether, which was the aim of this study. In addition, the API and the **NE** were investigated further by means of *in vitro* cell culture cytotoxic experiments, to establish the safety thereof towards human skin cells, and no cytotoxicity was found for both in low concentrations.

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**Conflict of Interest**

The authors declare that no conflict of interest exists.

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## Tables

Table 1: Ingredients used to formulate the nano-emulsions

| Phase                            | Excipient               | 4:1 surfactant ratio | 3:2 surfactant ratio |
|----------------------------------|-------------------------|----------------------|----------------------|
| <b>Oil Phase<br/>(phase A)</b>   | Artemether              | 0.4 g                | 0.4 g                |
|                                  | Lipophilic surfactant:  |                      |                      |
|                                  | Span <sup>®</sup> 60    | 0.5 g                | 1.0 g                |
|                                  | Safflower oil           | 2.3 ml               | 2.3 ml               |
| <b>Water phase<br/>(phase B)</b> | Hydrophilic surfactant: |                      |                      |
|                                  | Tween <sup>®</sup> 80   | 1.8 ml               | 1.5 ml               |
|                                  | Water                   | 45.0 ml              | 45.0 ml              |

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Table 2: Ingredients used to formulate the semi-solid dosage forms

| Phase              | Excipient               | NEG         | CEG         |
|--------------------|-------------------------|-------------|-------------|
| <b>Oil phase</b>   | Liquid Paraffin         | 20.0 g      | 20.0 g      |
|                    | Lipophilic surfactant:  |             |             |
|                    | Span® 60                | 0.5 g       | 0.5 g       |
|                    | Hydrophilic surfactant: |             |             |
|                    | Tween® 80               | 4.5 g       | 4.5 g       |
| <b>Water phase</b> | Nano-emulsion or        |             |             |
|                    | coarse emulsion         | 50.0 ml     | 50.0 ml     |
|                    | Xanthan gum             | 1.5 g       | 1.5 g       |
|                    | Water                   | ad 100.0 ml | ad 100.0 ml |

700 Table 3: Characterisation results of the formulations containing artemether and safflower oil

| <b>Characteristics<br/>determined</b> | <b>NE</b>    | <b>NEG</b>   | <b>CEG</b>   |
|---------------------------------------|--------------|--------------|--------------|
| <b>pH</b>                             | 6.82±0.03    | 5.14±0.02    | 5.86±0.02    |
| <b>Pdl</b>                            | 0.24±0.01    | 0.44±0.08    | 1.00±0.00    |
| <b>Droplet size (nm)</b>              | 156.34±2.96  | 229.59±98.00 | 401.30±13.26 |
| <b>Zeta-potential (mV)</b>            | - 41.93±1.43 | - 46.57±1.80 | - 43.90±1.20 |
| <b>Viscosity (cP)</b>                 | 1.64±0.02    | 795.87±3.40  | 884.53±2.01  |

Table 4: Percentage cell viability of the HaCaT cells after treatment

|                    | Concentration | PNE   | NE    | ArtS  | Control |
|--------------------|---------------|-------|-------|-------|---------|
| %cell<br>viability | 0.5%          | 86.33 | 90.03 | 84.94 | 100.00  |
|                    | 1.0%          | 86.50 | 90.95 | 87.29 | 100.00  |
|                    | 2.0%          | 56.97 | 63.18 | 57.00 | 100.00  |

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### Figure legends

Figure 1: Diagrammatic representation of the preparation of a nano-emulsion, nano-emulgel or a conventional emulgel

Figure 2: TEM micrographs of the **PNE** sonicated for 3 min and placed in an ultrasonication

710 bath for 15 min

## Figures

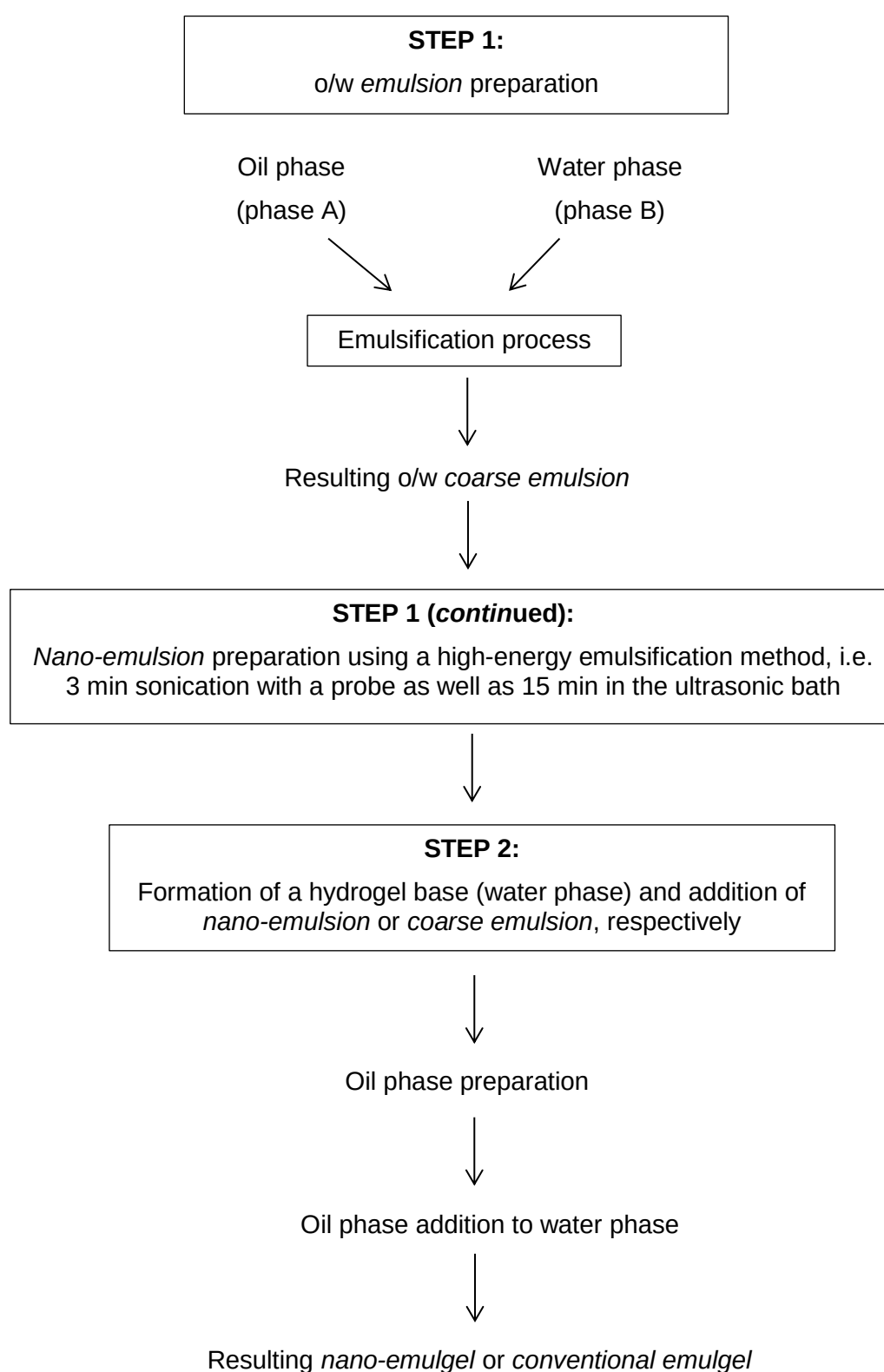


Figure 1: Diagrammatic representation of the preparation of a nano-emulsion, nano-emulgel

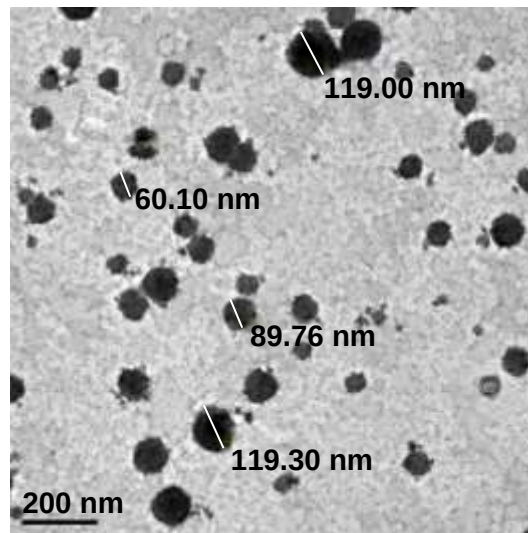


Figure 2: TEM micrographs of the **PNE** sonicated for 3 min and placed in an ultrasonication bath for 15 min

# CHAPTER 4:

## FINAL CONCLUSIONS AND FUTURE PROSPECTS

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Tuberculosis (TB), primarily caused through *M. tuberculosis*, can occur as pulmonary or extra-pulmonary presentations, the latter of which can present as CTB (Almaguer-Chávez *et al.*, 2009:562; Bravo & Gotuzzo, 2007:173). CTB is only one of the many extra-pulmonary presentations. CTB can present through a variety of inflamed, caseous, purulent and necrotic lesions, ranging from nodules, to abscesses and consequently, ulcers (Almaguer-Chávez *et al.*, 2009:564; Bravo & Gotuzzo, 2007:174-177; Van Zyl *et al.*, 2015:3). Currently there is no existing topical TB treatment and due to growing resistance, it is necessary to investigate the topical delivery route (Almaguer-Chávez *et al.*, 2009:563; Van Zyl *et al.*, 2015:630). Artemether, a derivative of artemisinin, which is usually employed as an anti-malarial treatment, possesses promising properties that can be used as effective TB treatment against *M. tuberculosis* (McIntosh & Oliaro, 2010:3; Miller *et al.*, 2011:2076). Although posing as an effective anti-TB API, it possesses physicochemical properties, which present as a challenge during topical drug delivery. Considering that the stratum corneum layer of the skin acts as a main barrier to external influences (due to its complex, impermeable and lipophilic structure), it can be viewed as the largest challenge to overcome during topical drug delivery (Subedi *et al.*, 2010:339; Venus *et al.*, 2011:472).

This study's aim was to investigate the topical delivery of artemether. Firstly to formulate an optimised nano-emulsion to contain artemether and a natural oil, i.e. safflower oil. Secondly, the optimised nano-emulsion was then formulated within a semi-solid dosage form; a nano-emulgel that was compared to a conventional emulgel. These formulations were characterised and evaluated for their topical delivery effects. In addition, the API and the nano-emulsion were further investigated by means of *in vitro* cell culture cytotoxic experiments to establish the safety thereof towards human skin cells.

In order to achieve the aforementioned aims, the following objectives were set:

- \* Validation of an HPLC analytical method for the quantitative determination of artemether concentrations in the formulations, as well as samples collected from diffusion studies.
- \* Determination of artemether's aqueous solubility and log D.
- \* Development and formulation of a nano-emulsion, a nano-emulgel as well as a conventional emulgel by incorporating artemether and safflower oil in the oil phase.

- \* Characterisation of the pre-formulated and optimised nano-emulsion in terms of morphology, droplet size, droplet size distribution, pH, zeta-potential, viscosity, entrapment efficiency and visual examination.
- \* Characterisation of the nano-emulgel and conventional emulgel in terms of morphology, droplet size, pH, zeta-potential, viscosity and visual examination.
- \* Determining the release of artemether from the optimised nano-emulsion, nano-emulgel and conventional emulgel through membrane diffusion studies.
- \* Determining the transdermal and topical delivery of artemether from the optimised nano-emulsion, nano-emulgel and conventional emulgel into the skin, by performing both Franz cell skin diffusion studies and tape stripping, respectively.
- \* Determining the cytotoxic effects of artemether, alone and in the optimised nano-emulsion, through conducting *in vitro* cell culture cytotoxic experiments on HaCaT cells.

The HPLC analysis method of artemether was validated under the supervision and the guidance of Prof JL du Preez, at the Analytical Technology Laboratory of the North-West University (NWU), Potchefstroom Campus, RSA. This analysis method was found to be sensitive, responsive and reliable for the quantification of the artemether. During this study, an optimised o/w nano-emulsion ((**NE1**), known as an o/w nano-emulsion with a Tween<sup>®</sup> 80:Span<sup>®</sup> 60 ratio of 4:1), was formulated, where after it was formulated within a semi-solid form. A (**NEG**) (nano-emulgel containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil) and a (**CEG**) (conventional emulgel containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil) were formulated and both were represented by a white, smooth and homogenous semi-solid. Consequently, these three formulations were used as test formulations during *in vitro* Franz cell diffusion studies and this analysis method was used to determine the concentrations of artemether within the samples as collected during the diffusion studies.

Ideal physicochemical properties of an API are needed to successfully achieve skin permeation. The aqueous solubility and log D are two important physicochemical properties of the API that need to be determined. In this study, it was determined that artemether presented with an aqueous solubility of  $0.1053 \pm 0.0022$  mg/ml (water) and  $0.090 \pm 0.0030$  mg/ml (PBS (pH 7.4)), as well as a log D value of  $2.35 \pm 0.1170$ . In terms of aqueous solubility, artemether would not be optimal for skin permeation compared to its ideal log D value, being less than 3 (which is viewed as ideal for skin permeation).

During pre-formulation, an optimised (**NE1**) with ideal characteristics was formulated. The optimised (**NE1**) measured nano-sized droplets ranging between 20 – 200 nm within a relatively monodispersed dispersion. These nano-sized droplets of the (**NE1**), together with its highly negative zeta-potential, could have effective skin permeation (Abolmaali *et al.*, 2011:140;

Duangjit *et al.*, 2011:6). The **(NE1)** was safe and non-irritating, with a skin acceptable pH and was also greatly entrapped within the oil phase of the **(NE1)**.

Semi-solid dosage forms of the optimised **(NE1)** were also formulated, since the **(NE1)** had a low viscosity making the application thereof to the skin difficult. A **(NEG)** and a **(CEG)** was consequently formulated. The **(NEG)** and the **(CEG)** presented with nano-sized droplets larger than the **(NE1)**, and the **(CEG)** contained larger droplets compared to those of the **(NEG)**, proposing a challenge during skin permeation. It was observed that both semi-solids, **(NEG)** and **(CEG)**, was relatively polydispersed, yet the **(NEG)** posed less polydispersed. Adequate and acceptable pH values were measured for the **(NEG)** and the **(CEG)**. The aim of increasing the viscosity of the optimised **(NE1)** was achieved, as the **(NEG)** and the **(CEG)** measured with a large average viscosity, indicating a completely viscous formulation. Both the **(NEG)** and the **(CEG)** had highly negative zeta-potential values.

Following this characterisation, all three formulations were investigated to determine whether the API was released from the formulations. The release of artemether from the **(NE1)**, the **(NEG)** and the **(CEG)** was determined through membrane diffusion studies, respectively. Artemether was released from all three formulations. The optimised **(NE1)** presented with the highest average flux ( $1\,011.4 \pm 66.968 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), followed by the **(NEG)** ( $127.57 \pm 12.178 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ) and lastly, the **(CEG)** ( $100.39 \pm 9.294 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ) after 6 h. Consequently, it can be said that the **(NE1)** presented with the highest average flux, compared to the very low average flux values of the semi-solids. When the two semi-solids were compared, the **(NEG)** presented with a higher average flux than the **(CEG)**. Following these studies, transdermal skin diffusion studies and tape stripping were performed.

Topical and transdermal delivery through the skin was investigated through *in vitro* skin diffusion studies and tape stripping, respectively. Artemether was found in the stratum corneum-epidermis and in the receptor phase when treated with the **(NE1)**, yet concentrations for the receptor phase was lower than both the limit of detection and lower limit of quantification, thus proposing it was not found in the systemic circulation. No results were found topically or transdermally when treated with the semi-solid dosage forms (the **(NEG)** and the **(CEG)**). No concentrations of artemether were quantified for any of the three formulations in the epidermis-dermis; only the optimised **(NE1)** resulted in topical delivery of artemether, which was the aim of this study.

It can be concluded that in this study, the optimised **(NE1)** was the most successful of all three formulations. It presented with optimal characteristics together with membrane and skin diffusion study results, whilst the semi-solids (**(NEG)** and **(CEG)**) presented with optimal characteristics and only membrane release results.

Artemether and the optimised **(NE1)** containing artemether were also investigated in terms of their toxicity through cytotoxicity studies. By conducting a methylthiazol tetrazolium (MTT) assay on HaCaT cell cultures, artemether and the **(NE1)** were determined to be non-cytotoxic, as they caused little cell death in a 0.5% and a 1.0% treatment. A 90.032% and a 90.951% cell viability was obtained for **(NE1)** and 84.938% and 87.288% cell viability was yielded for the artemether stock solution. These results were obtained for treatments with a 0.5% and a 1.0% concentration. Consequently, the API and the **(NE1)** can be viewed as non-cytotoxic and safe to be utilised as a novel topical CTB treatment.

To treat CTB, the topical delivery of artemether had to be achieved, thus permeation through the skin was not necessarily required. The **(NE1)** was the only formulation that succeeded in being retained within the stratum corneum, i.e. the epidermis. The semi-solids **((NEG)** and **(CEG))** were not successful in reaching the set aim.

Future prospects include:

- \* Other artemisinin derivatives may be used as API for the treatment of CTB topically.
- \* Other natural oils may also be investigated as permeation enhancers and with more favourable compatibility profiles with the artemether.
- \* Formulating artemether at higher concentrations in the nano-emulsion, the nano-emulgel and the conventional emulgel.
- \* Exploration of other semi-solids, such as a lotion, to see if it would result in optimal topical delivery of artemether.
- \* Investigation of the cytotoxicity effect of artemether and/or the nano-emulsion through other assays.
- \* Investigation of the efficacy studies of artemether and/or the nano-emulsion against *M. tuberculosis* cells.

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# APPENDIX A:

## VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF ARTEMETHER

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### A.1 Objective

The objective was to validate an HPLC assay in order to determine if the analytical method was suitable and sensitive enough with regards to the quantification of artemether in a topical o/w nano-emulsion and two semi-solid dosage forms, containing safflower oil. In order to validate this analytical method, certain validation criteria needed to be investigated (ICH, 2005:2). These criteria include linearity, accuracy, precision, ruggedness, detection limit and quantification limit (ICH, 2005:2). This HPLC assay was also used to determine and quantify the concentration of artemether in the receptor phase, as collected during *in vitro* diffusion studies.

### A.2 Chromatographic conditions

This HPLC analytical method for artemether was validated under the supervision and the guidance of Prof JL du Preez at the Analytical Technology Laboratory of the North-West University (NWU), Potchefstroom Campus, RSA. The validation of this method was done in conjunction with Me C van der Merwe, as both studies included artemether as an API. It should hereby be noted that the recording of data was done separately by each student.

Various chromatographic conditions need to be controlled during the validation process of an analytical method. These conditions include the analytical instrument used, the column, mobile phase, detection wavelength, injection volume and the flow rate. The following chromatographic conditions were used during the validation of an HPLC assay of artemether:

**Analytical instrument:** The artemether HPLC analysis was performed by making use of an Agilent® 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1100 gradient pump. A UV detector, as well as an autosampler injection mechanism, was fitted to the system. Chromatograms were analysed by making use of ChemStation Rev. A.10.02 acquisition and analysis software. A laboratory environment with a controlled temperature of 25 °C was maintained.

**Column:** A Venusil® XBP C<sub>18</sub> (2) reverse phase column (100 Å pores, 150 x 4.6 mm) with a particle size of 5 µm was used (Agela Technologies, Newark, DE).

|                          |                                                                                                                                                                                                                                                                                                           |
|--------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Mobile phase:</b>     | The mobile phase consisted of 70 vol (70%) of acetonitrile (ACN) and 30 vol (30%) of Milli-Q® water (Milli-Q® Academic water purification system, Merck-Millipore, Midrand, RSA). Before using the mobile phase it was filtered through a 0.45 µm nylon membrane filter (Agela Technologies, Newark, DE). |
| <b>Solvent:</b>          | Mixture of ACN and Milli-Q® water in a ratio of 70:30. This mixture, used as a solvent, was filtered before use.                                                                                                                                                                                          |
| <b>Injection volume:</b> | An injection volume of 50 µl was chosen.                                                                                                                                                                                                                                                                  |
| <b>Detection:</b>        | For the detection of artemether the UV detector was set at 216 nm.                                                                                                                                                                                                                                        |
| <b>Flow rate:</b>        | A flow rate of 1.0 ml/min was set.                                                                                                                                                                                                                                                                        |
| <b>Retention time:</b>   | Artemether's retention time is approximately ± 9.355 min.                                                                                                                                                                                                                                                 |
| <b>Run time:</b>         | Artemether's run time is 15 min.                                                                                                                                                                                                                                                                          |

### A.3 Validation criteria

#### A.3.1 Linearity

Linearity can be defined as a measurement of an analytical procedure's ability to obtain test results that are directly equivalent to the analyte concentration in the sample (APVMA, 2004:5; ICH, 2005:5). To determine the linearity, a linear regression analysis of the data plots, formed by the peak areas against the analyte concentration (µg/ml) on the linear regression curve, should be done. The plots should form a straight line ( $R^2 \geq 0.98$ ) (see Figure A.1) (Snyder *et al.*, 1997b:691). This value is indicative of a high degree of linearity, thus there is a direct relationship between the response and the concentration of the analyte (APVMA, 2004:4).

The regression curve was interpreted in terms of the peak area *versus* the analyte concentration (µg/ml). Consequently, the peak areas (y-axis) were plotted against the determined API concentrations (x-axis). The linearity of the data can best be described by the linear regression equation (Equation A.1), where *y* represents the peak area of the analyte (artemether), *m* represents the slope, *x* is the concentration of the analyte (artemether) and *c* is the y-intercept (Snyder *et al.*, 1997a:644).

$$y = mx + c$$

**Equation A.1**

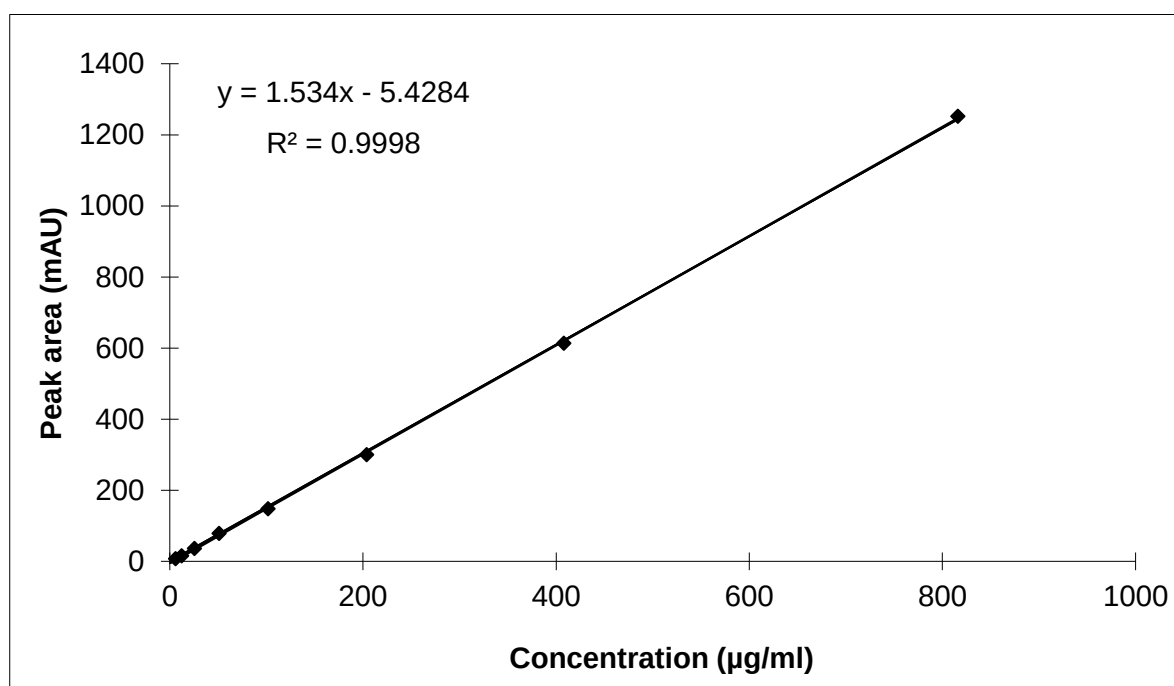
Linearity of the HPLC analysis was determined by preparing a stock solution and making seven dilutions thereof. Thus, 20.40 mg of artemether was weighed and dissolved in a 25 ml volumetric flask and made up to volume by adding the solvent (ACN:water = 70:30). Of the stock solution, 10 ml was transferred to a 20 ml volumetric flask and made up to volume with solvent (ACN:water = 70:30). This process was repeated six more times in order to obtain

different concentrations ranging from 6.13 – 816.00 µg/ml. These dilutions, together with the stock solution, were injected into the HPLC in duplicate, at a default injection volume of 50 µl.

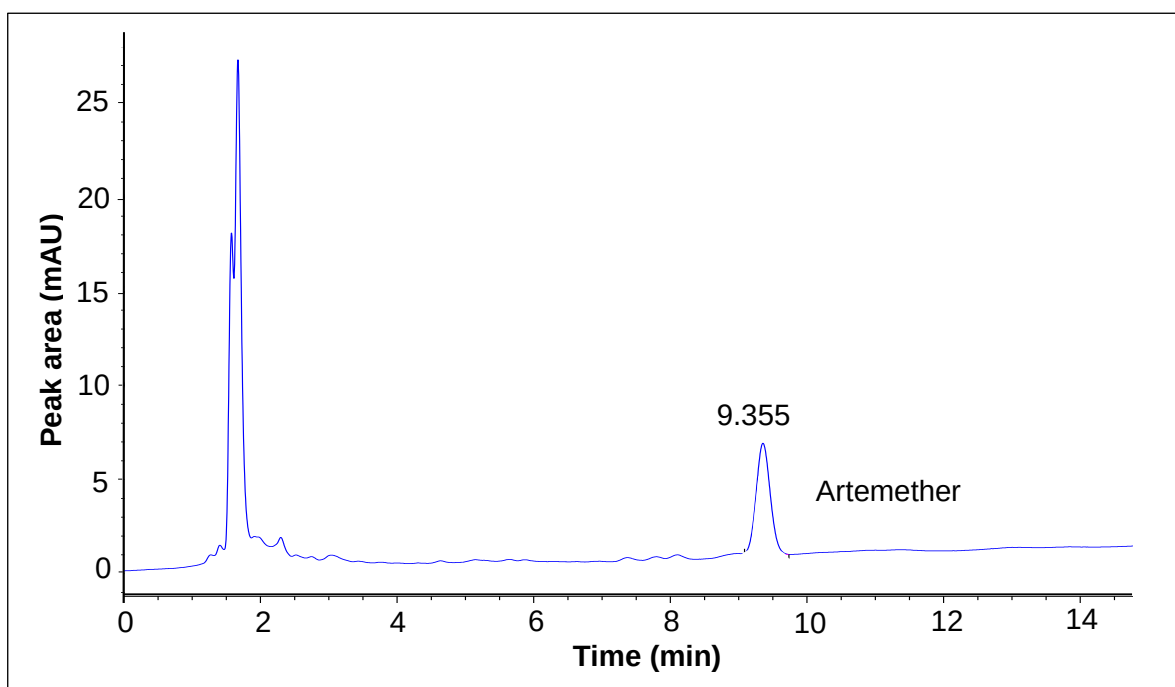
Table A.1 and Figure A.1 demonstrates the linearity data of artemether over a concentration range of 6.13 – 816.00 µg/ml. Artemether demonstrated an  $R^2$  value of 0.9998, obtained from the linear regression curve (see Table A.1 and Figure A.1), indicating a perfectly positive linear relationship. Figure A.2 represents an artemether HPLC chromatogram with a retention time of  $\pm 9.355$  min.

**Table A.1:** Linearity results of standard solution of artemether

| Sample         | Concentration (µg/ml) | Peak area 1 (mAU) | Peak area 2 (mAU) | Average peak area (mAU) |
|----------------|-----------------------|-------------------|-------------------|-------------------------|
| Dilution 7     | 6.13                  | 7.00              | 6.60              | 6.80                    |
| Dilution 6     | 12.25                 | 15.60             | 15.50             | 15.55                   |
| Dilution 5     | 25.50                 | 35.40             | 36.70             | 36.05                   |
| Dilution 4     | 51.00                 | 78.10             | 78.10             | 78.10                   |
| Dilution 3     | 102.00                | 149.40            | 146.50            | 147.95                  |
| Dilution 2     | 204.00                | 298.60            | 300.90            | 299.75                  |
| Dilution 1     | 408.00                | 613.20            | 612.80            | 613.00                  |
| Stock solution | 816.00                | 1252.90           | 1250.80           | 1251.85                 |
| $R^2$          |                       |                   |                   | 0.9998                  |
| y-intercept    |                       |                   |                   | - 5.4284                |
| Slope          |                       |                   |                   | 34.6639                 |



**Figure A.1:** Linear regression curve of artemether standard solutions



**Figure A.2:** HPLC chromatogram representing an artemether standard solution peak

### A.3.2 Accuracy

Accuracy of an analytical procedure can be viewed as the closeness of the test results, obtained by the procedure, to that of the true value (APVMA, 2004:4; ICH, 2005:4; USP, 2011:878). To determine the accuracy, it is recommended that a minimum of nine determinations over a range of three different concentrations, representing the full calibration curve range, be analysed (Bansal & DeStefano, 2007:E111; Snyder *et al.*, 1997b:691).

Accuracy was determined using one point on the linear regression curve (seen on Figure A.1) in order to prepare three samples. A point on the regression curve was chosen with a known concentration of 408 µg/ml. By means of calculations, it was determined that the following three samples should be prepared with the following quantities of artemether and solvent (ACN:water = 70:30):

- \* Sample 1 contained 10.22 mg artemether and 25 ml solvent.
- \* Sample 2 contained 10.16 mg artemether and 25 ml solvent.
- \* Sample 3 contained 10.25 mg artemether and 25 ml solvent.

For Samples 1 to 3, artemether was weighed and dissolved in solvent (ACN:water = 70:30), which was filled to volume of a 25 ml volumetric flask. To ensure adequate dissolving, the volumetric flasks were placed in an ultrasonication bath. All three samples were diluted twice, firstly by removing 10 ml of the stock solutions and placing it in a 20 ml volumetric flask and made up to volume with solvent (ACN:water = 70:30). From the first dilution, 10 ml was placed

in a 20 ml volumetric flask and made up to volume with solvent (ACN:water = 70:30). This resulted in nine determinations which were injected into the HPLC in duplicate.

A standard solution was also prepared to be used as a standard curve. This was done by weighing 11.56 mg of artemether and placing it in a 25 ml volumetric flask, made up to volume with solvent (ACN:water = 70:30) after which five dilutions were made. All the dilutions were prepared by taking 5 ml of the stock solution and placing it in a 10 ml volumetric flask, then made up to volume with solvent (ACN:water = 70:30). The stock solution plus five dilutions were injected, in duplicate, into the HPLC and the resulting peak areas are given in Table A.2.

**Table A.2:** Peak areas of a standard solution and five dilutions

| Sample (Standard) | Concentration spiked ( $\mu\text{g/ml}$ ) | Peak area (mAU) |        |         |
|-------------------|-------------------------------------------|-----------------|--------|---------|
|                   |                                           | 1               | 2      | Average |
| Dilution 5        | 14.45                                     | 21.40           | 13.80  | 17.60   |
| Dilution 4        | 28.90                                     | 41.10           | 40.90  | 41.00   |
| Dilution 3        | 57.80                                     | 86.60           | 86.80  | 86.70   |
| Dilution 2        | 115.60                                    | 162.30          | 160.90 | 161.60  |
| Dilution 1        | 231.20                                    | 360.90          | 359.10 | 360.00  |
| Standard solution | 462.40                                    | 713.70          | 713.20 | 713.50  |

Table A.3 establishes the accuracy data as well as the standard deviation (SD) and percentage relative standard deviation (%RSD).

**Table A.3:** Artemether accuracy parameters

| Concentration spiked ( $\mu\text{g/ml}$ ) | Peak area (mAU) |        |         | Recovery    |               |
|-------------------------------------------|-----------------|--------|---------|-------------|---------------|
|                                           | 1               | 2      | Average | (mg/100 ml) | %             |
| 410.00                                    | 629.10          | 628.70 | 628.90  | 407.75      | 99.45         |
| 408.80                                    | 638.60          | 639.00 | 638.80  | 414.11      | 101.30        |
| 406.40                                    | 617.00          | 617.00 | 617.00  | 400.11      | 98.45         |
| 205.00                                    | 311.20          | 311.00 | 311.10  | 203.75      | 99.39         |
| 204.00                                    | 317.60          | 317.00 | 317.30  | 207.73      | 101.83        |
| 203.20                                    | 304.50          | 304.10 | 304.30  | 199.38      | 98.12         |
| 102.50                                    | 153.70          | 153.70 | 153.70  | 107.71      | 100.20        |
| 102.20                                    | 158.80          | 158.80 | 158.80  | 105.98      | 103.70        |
| 101.60                                    | 149.20          | 149.50 | 149.35  | 99.92       | 98.34         |
|                                           |                 |        |         | <b>Mean</b> | <b>100.98</b> |
|                                           |                 |        |         | <b>SD</b>   | <b>1.51</b>   |
|                                           |                 |        |         | <b>%RSD</b> | <b>1.50</b>   |

The acceptable accuracy criteria are when the recovery results fall in a range of 98.00 – 102.00% (APVMA, 2004:5). Recovery, expressed as a percentage, can be defined as the ratio between the observed results and the estimated results (APVMA, 2004:5). Therefore, this percentage recovery serves as an indicator of the system's accuracy. The recovery of artemether ranged between 98.12 – 103.70% and a mean percentage recovery of 100.98% was determined. Consequently, an acceptable mean percentage recovery was yielded.

### A.3.3 Precision

An analytical procedure's precision can be viewed as the closeness of agreement between a series of individual measurements obtained from numerous sampling of the same standardised sample under prescribed conditions (ICH, 2005:4; Snyder *et al.*, 1997b:690). The precision of an analytical method can be investigated in terms of intra-day precision (repeatability), inter-day precision (reproducibility) and intermediate precision (ICH, 2005:4). In this study, only the repeatability and reproducibility was investigated and summarised in Tables A.4 and A.5.

#### A.3.3.1 Repeatability (intra-day precision)

Repeatability of an analytical procedure can be defined as the precision under the same operating conditions over a short period of time (ICH, 2005:4). Repeatability can be conducted by preparing at least nine determinations, e.g. three different samples of three different concentrations and analysing these samples on the HPLC on the same day (ICH, 2005:4). The samples were prepared by using the sample preparation method as used in Section A.3.2. A RSD of 2.00% or less is viewed as acceptable for repeatability (APVMA, 2004:5; Snyder *et al.*, 1997b:691).

**Table A.4:** Repeatability (intra-day precision) results of artemether

| Concentration spiked (µg/ml) | Peak area |        |         | Recovery    |               |
|------------------------------|-----------|--------|---------|-------------|---------------|
|                              | 1         | 2      | Average | (mg/100 ml) | %             |
| 410.00                       | 629.10    | 628.70 | 628.90  | 407.75      | 99.45         |
| 408.80                       | 638.60    | 639.00 | 638.80  | 414.11      | 101.30        |
| 406.40                       | 617.00    | 617.00 | 617.00  | 400.11      | 98.45         |
| 205.00                       | 311.20    | 311.00 | 311.10  | 203.75      | 99.39         |
| 204.00                       | 317.60    | 317.00 | 317.30  | 207.73      | 101.83        |
| 203.20                       | 304.50    | 304.10 | 304.30  | 199.38      | 98.12         |
| 102.50                       | 153.70    | 153.70 | 153.70  | 107.71      | 100.20        |
| 102.20                       | 158.80    | 158.80 | 158.80  | 105.98      | 103.70        |
| 101.60                       | 149.20    | 149.50 | 149.35  | 99.92       | 98.34         |
|                              |           |        |         | <b>Mean</b> | <b>100.98</b> |
|                              |           |        |         | <b>SD</b>   | <b>1.51</b>   |
|                              |           |        |         | <b>%RSD</b> | <b>1.50</b>   |

In order to determine the repeatability of artemether, the preparations and results of the samples used is described in Section A.3.2. Table A.4 represents the repeatability results and illustrates that artemether had a RSD of 1.50%, which is acceptable.

### **A.3.3.2 Reproducibility (inter-day precision)**

Reproducibility can be defined as the agreement between numerous measurements that have been collected under sample conditions within a laboratory using the same method (ICH, 2005:5). The reproducibility of an analytical method expresses the method precision between different laboratories. This was determined by performing analysis on three homogenous samples, with approximately the same known concentration on three consecutive days, with the HPLC.

#### **Day 1:**

The %recovery results from the repeatability data were used (Section A.3.3.1).

#### **Day 2:**

The following samples were prepared and analysed with the HPLC:

- \* Sample 1 contained 10.18 mg artemether and 25 ml solvent.
- \* Sample 2 contained 10.15 mg artemether and 25 ml solvent.
- \* Sample 3 contained 10.18 mg artemether and 25 ml solvent.

A standard solution was prepared with artemether (5.13 mg) and solvent (ACN:water = 70:30) in a 25 ml volumetric flask.

#### **Day 3:**

The following samples were prepared and analysed with the HPLC:

- \* Sample 1 contained 10.17 mg artemether and 25 ml solvent.
- \* Sample 2 contained 10.15 mg artemether and 25 ml solvent.
- \* Sample 3 contained 10.19 mg artemether and 25 ml solvent.

A standard solution was prepared with artemether (5.10 mg) and solvent (ACN:water = 70:30) in a 25 ml volumetric flask.

All the samples were diluted using the same method as described in Section A.3.2 and injected into the HPLC, in duplicate, for analysis. Acceptable reproducibility criteria are a %RSD equal or less than 3.00% (Rafael *et al.*, 2007:100). The inter-day precision with a RSD value of 1.95% was within the acceptable limits as is illustrated by Table A.5.

**Table A.5:** Inter-day precision results of artemether

| Days          | %Recovery |        |       | Mean   | SD   | %RSD |
|---------------|-----------|--------|-------|--------|------|------|
| Day 1         | 100.66    | 103.13 | 99.37 | 101.05 | 1.56 | 1.54 |
| Day 2         | 99.08     | 97.59  | 99.36 | 98.68  | 0.78 | 0.79 |
| Day 3         | 100.42    | 96.07  | 97.71 | 98.06  | 1.79 | 1.83 |
| Between days: |           |        |       | 99.26  | 1.94 | 1.95 |

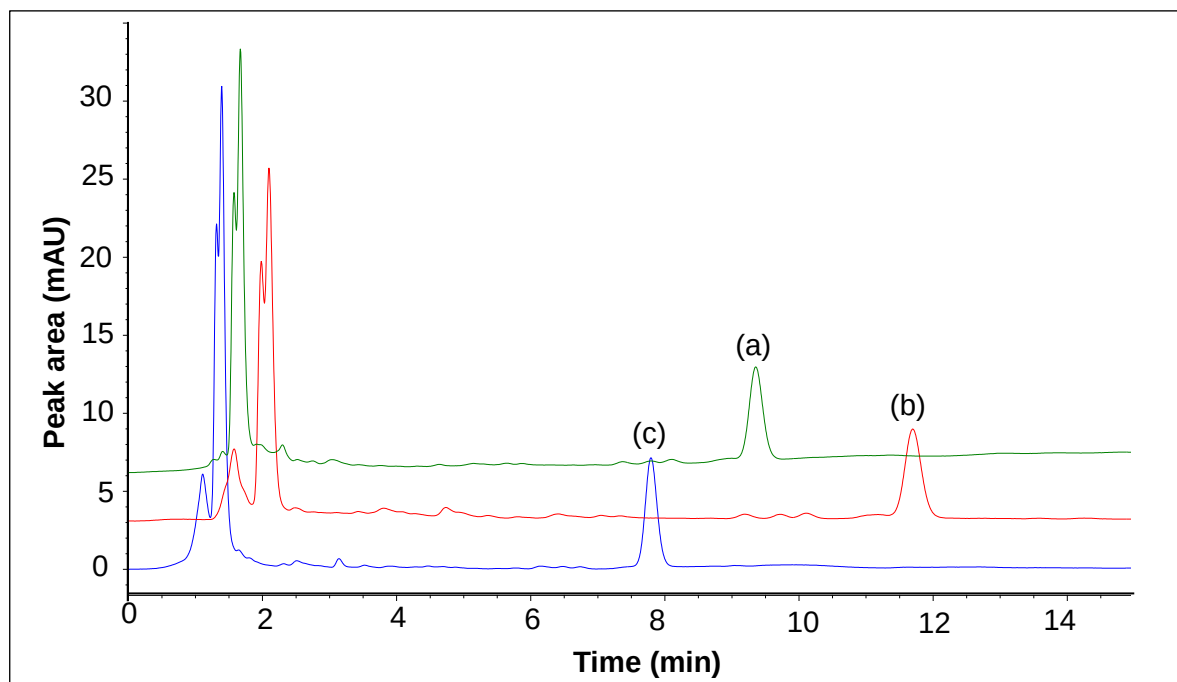
#### A.3.4 Robustness

Robustness can be viewed as the measurement to determine whether the analytical procedure stays constant even though deliberate changes have been made to certain test parameters. Deliberate changes can be made to the UV wavelength, the injection volume or the flow rate (USP, 2011:881). This measurement is therefore an indication of the effect of these small parameter changes on the retention time of the peaks on the chromatograms (Snyders *et al.*, 1997b:702). To determine the robustness of the analytical procedure, the sample chromatograms acquired under the altered parameters must be compared to the sample chromatogram obtained under normal test parameters; therefore indicating whether or not the analytical method is reliable (ICH, 2005:5). Robustness was determined by preparing a standard solution containing 5.00 mg artemether dissolved in a 100 ml volumetric flask containing ACN:water (70:30) solvent and placed in the ultrasonication bath to ensure adequate mixing.

A volume of the standard solution was placed in an HPLC vial and injected into the HPLC under normal chromatogram parameters (50 µl injection volume, 1.00 ml/min flow rate at a wavelength of 216 nm) – this was used as reference chromatogram (Figure A.3.a). The standard solution was injected for a second time (SROB1; Figure A.3.c) under varied parameters (45 µl injection volume, flow rate of 1.20 ml/min at a wavelength of 210 nm) and again (SROB2; Figure A.3b) under varied parameters (55 µl injection volume, 0.80 ml/min flow rate at a wavelength of 220 nm). Table A.6 represents the data obtained during the robustness test and in Figure A.3; the chromatograms obtained from these injections were compared. Hence, the peak areas and retention times of SROB1 and SROB2 were compared to the reference chromatogram of the standard solution. It can be seen that the changes in the peak areas and retention times, due to the varied HPLC parameters, was minimal (artemether peak shifted between 7 and 12 min) and therefore the method can be viewed as reliable.

**Table A.6:** Robustness data for artemether HPLC method

| Sample                | HPLC parameters       |                    |                 |                 |                      |
|-----------------------|-----------------------|--------------------|-----------------|-----------------|----------------------|
|                       | Injection volume (µl) | Flow rate (ml/min) | Wavelength (nm) | Peak area (mAU) | Retention time (min) |
| Standard solution (a) | 50                    | 1.00               | 216             | 83.549          | 9.355                |
| SROB1 (c)             | 45                    | 1.20               | 210             | 86.474          | 7.792                |
| SROB2 (b)             | 55                    | 0.80               | 220             | 96.573          | 11.696               |



**Figure A.3:** An HPLC chromatogram representing the robustness data of an artemether standard solution injected at different test parameters: a) normal conditions of 50 µl injection volume, 1.0 ml/min flow rate and wavelength of 216 nm; b) at 55 µl injection volume, 0.8 ml/min flow rate and a wavelength of 220 nm and c) at 45 µl injection volume, flow rate of 1.2 ml/min and at a wavelength of 210 nm

### A.3.5 Ruggedness

Ruggedness is the ability to reproduce results when the method is performed under actual conditions used; therefore it determines the method's variability (Snyder *et al.*, 1997b:701). It can be investigated in terms of system stability and system repeatability.

#### A.3.5.1 System stability

A 50.8 µg/ml sample of artemether was prepared by weighing 5.08 mg of artemether, which was dissolved in 100 ml of solvent (ACN:water = 70:30). A volume was transferred to an HPLC

vial with a micropipette and left in the HPLC autosampler tray. With the HPLC, the sample was investigated at hourly intervals for 24 h in order to determine the stability and method. Sample solutions should not be used for a period longer than what it takes to degrade by 2.00% (Cowley, 2012:113). From Table A.7, it can be seen that artemether was stable for a period of 24 h with a %RSD of 1.02, therefore no degradation was observed.

**Table A.7:** Sample stability parameters for artemether

| <b>Time (h)</b> | <b>Peak area</b> | <b>%Remaining</b> |
|-----------------|------------------|-------------------|
| <b>0</b>        | 74.90            | 100.00            |
| <b>1</b>        | 75.50            | 100.80            |
| <b>2</b>        | 74.50            | 99.47             |
| <b>3</b>        | 74.70            | 99.73             |
| <b>4</b>        | 75.00            | 100.13            |
| <b>5</b>        | 74.60            | 99.60             |
| <b>6</b>        | 74.50            | 99.47             |
| <b>7</b>        | 73.90            | 98.66             |
| <b>8</b>        | 73.70            | 98.40             |
| <b>9</b>        | 73.80            | 98.53             |
| <b>10</b>       | 73.90            | 98.66             |
| <b>11</b>       | 74.30            | 99.20             |
| <b>12</b>       | 74.70            | 99.73             |
| <b>13</b>       | 74.70            | 99.73             |
| <b>14</b>       | 74.70            | 99.73             |
| <b>15</b>       | 74.90            | 100.00            |
| <b>16</b>       | 74.90            | 100.00            |
| <b>17</b>       | 75.30            | 100.53            |
| <b>18</b>       | 74.90            | 100.00            |
| <b>19</b>       | 75.00            | 100.13            |
| <b>20</b>       | 75.20            | 100.40            |
| <b>21</b>       | 75.30            | 100.53            |
| <b>22</b>       | 75.80            | 101.20            |
| <b>23</b>       | 76.60            | 102.27            |
| <b>24</b>       | 77.00            | 102.80            |
| <b>Mean</b>     | <b>74.89</b>     | <b>99.99</b>      |
| <b>SD</b>       | <b>0.76</b>      | <b>1.02</b>       |
| <b>%RSD</b>     | <b>1.02</b>      | <b>1.02</b>       |

### A.3.5.2 System repeatability

In order to determine the repeatability of the system in terms of accuracy and precision, a sample with a concentration of 50.80 µg/ml was prepared as described in Section A.3.5.1. This sample was injected into the HPLC seven consecutive times, which evaluated the repeatability of the peak areas as well as the retention times of the sample on the same day and under the same conditions. A %RSD of less than 2.00% is viewed as acceptable criteria for the peak area and retention times (Cowley, 2012:114). Table A.8 reflects the performance of the system, which was acceptable as the %RSD values for the peak area and the retention time was 0.29% and 0.54%, respectively.

**Table A.8:** System repeatability of artemether

| Injection | Peak area | Retention time (min) |
|-----------|-----------|----------------------|
| 1         | 75.50     | 11.96                |
| 2         | 76.00     | 12.00                |
| 3         | 76.10     | 12.03                |
| 4         | 76.10     | 12.07                |
| 5         | 75.90     | 12.10                |
| 6         | 75.90     | 12.13                |
| 7         | 75.60     | 12.16                |
| Mean      | 75.87     | 12.06                |
| SD        | 0.22      | 0.07                 |
| %RSD      | 0.29      | 0.54                 |

### A.3.6 Specificity

Specificity is a measurement that can be used to accurately investigate the concentration analyte in the presence of other components (ICH, 2005:4). Specificity is needed to consider an analytical procedure as acceptable (Snyders *et al.*, 1997b:696).

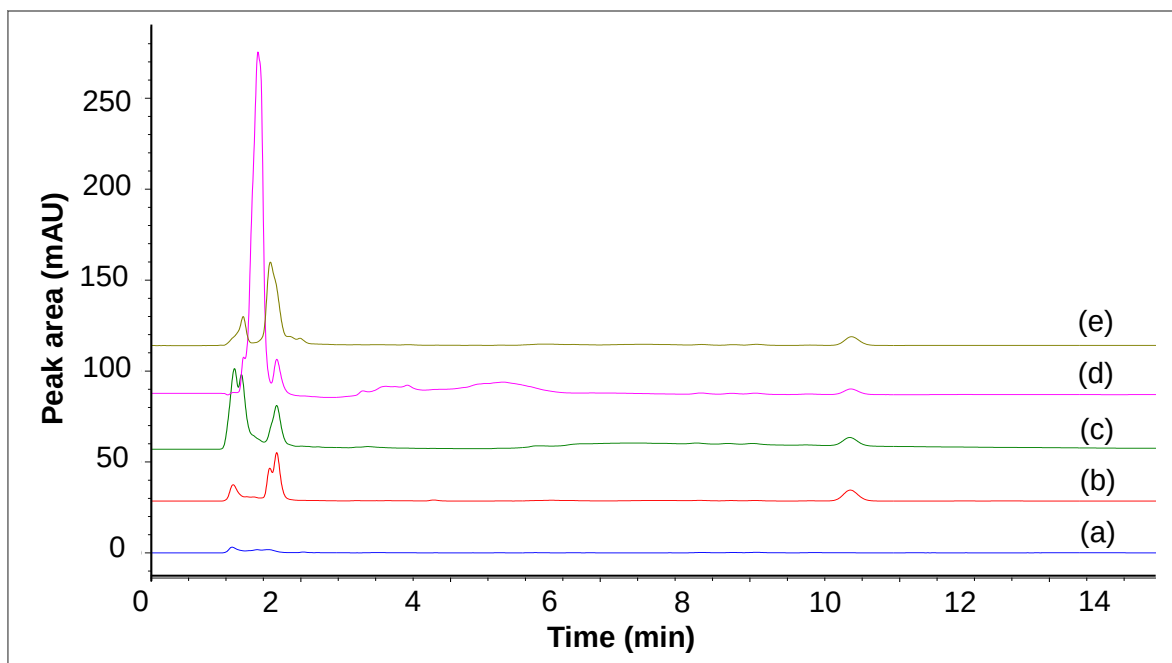
Specificity was determined by preparing five samples: a standard solution of 5.00 mg artemether dissolved in 100 ml volumetric flask containing ACN:water (70:30) solvent (Figure A.4.b). After placing the standard solution in the ultrasonication bath, 1 ml of the solution was placed into three individual test tubes. To one of the test tubes, 200 µl ammonia (NH<sub>4</sub>OH) was added (Figure A.4.c), to another test tube 200 µl hydrochloric acid (HCl) was added (Figure A.4.d) and to the third test tube 200 µl hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added (Figure A.4.e). These test tubes were shaken and left to stand for a few minutes. Thereafter, a volume of each tube was transferred to a corresponding HPLC vial. A volume of the standard solution was also placed into an HPLC vial. An HPLC vial containing only ACN:water (70:30)

mobile phase was used as a placebo test sample (Figure A.4.a). All sample vials were analysed in duplicate by means of HPLC.

The method can be viewed as specific when there are no peaks interfering with the retention time of the API (Snyders *et al.*, 1997b:700). Table A.9 represents the peak areas and retention times of the five samples injected into the HPLC; the retention time of artemether was not affected and therefore the method can be viewed as specific. Figure A.4 represents the HPLC chromatogram of the samples: the placebo (a) injection was represented by a flat line on the chromatogram in comparison to the standard solution (b), the  $\text{NH}_4\text{OH}$  (c), the HCl (d) and the  $\text{H}_2\text{O}_2$  (e) chromatograms presenting with a retention time of  $\pm 9.3$  min. As seen on Figure A.4, no peak interference was visible therefore the artemether peak was not obstructed in any way.

**Table A.9:** Specificity data for artemether

| Sample                     | Peak area (mAU) |       |         | Retention time (min) |
|----------------------------|-----------------|-------|---------|----------------------|
|                            | 1               | 2     | Average |                      |
| Placebo (a)                | 0.00            | 0.00  | 0.00    | 0.00                 |
| Standard solution (b)      | 83.70           | 84.20 | 83.95   | 9.34                 |
| $\text{NH}_4\text{OH}$ (c) | 63.20           | 63.40 | 63.30   | 9.33                 |
| HCl (d)                    | 41.20           | 41.10 | 41.15   | 9.35                 |
| $\text{H}_2\text{O}_2$ (e) | 65.60           | 65.60 | 65.60   | 9.36                 |



**Figure A.4:** HPLC chromatogram showing specificity data for artemether: a) the placebo solution; b) the standard solution of artemether; c) the sample solution of artemether stressed in 200  $\mu\text{l}$   $\text{NH}_4\text{OH}$ ; d) the sample solution stressed in 200  $\mu\text{l}$  HCl and e) the sample solution stressed in 200  $\mu\text{l}$   $\text{H}_2\text{O}_2$

### A.3.7 Limit of detection and lower limit of quantification

#### A.3.7.1 Limit of detection

The limit of detection (LOD) is a representative value of sensitivity and can be viewed as the analyte with the lowest concentration that can be detected in the sample matrix under specific analytical conditions (APVMA, 2004:4; Snyder *et al.*, 1997b:659).

In order to determine the LOD of artemether, a stock solution was prepared by weighing 5.08 mg of artemether and placing it in a 100 ml volumetric flask. This was made up to volume by adding solvent (ACN:water = 70:30) to the powder. To prepare the dilution, 5 ml of the stock solution was withdrawn with a micropipette and placed into a 50 ml volumetric flask, made up to volume by adding solvent (ACN:water = 70:30) to it, after which it was placed in the ultrasonic bath to ensure full dissolving. This was transferred to an HPLC vial. The sample was injected at different injection volumes ranging from 10 – 50 µl (increasing with increments of 10 µl at a time). For each injection volume, the sample was injected into the HPLC seven times, resulting in thirty-five injections. A %RSD of no more than 15.00% is seen as acceptable criteria for LOD (FDA, 2001:10; Rathmann *et al.*, 2015:54). The LOD of artemether was determined to be 10.16 µg/ml with an acceptable %RSD of 13.35, as represented by Table A.10.

#### A.3.7.2 Lower limit of quantification

**Table A.10:** LOD and LLOQ results of artemether

|                       | LOD   | LLOQ  |
|-----------------------|-------|-------|
| Injection volume (µl) | 10.00 | 20.00 |
| Concentration (µg/ml) | 10.16 | 20.32 |
| Peak area             | 1.20  | 2.40  |
|                       | 1.00  | 2.40  |
|                       | 1.50  | 2.30  |
|                       | 1.40  | 2.50  |
|                       | 1.10  | 2.30  |
|                       | 1.40  | 2.40  |
|                       | 1.20  | 2.20  |
| Mean                  | 1.26  | 2.36  |
| SD                    | 0.17  | 0.09  |
| %RSD                  | 13.35 | 3.83  |

The lower limit of quantification (LLOQ) is a representative value of sensitivity and can be viewed as the least amount of analyte in a sample matrix that can be quantified with acceptable accuracy and precision (APVMA, 2004:9). The same dilution, as prepared in Section A.3.4.1, was used to determine LLOQ. The acceptable criteria for the LLOQ can be viewed as a %RSD of less than 20.00% (FDA, 2001:10; Rathmann *et al.*, 2015:53). As represented by Table A.10, the LLOQ of artemether was 20.32 µg/ml and it was determined to have a %RSD of 3.83.

#### **A.4 Conclusion**

In conclusion, the HPLC method for artemether was successfully validated; the method was found to be sensitive, responsive and reliable for the quantification of the artemether concentration in the formulated nano-emulsion, as well as in the semi-solid dosage forms. The method performed according to acceptable criteria and should therefore be appropriate to determine and analyse the API in the formulations. Consequently, this analysis method was also used for the quantification of artemether during *in vitro* skin diffusion studies (discussed further in Appendix F).

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ICH **see** International Conference of Harmonisation

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USP **see** United States Pharmacopeia

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[http://www.drugfuture.com/Pharmacopoeia/usp35/PDF/08770882%20\[1225\]%20VALIDATION%20OF%20COMPENDIAL%20PROCEDURES.pdf](http://www.drugfuture.com/Pharmacopoeia/usp35/PDF/08770882%20[1225]%20VALIDATION%20OF%20COMPENDIAL%20PROCEDURES.pdf) Date of access: 20 Sept. 2015.

# APPENDIX B:

## FORMULATION OF AN O/W NANO-EMULSION CONTAINING ARTEMETHER AND SAFFLOWER OIL

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### B.1 Introduction

Although it is known that the skin provides protection against any external factors, it is easier for microorganisms, such as *M. tuberculosis*, to penetrate the skin when the barrier is compromised (Almaguer-Chávez *et al.*, 2009:563). This could describe one of the occurrences of extra-pulmonary TB, better known as CTB. Topical drug delivery focuses on the treatment of skin disorders by directly applying an API to the site, resulting in the API being retained within the skin (Williams, 2013:676). Currently there is no available topical treatment for CTB and as a result, an opportunity for the development thereof exists (Van Zyl *et al.*, 2015:630). Systemic TB treatment together with topical treatment could result in an efficient treatment with better results.

Although topical drug delivery offers many advantages it presents one unavoidable disadvantage – the skin, which acts as a barrier (Lovelyn & Attama, 2011:630; Naik *et al.*, 2000:319). This barrier is due to the stratum corneum layer acting as an API flux regulator and as a rate-limiting step when an API crosses the skin layers (El Maghraby *et al.*, 2008:204; Williams, 2003:28). Many attempts have been made to overcome this barrier and to increase API flux (Shakeel *et al.*, 2007:E1).

Properties of the skin, physicochemical properties of the API and characteristics of the delivery system used to deliver the API can influence skin penetration and absorption (Wiechers & Watkinson, 2008:63). Therefore, the physicochemical properties of the API should be thoroughly investigated since it is important to formulate a product that is acceptable and ideal for topical drug delivery (Williams, 2013:685). The delivery system is just as important as it should be compatible with the chosen API; it should not present any irritation and should be acceptable as a topical treatment (Weiss, 2011:471). A nano-emulsion have been chosen as a possible delivery system, given the promising permeation enhancement effects and many unique advantages due to nano-sized droplets and by being a two-phase dispersion consisting of a water and an oil phase, which could help facilitate absorption and permeation (Abolmaali *et al.*, 2011:140; Shakeel *et al.*, 2007:E1). Lipophilic APIs, such as artemether, can be delivered through nano-emulsions, especially o/w nano-emulsions (Chime *et al.*, 2014:98).

## **B.2 Intended purpose of the formulation**

During the development and formulation process of a new formulation, the intended purpose should be kept in mind. The aim of this study was to investigate a novel topical CTB treatment with artemether as the API. Although artemether presents with an ideal low molecular weight, it has less ideal solubility and ionisation properties therefore hindering the utilisation thereof as a topical API. Consequently, a nano-emulsion as a drug delivery system has been proposed to improve these unfavourable properties (Silva *et al.*, 2012:864). To achieve the aim of this study and to overcome unfavourable physicochemical properties, an o/w nano-emulsion containing artemether and safflower oil was formulated and investigated, firstly, to be effective as a novel topical treatment of CTB and secondly, be successful in overcoming the barrier presented by the skin. It should be noted that CTB would still be treated systemically and that the topical formulation would be used concurrently.

## **B.3 Delivery system selection**

A variety of properties can influence topical drug delivery and especially drug flux; one property being the API release from the selected delivery system (Barry, 2002:508). A topical drug delivery system can also be referred to as a vehicle (Weiss, 2014:472). It is important that the excipients within the delivery system should result in effective drug release and should be suitable for topical drug delivery (Weiss, 2014:472). Delivery system selection is therefore important, as they are responsible for bringing an API in contact with the target (Weiss, 2014:472). Consequently, the API must diffuse within the selected delivery system from where it moves through the skin layers, complicating the delivery process further (Weiss, 2014:471; Wiechers, 2008:7).

In this study, a nano-emulsion was used as delivery system for the topical delivery of artemether. Selection was based on the fact that the skin was the target and nano-emulsions proposed effective topical delivery (Shakeel *et al.*, 2007:E1). Effective topical delivery can be ascribed to the many advantages that nano-emulsions pose when being used as delivery system (Section 2.9.2.1).

## **B.4 Excipients used to formulate a nano-emulsion**

The main excipients used when formulating a nano-emulsion consists of oils, aqueous material, surfactant(s) and co-surfactant(s) (Basera *et al.*, 2015:1878; Chime *et al.*, 2014:90; Reddy *et al.*, 2013:87; Thakur *et al.*, 2012:222). These excipients, especially the oils, can influence the other selected ingredients, therefore oils should facilitate the intended purpose, be compatible with the selected API and aid the formation of nano-emulsions (Reddy *et al.*, 2013:87). Surfactants also play an important role during the formation process of the nano-emulsion by decreasing the

interfacial tension between the oil and water phase and consequently, any coalescence (Chime *et al.*, 2014:92; Reddy *et al.*, 2013:87; Tadros *et al.*, 2004:305). The type of nano-emulsion, either an o/w or w/o, is generally determined by the API that needs to be delivered or by the aim of the study. The API is as a result of being combined within the core component, either being part of the water or the oil phase (Chime *et al.*, 2014:77; Kela & Kaur, 2013:9203).

The aim of this study was to formulate an o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil as a possible novel treatment of CTB. Dispersions with different surfactant ratios were formulated and the most optimal was chosen accordingly. The excipients used during the formulation of an o/w nano-emulsion containing artemether and safflower oil are listed in Table B.1.

**Table B.1:** Excipients, function, suppliers and batch numbers as used during the formulation of the o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil

| Excipient      | Function                             | Supplier                        | Batch number   |
|----------------|--------------------------------------|---------------------------------|----------------|
| Artemether     | API                                  | DB Fine Chemicals               | 120702         |
| Safflower oil  | Natural oil and penetration enhancer | Sharon Bolel Chemical Marketing | P-0810B13      |
| Span® 60       | Lipophilic surfactant                | Fluka                           | 423065/1 41002 |
| Tween® 80      | Hydrophilic surfactant               | Merck Chemicals                 | 1043695        |
| Milli-Q® water | Solvent                              | In house                        | Direct Pure UP |

#### B.4.1 Artemether

Artemether presents as a lipophilic API (as described in Section 2.3.3.2). According to Kela and Kaur (2013:9203), lipophilic APIs are generally formulated within an o/w nano-emulsion. Consequently, in this study, artemether was incorporated within the oil phase of the nano-emulsion for adequate formulation and delivery, resulting in the formulation of an o/w nano-emulsion. The concentration of artemether was kept at 0.8% (w/v) throughout all the dispersions.

#### B.4.2 Safflower oil

A variety of oils, ranging from natural or synthetic lipids, fatty acids, triglycerides or mineral oils, with medium to long chain triglycerides, can be used as the oil phase (Abolmaali *et al.*, 2011:140; Baibhav *et al.*, 2011:68). It is important that the oil used should also solubilise the lipophilic API incorporated within and facilitate the formation of a nano-emulsion (Debnath *et al.*, 2011:74; Reddy *et al.*, 2013:87; Setya *et al.*, 2014:2218). Through adequate solubility of the API, maximum drug loading can also be achieved (Debnath *et al.*, 2011:74; Reddy *et al.*,

2013:87). In this study, safflower oil (a natural oil), which is composed mainly of linoleic acid, a C<sub>18</sub>-unsaturated fatty acid that presents with ideal penetration enhancement effect, was used to form the core of the oil phase of the nano-emulsion (Williams & Barry, 2012:132). The incorporation of a penetration enhancer is used to lead to the disruption of the stratum corneum barrier, resulting in better API delivery (Baibhav *et al.*, 2011:68).

#### **B.4.2.1 Safflower oil solubility of artemether**

As mentioned, when selecting an oil for the oil phase of the nano-emulsion, the compatibility as well as the solubility of the oil and the selected API is a very important factor (Chime *et al.*, 2014:91). To determine the maximum amount of API that can be incorporated within the nano-emulsion, the solubility of artemether within the safflower oil was determined. This was done by preheating a water-shaker bath to 32 °C; 4 ml safflower oil was placed into a clean test tube, then an excess amount of artemether was added, the tube was sealed tightly and placed in the water-shaker bath for 24 h where after it was removed. With a micropipette, 2 ml was removed from the test tube, placed into a 2 ml microcentrifuge tube and centrifuged. After centrifugation, 2 µl of the oily supernatant was removed by means of a micropipette and placed into a clean test tube to which 5 ml of tetrahydrofuran (THF) was added. Another 1 ml was transferred to a volumetric flask and diluted further with 100 ml THF. A volume of the second dilution was then transferred to an HPLC vial and injected into the HPLC to determine the API concentration in the oil.

It was calculated that artemether presented with a safflower oil solubility of 191.680 mg/ml. As the o/w nano-emulsion contained 2.3 ml of safflower oil within the 50 ml formula, it was determined the formula could contain a maximum of 440.864 mg artemether within the safflower oil.

#### **B.4.3 Emulsifiers**

Emulsifiers are also better known as surfactants, or surface acting agents, which also clearly describe their function (Setya *et al.*, 2014:2218). They are therefore incorporated within a formulation to decrease interfacial tension, which exists between the two phases of the nano-emulsion (Setya *et al.*, 2014:2218). Emulsifying agents are also incorporated within a nano-emulsion to promote emulsification during manufacturing (Baibhav *et al.*, 2011:68; Hyma *et al.*, 2014:4). Nano-emulsions are therefore formed through a combination of a lipophilic surfactant and a hydrophilic surfactant (Magdassi & Garti, 1999:156; Reddy *et al.*, 2013:87). It can be concluded that a hydrophilic-lipophilic balance (HLB) (between 9 – 18) of the surfactant(s) used are required for the formulation of an o/w dispersion (Eid *et al.*, 2014:4; Setya *et al.*, 2014:2218). It has been proposed that a more stable nano-emulsion should be produced through the

combination of surfactants (Setya *et al.*, 2014:2219). Although various surfactants exist, in this study Span® 60 and Tween® 80 were used.

#### **B.4.3.1 Span® 60**

Span® 60, also referred to as sorbitan monostearate, is a sorbitol monostearate ester (Zhang, 2009b:675), which possesses the function of a dispersing, emulsifying, suspending and wetting agent and is classified as a non-ionic surfactant (Zhang, 2009b:675). It also leads to better stability over time (Baibhav *et al.*, 2011:68). Span® 60 has an HLB value of 4.7 and if the HLB value is less than 10, it indicates it is a lipophilic surfactant and should consequently be incorporated within the oil phase of the o/w nano-emulsion (Reddy *et al.*, 2013:87; Zhang, 2009b:6780). Due to its lipophilic nature, it presents with good solubility in oils or any organic solvents (Zhang, 2009b:676). Span® 60 was used as surfactant during the formulation of the o/w nano-emulsion, which consequently resulted in enhanced emulsification during the formulation process (Baibhav *et al.*, 2011:68). Span® 60 was therefore incorporated within the oil phase, together with the other lipophilic excipients of the nano-emulsion.

#### **B.2.4.2 Tween® 80**

Tween® 80, also known as polysorbate 80, acts as surfactant in the nano-emulsion and is classified as a polyoxyethylene 20 sorbitan fatty acid ester (Zhang; 2009a:549). Tween® 80 represents a non-ionic hydrophilic surfactant, which is widely used during the formulation of cosmetics, topical formulations and o/w emulsions (Zhang; 2009a:550). It is known that Tween® 80 has a HLB value of 15 (Zhang; 2009a:551), consequently, when a HLB value is above 10, for instance with Tween® 80, the surfactant can be viewed as hydrophilic and should be incorporated within the water phase of the o/w nano-emulsion (Reddy *et al.*, 2013:87). The purpose of the incorporation of hydrophilic surfactants is to decrease the amount of energy needed to create the nano-emulsion and as a result, contributes to stable formulations (Chime *et al.*, 2014:91). In this study, Tween® 80 was incorporated within the water phase of the nano-emulsion.

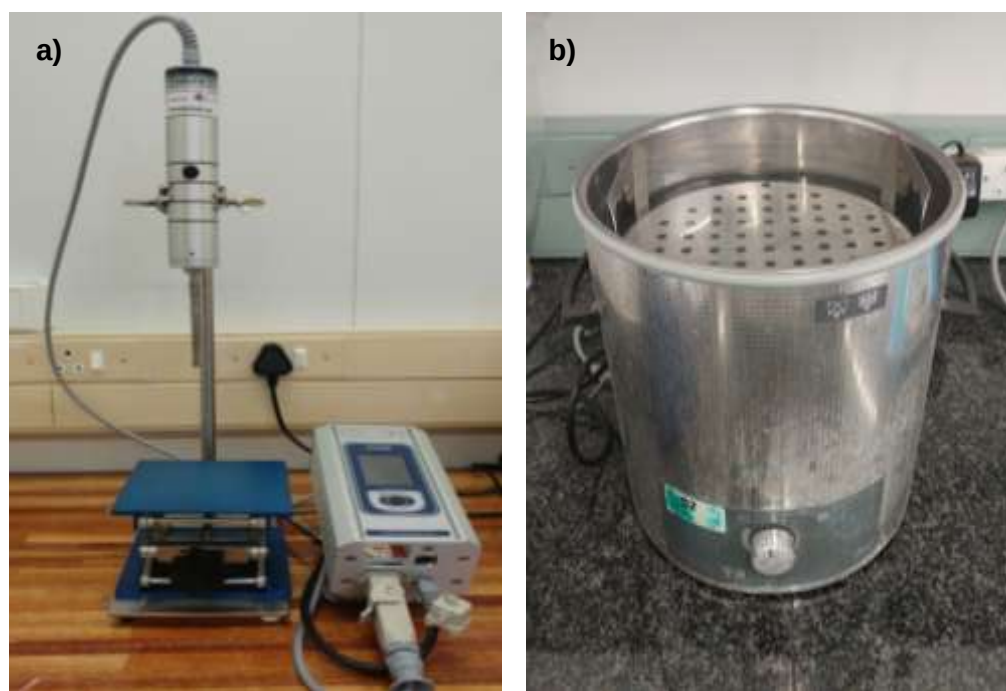
#### **B.4.4 Water**

The water or hydrophilic phase of a nano-emulsion consists of an aqueous material such as water or any alcohols (Hyma *et al.*, 2014:4). Solvents, such as water, perform an important role during the formulation of nano-emulsions (Silva *et al.*, 2012:857). When an o/w nano-emulsion is formulated, the water phase constitutes the largest part of the nano-emulsion, consequently Milli-Q® water was used as a solvent and constituted the water phase of the nano-emulsion in this study.

## B.5 Formulation of nano-emulsions

Knowledge and information about nano-emulsions and the formulation thereof was sourced during the literature study completion. Methods and formulas gathered from literature were thus altered in accordance with the properties of the API and the optimal effect that was desired. A trial-and-error approach was applied utilising existing approaches to develop the desired formulation.

To achieve the aims as set out in this study, it was decided that a high-energy emulsification method would be implemented to formulate the nano-emulsions. This was decided after a low-energy emulsification method, i.e. solvent displacement method, was tested and found to contain too much organic solvent remaining in the dispersion, which could potentially influence the integrity of the skin during future *in vitro* skin diffusion studies. Hence, ultrasonication of a coarse emulsion was chosen as the method to formulate the nano-emulsion instead. Before the surfactant ratios were explored, various ultrasonication times were investigated. It was found the ideal ultrasonication times were 3 min with an ultrasonic probe (1 min intervals), followed by 15 min in the ultrasonication bath. These times resulted in the most stable dispersions.



**Figure B.1** High-energy emulsification methods: a) ultrasonicator (Model UP200St) and b) Elma Transsonic EL540 ultrasonic bath

In this study, an ultrasonicator (Model UP200St, Hielscher Ultrasonics, Teltow, DE) (Figure B.1.a) and an Elma Transsonic EL540 ultrasonic bath (Elma Electronic GmbH, Pforzheim, DE) (Figure B.1.b) were used to create the nano-sized droplets. These mechanical

devices are based on high-energy forces, which break up the oil and water phase into nano-sized droplets (Bhatt & Madhav, 2011:2294; Debnath *et al.*, 2011:77).

With the intended purpose in mind, an o/w nano-emulsion was formulated through a high-energy emulsification method (ultrasonication) to contain 0.8% (w/v) artemether and 5% (w/v) safflower oil. Two different surfactant ratios were investigated: 1) Tween® 80:Span® 60 in a ratio of 4:1 and 2) Tween® 80:Span® 60 in a ratio of 3:2. The optimised o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil was also used for the development of semi-solid forms (which is discussed further in Appendix C and Appendix D). Consequently, the optimised nano-emulsion and semi-solid dosage forms were investigated during *in vitro* diffusion studies (Appendix F).

## **B.6 Characterisation of the pre-formulated nano-emulsions**

The characteristics of a dispersion is indicative of its effectiveness of reaching the set aim(s). Therefore, evaluating and comparing these properties would help to determine which surfactant ratio would be the best to use as an optimal o/w nano-emulsion. The final formulation with an optimal surfactant ratio was chosen once the characteristics of the different ratios were compared to each other and was consequently used during further investigations.

The following characteristics were tested of the two dispersions with different surfactant ratios:

- \* morphology,
- \* droplet size and distribution,
- \* pH,
- \* zeta-potential and
- \* viscosity.

## **B.7 Methods**

### **B.7.1 General method used to formulate a nano-emulsion**

Various methods exist by which nano-emulsions can be formulated. Primarily a nano-emulsion is formulated as a heterogeneous, two-phase dispersion formed through the mixture of the main components, consisting of oil and water, which are stabilised by surfactants (Abolmaali *et al.*, 2011:140; Chime *et al.*, 2014:77). As nano-emulsions are not very thermodynamically stable, energy is required to form the nano-sized droplets (Abolmaali *et al.*, 2011:141; Tadros *et al.*, 2004:303). Energy can be applied through a high-energy emulsification method, a low-energy emulsification method or a combination of the two (Abolmaali *et al.*, 2011:140-141; Tadros *et al.*, 2004:307).

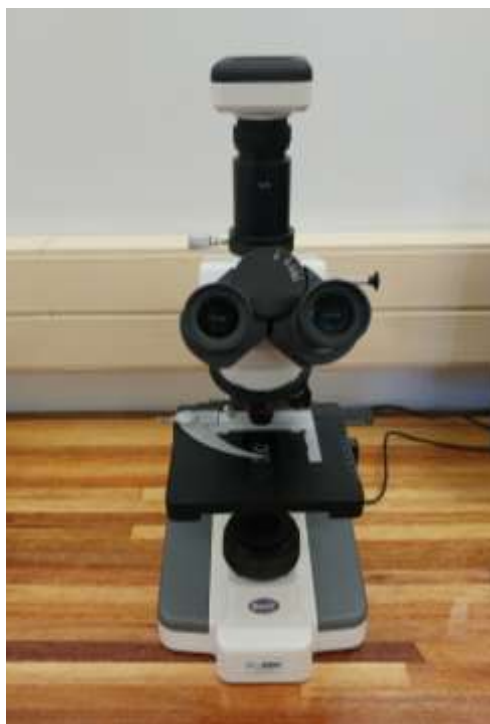
Of the two methods, the high-energy emulsification method is the most favourable as very small droplets can be created (Debnath *et al.*, 2011:77). High-energy emulsification methods are achieved through mechanical devices, such as ultrasonicators, microfluidisers or high-pressure homogenisers (Bhatt & Madhav, 2011:2294; Lovelyn & Attama, 2011:627; Tadros *et al.*, 2004:308). In contrast, low-energy emulsification methods are based on using the energy stored within the delivery system and focuses on transitions within the phases during emulsification (Chime *et al.*, 2014:92; Kela & Kaur, 2013:9203; Lovelyn & Attama, 2011:627). Low-energy methods can be divided into three methods: self-nano-emulsification, solvent displacement method, phase transition, as well as phase inversion temperature method (Chime *et al.*, 2014:92; Lovelyn & Attama, 2011:627).

## **B.7.2 Characterisation methods**

The various methods and equipment used to determine the characteristics of the pre-formulated nano-emulsions are discussed in the following section. It must be noted that from this point the o/w nano-emulsion with a Tween® 80:Span® 60 ratio of 4:1 will be referred to as **(NE1)** and the o/w nano-emulsion with a Tween® 80:Span® 60 ratio of 3:2 as **(NE2)**.

### **B.7.2.1 Morphology**

#### **B.7.2.1.1 Light microscopy**



**Figure B.2:** A Nikon Eclipse E4000 microscope

To determine whether nano-sized droplets formed within the dispersion, light microscopy was conducted before any other microscopic studies were done. Light microscopy was performed using a Nikon Eclipse E4000 microscope, fitted with a Nikon DSFi1 camera (Nikon, Japan Linkam THMS600) equipped with a T95 LinkPad temperature controller (Surrey, ENG) (Figure B.2). Motic Images Plus software was used to capture the micrographs. Slides were prepared by placing a small volume of the different dispersions ((**NE1**) and (**NE2**)) respectively, on a microscope slide and covering them with a 16 mm cover slip. The slides were then investigated, respectively, under the microscope making use of various magnifications. Various magnifications were used but finally, the 40x magnification was used to evaluate the morphology of the (**NE1**) and (**NE2**).

#### **B.7.2.1.2 Transmission electron microscopy**

The morphological characteristics, such as the droplet size and structural shape of the (**NE1**) and the (**NE2**) were studied further through transmission electron microscopy (TEM) (Chime *et al.*, 2014:96; Gaur *et al.*, 2014:47). The TEM was operated and images were captured by Dr A Jordaan (Electron Microscopy Laboratory of the North-West University (NWU), Potchefstroom) using a FEI Tecnai G2 20S-Twin 200 kV high-resolution transmission electron microscope (HRTEM) (Czech Republic, EU), with an Oxford INCA X-Sight EDS System. TEM observations were only conducted on the nano-emulsions as they formed the core focus of this study.

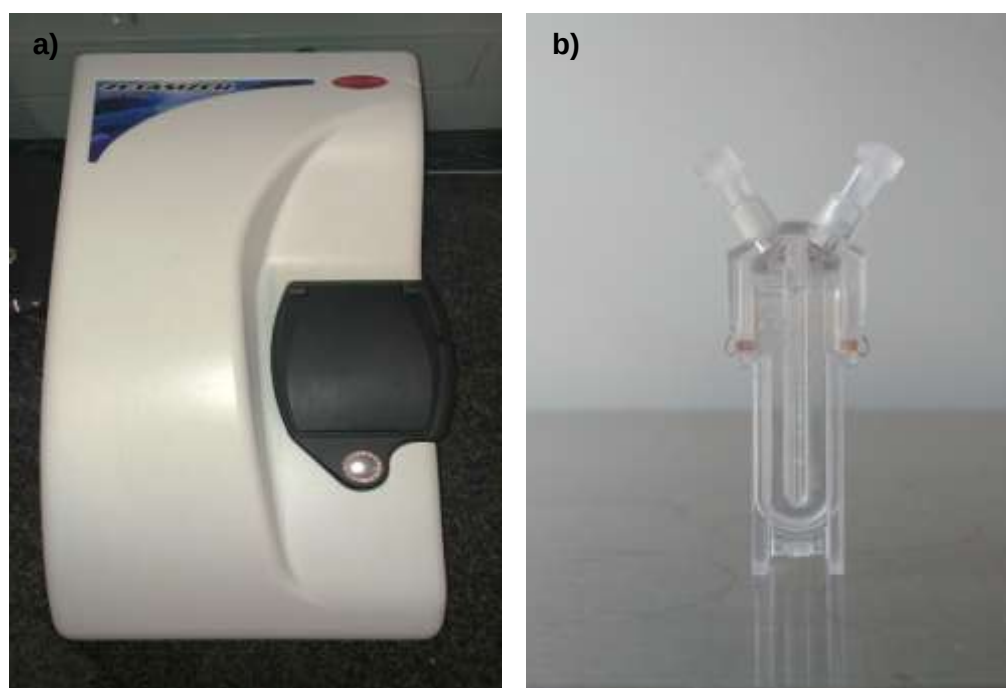
To perform TEM observations and not to cause any harm to the HRTEM, samples were prepared without any API. Samples of the dispersions were diluted and a small volume of the dilution was placed on a microscopic carbon-coated 300 mesh copper grid using a micropipette, which was then stained with osmium tetroxide (Chime *et al.*, 2014:96), which is used to stain the samples to result in images of high-contrast quality (Nomaki *et al.*, 2015:33). The oil droplets in the o/w nano-emulsion could therefore be viewed with ease as the osmium tetroxide was able to preserve any unsaturated fatty acid present (Nomaki *et al.*, 2015:33). After allowing it to dry for 10 – 15 min, the osmium stained carbon-coated grid was investigated at the adequate voltage under the TEM. A Gatan bottom mount camera was used to capture the micrographs in conjunction with digital micrograph software.

#### **B.7.2.2 Droplet size and distribution**

Instruments based on photon correlation spectroscopy (PCS), such as a Zetasizer, are usually used to determine the droplet size and droplet distribution of a dispersion. PCS is based on measuring light fluctuations between the dispersed droplets caused by Brownian motion (Gaur *et al.*, 2014:40; Malvern Instruments Limited, 2015:15). By evaluating these light scatterings, the size of the droplets could be determined by measuring the volume, i.e. diameter of the

droplet (Malvern Instruments Limited, 2015:15). The droplet distribution is measured and expressed by the polydispersity index (Pdl), as measured through PCS.

Determining the droplet size and the distribution of the droplets is a crucial characteristic of an ideal formulation (Gaumet *et al.*, 2008:2). The determination was thus important, as information such as appearance, release efficacy, stability, texture (i.e. spreadability) and even viscosity can be provided (Malvern Instruments Limited, 2015:2). Stability of the dispersion can be directly evaluated by the droplet size and it is stated that very small droplets can present without sedimentation or creaming effects (Solans *et al.*, 2005:105). The droplet size can also have an influence on the distribution of the droplets throughout the dispersion (Gaumet *et al.*, 2008:2).



**Figure B.3:** a) Malvern Zetasizer Nano ZS 2000 and b) a clear disposable DTS1070 folded capillary zeta cell

Both the droplet size and Pdl were measured using a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK) (Figure B.3.a). Diluted samples of the **(NE1)** and the **(NE2)** were used during this characterisation by placing 10 ml of Milli-Q® water into two polytops. Two drops of the **(NE1)** and the **(NE2)** were added to the 10 ml Milli-Q® water, respectively, and mixed thoroughly. Since dilution decreases the extent of interaction between the droplets, and to ensure improved readings, diluted samples were prepared (Gaumet *et al.*, 2008:7). A clear disposable zeta cell (DTS1070 folded capillary cell) (Figure B.3.b), used during analysis, was filled with 2 ml of the diluted sample by means of a syringe. Each sample was measured in triplicate and the average, of both the droplet size and Pdl, was calculated respectively.

### B.7.2.3 pH

It was important to determine the safety level of the topical formulation to ensure it did not present with an irritating nature, and this was done by measuring the pH value of the dispersion (Basera *et al.*, 2015:1881). A pH reading provides information about how acidic or basic a formulation or dispersion is (Hach Company, 2010:6). The pH value is consequently expressed in terms of the amount of hydrogen ions  $[H^+]$  to hydroxide ions  $[OH^-]$  (Hach Company, 2010:6). As a result, a stable dispersion can be achieved when the concentration of these hydrogen and hydroxide ions remains unchanged (Hach Company, 2010:6).

The pH of the **(NE1)** and the **(NE2)** were measured by means of a Mettler Toledo® pH meter (Mettler Toledo, CU) equipped with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) (Figure B.4). Each dispersion's pH was measured, in triplicate, by placing the electrode into the freshly prepared **(NE1)** and **(NE2)**, respectively, where after the average pH for each dispersion was calculated.



**Figure B.4:** A Mettler Toledo® pH meter with a Mettler Toledo® InLab® 410 electrode

### B.7.2.4 Viscosity

A nano-emulsion's viscosity can be seen as an overview of the excipients, such as the surfactants, oil and water parts, used to formulate the o/w nano-emulsion (Chime *et al.*, 2014:97). A viscosity reading can also be indicative of the stability of the formulation, as well as how the API is released from the delivery system (Chime *et al.*, 2014:97). Visually, nano-

emulsions present with a watery appearance and normally with very low viscosity values (Thakur *et al.*, 2012:223).

A Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA), connected to a thermostatic water bath (controlled at  $\pm 25\text{ }^{\circ}\text{C}$ ) (Figure B.5), was used to determine the viscosity of the **(NE1)** and the **(NE2)**. The Viscometer was equipped with a T-bar 18 spindle. The **(NE1)** and the **(NE2)** were placed into the thermostatic water bath (at  $\pm 25\text{ }^{\circ}\text{C}$ ) an hour prior to the conduction of the experiment in order to acclimatise to  $\pm 25\text{ }^{\circ}\text{C}$ . Approximately 6.7 ml of the sample dispersion was transferred to the sample chamber and fitted to the small sample adapter of the viscometer. The T-bar cylindrical spindle, SC4-18, was inserted into the sample formulation and connected to the viscometer. The small sample adapter was rotated at a specified speed of 200 rpm to measure the viscosity. Viscosity readings were described in terms of centipoise (cP), at room temperature ( $\pm 25\text{ }^{\circ}\text{C}$ ). A percentage torque was achieved around  $\pm 10.48$  during the measurements. Multipoint data was collected on Rheocalc T 1.2.19 software and was programmed to take readings at 10 s intervals for one minute. The average viscosity values were calculated thereafter.



**Figure B.5:** A Brookfield Viscometer DV2T LV Ultra connected to a water bath

#### **B.7.2.5 Zeta-potential**

Zeta-potential determination is based on the measurement of the surface charge of a dispersion (Eid *et al.*, 2014:2; Thakur *et al.*, 2012:223). Malvern Instruments Limited (2015:10) stated the zeta-potential of a dispersion provides an overview of the electrostatic charges (either repulsion or attraction) present between the droplets. Typically, these electrostatic charges between the nano-sized droplets are caused through Van der Waals attraction forces and Brownian motion (Cao & Wang, 2011:43). PCS is a useful technique to determine the zeta-potential as the light fluctuations between the dispersed droplets, caused by Brownian motion, can be successfully

measured (Gaur *et al.*, 2014:40). It is important to investigate the surface charge of a formulation, since this characteristic has an effect on the stability and entrapment of the API within the formulation (Bhatt & Madhav, 2011:2296; Eid *et al.*, 2014:2; Klang *et al.*, 2015:262; Malvern Instruments Limited, 2015:10). This property could also affect the manner in which the formulation and the skin interact with each other and as a result influence the penetration of the API (Klang *et al.*, 2015:262).

The zeta-potential of the **(NE1)** and the **(NE2)** was measured with a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK) (Figure B.3.a), which is based on PCS (Gaur *et al.*, 2014:40). A dilution of the freshly prepared **(NE1)** and **(NE2)** were made respectively, by adding two drops of the dispersion to a polytop containing 10 ml Milli-Q® water. With the use of a syringe, 2 ml of the diluted sample was injected into a clear disposable zeta cell (DTS1070 folded capillary cell) (Figure B.3.b). The light scattering between the droplets were evaluated at  $\pm 25$  °C and at an angle of 90°. The measurements of the zeta-potential, done in triplicate, were taken on the day of sample preparation.

## **B.8 Formulation and characterisation for the determination of an optimised nano-emulsion**

### **B.8.1 Formulation of nano-emulsion**

The formulas for the formulation of an o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil, either with a surfactant ratio (Tween® 80:Span® 60) of 4:1 **(NE1)** or 3:2 **(NE2)**, are outlined in Tables B.2 and B.3, respectively.

**Table B.2:** Formula of **(NE1)** (50 ml)

| Phase                  | Ingredient     | Function                             | Amount      |
|------------------------|----------------|--------------------------------------|-------------|
| <b>Oil phase (A)</b>   | Artemether     | API                                  | 0.4 g       |
|                        | Safflower oil  | Natural oil and penetration enhancer | 5% (2.3 ml) |
|                        | Span® 60       | Lipophilic surfactant                | 0.5 g       |
| <b>Water phase (B)</b> | Tween® 80      | Hydrophilic surfactant               | 1.8 ml      |
|                        | Milli-Q® water | Solvent                              | 45.0 ml     |

**Table B.3:** Formula of (NE2) (50 ml)

| Phase           | Ingredient     | Function                             | Amount      |
|-----------------|----------------|--------------------------------------|-------------|
| Oil phase (A)   | Artemether     | API                                  | 0.4 g       |
|                 | Safflower oil  | Natural oil and penetration enhancer | 5% (2.3 ml) |
|                 | Span® 60       | Lipophilic surfactant                | 1.0 g       |
| Water phase (B) | Tween® 80      | Hydrophilic surfactant               | 1.5 ml      |
|                 | Milli-Q® water | Solvent                              | 45.0 ml     |

**B.8.1.1 Formulation method of a nano-emulsion**

For the formulation of an o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil, a combination of methods were used: firstly, a coarse emulsion was prepared through self-emulsification, then ultrasonication, a high-energy emulsification method, was applied to create the nano-sized droplets.

The method of formulation of the o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil is listed below and diagrammatically represented in Figure B.6. The same method was used for both dispersions, each containing just a different surfactant ratio (as seen in Tables B.2 and B.3). The oil phase is constituted by all the ingredients listed under phase A and the water phase consisted of the excipients listed under phase B, respectively, as provided in Tables B.2 and B.3. The formulation method was as follow:

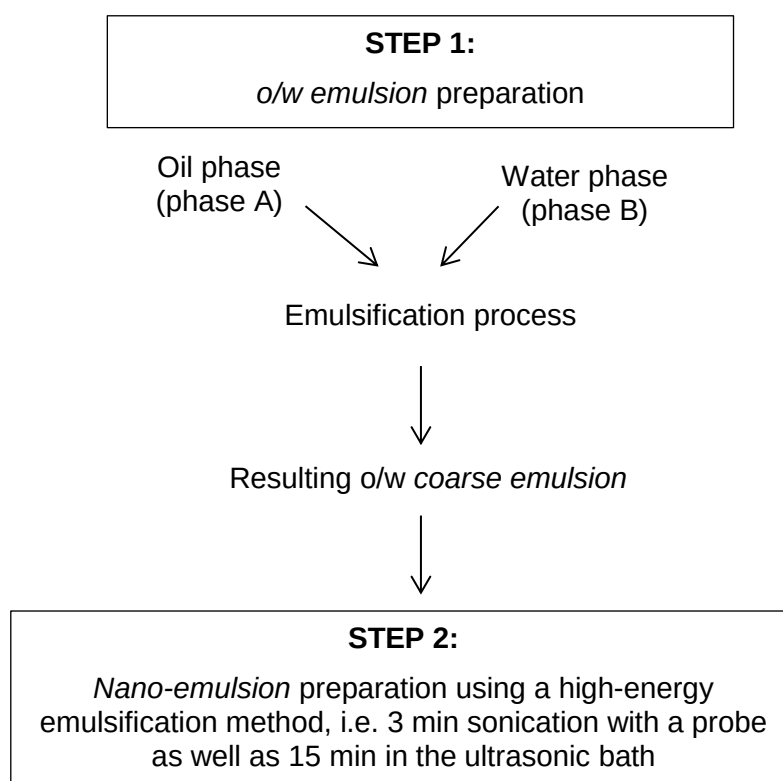
**STEP 1:**

- \* Milli-Q® water was measured in a measuring cylinder.
- \* The hydrophilic surfactant (Tween® 80) was measured by making use of a micropipette and placed in a beaker.
- \* The Milli-Q® water was added to the Tween® 80 (referred to as phase B). A magnetic stirring rod was added to the beaker and placed on a hot plate with continuous magnetic stirring.
- \* It was mixed until the Tween® 80 was completely dissolved.
- \* The lipophilic surfactant (Span® 60) was weighed in a weighing boat.
- \* The natural oil (i.e. safflower oil) was measured by making use of a micropipette and placed in a beaker and the Span® 60 was added to the safflower oil (referred to as phase A).
- \* A magnetic stirring rod was added to phase A, which was then placed on the hot plate, ensuring the plate was hot enough to warrant adequate dissolving.

- \* The artemether was added to the Span® 60-safflower oil mixture (phase A) and mixed until dissolved.
- \* The magnetic stirring rod was removed from the mixtures and phases A and B were placed back on the hot plate (the heat was turned off while stirring continued, as the plate remained warm).
- \* By making use of a syringe, phase A was drawn up and added drop wise to phase B.
- \* After all of phase A was added, the final dispersion was left on the hot plate to mix for a few minutes (this was referred to as the coarse emulsion).

## STEP 2:

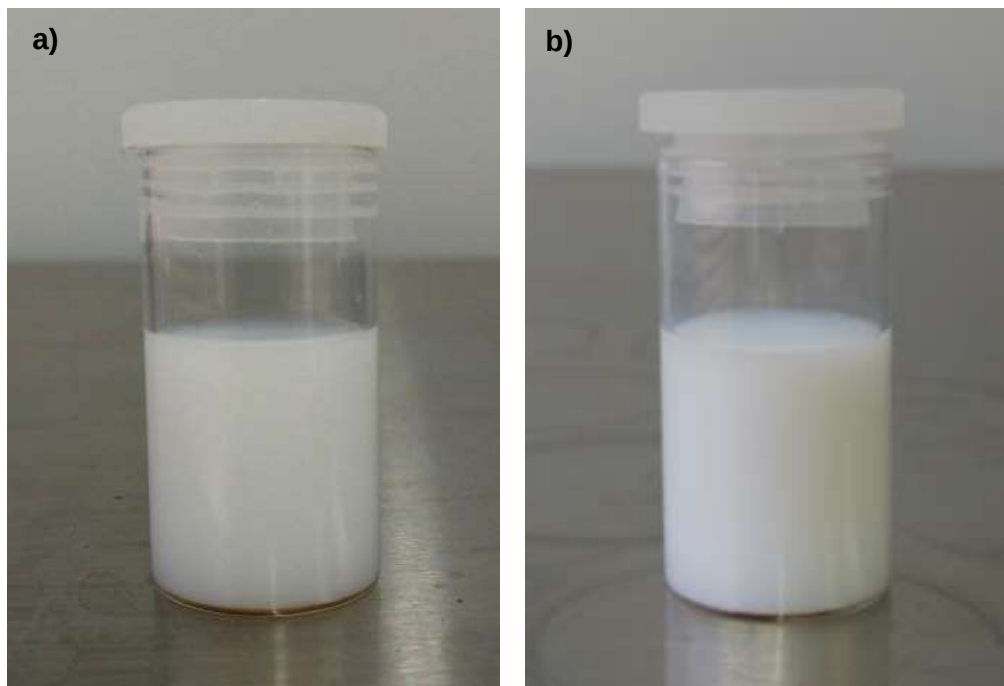
- \* The beaker was removed from the hot plate and placed under the ultrasonic probe for 3 min (three 1 min intervals, few seconds rest between each probe session).
- \* After sonication with the ultrasonic probe, the dispersion was placed in the ultrasonic bath for 15 min.
- \* Nano-sized droplets were created due to energy forces.



**Figure B.6:** Diagrammatic representation of the formulation of a nano-emulsion

### B.8.1.2 Outcome

From Figure B.7, both **(NE1)** and **(NE2)** presented as white translucent-like liquids. Figure B.7.a is representative of **(NE1)** and Figure B.7.b represents **(NE2)**. No sedimentation or aggregation was present in either dispersion. Since no oil droplets were visible, it could be said successful dispersion of the oil droplets within in the water phase was achieved. Although both dispersions presented milky, Figure B.7.a presented a translucent shade (less milky), whereas Figure B.7.b was milkier.



**Figure B.7:** Outcome of the formulations: a) **(NE1)** and b) **(NE2)**

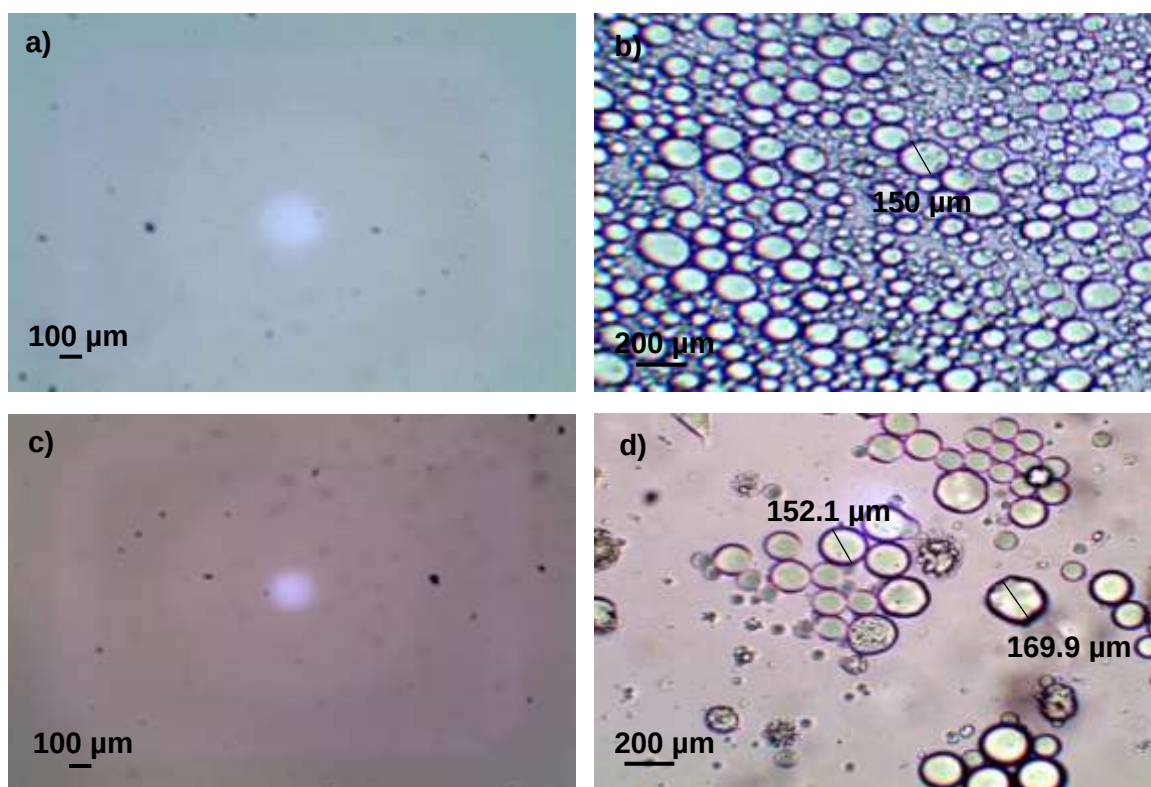
## B.8.2 Results and discussion for the characterisation of the dispersions

### B.8.2.1 Morphology

#### B.8.2.1.1 Light microscopy

Light microscopy was used to investigate the droplets of the two dispersions (**(NE1)** and **(NE2)**). Reportedly, nano-emulsions, due to their very small droplet size, are viewed with difficulty through normal microscopy (Graves, 2008:44). Despite this, micrographs were taken of the various dispersions (represented by Figure B.8) as confirmation of the formation of small droplets. From Figures B.8.a and B.8.c, it is observed that very small droplets are visibly present in both dispersions (**(NE1)** and **(NE2)**) and that the droplets were surrounded by a clear solution. This indicated nano-sized droplets were possibly formed; since it was not possible to measure their size accurately through normal light microscopy. As a result, further investigation by means of TEM was conducted on the two dispersions. Micrographs, Figures B.8.b and

B.8.d, represent the coarse emulsions of the **(NE1)** and the **(NE2)**, respectively, prior to the application of high-energy emulsification (ultrasonication). Figures B.8.b and B.8.d indicated the coarse emulsion presented with large droplets as their droplets could be measured with ease compared to the droplets of the nano-emulsions **(NE1)** and **(NE2)**. These micrographs therefore support the value of nano-sized droplets as well as the success of using the high-energy emulsification methods (ultrasonication) to formulate a nano-emulsion.



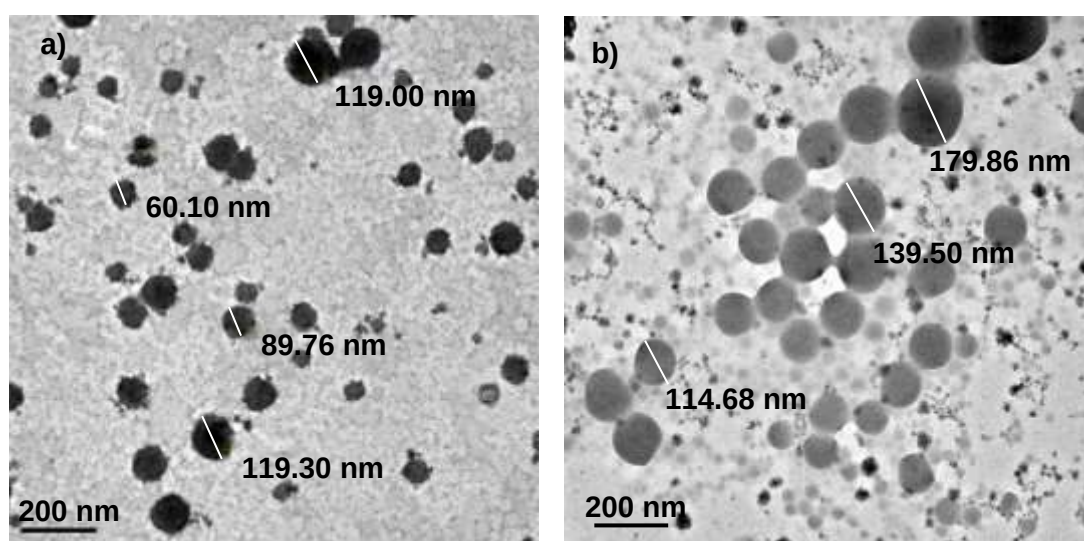
**Figure B.8:** Micrographs of the dispersions: a) **(NE1)**; b) **(NE1)** before sonication (coarse emulsion **(NE1)**); c) **(NE2)** and d) **(NE2)** before sonication (coarse emulsion **(NE2)**)

#### B.8.2.1.2 Transmission electron microscopy

To be classified as a nano-emulsion, the dispersed droplets within the aqueous phase need to fall within a nanometric range of 20 – 200 nm (Abolmaali *et al.*, 2011:139). Figure B.9 displays the HRTEM micrographs (Figures B.9.a and B.9.b) for the formulated dispersions (**(NE1)** and **(NE2)**) indicating the morphology of the dispersed droplets. Consequently, the droplet size and shape of the dispersions could be determined. Figure B.9.a exhibited droplets ranging from 60.10 – 119.30 nm for the **(NE1)**, whereas Figure B.9.b had droplet sizes ranging from 114.68 – 179.86 nm for the **(NE2)**. Figure B.9.a, **(NE1)**, recorded small droplets with a spherical shape. Figure B.9.b, **(NE2)**, recorded also small droplets with a spherical shape, yet coalescence of the dispersed droplets started to occur. No coalescence of the droplets was visible in Figure B.9.a, which made the **(NE1)** the more successful and possibly stable

dispersion. The droplets in Figure B.9.a appeared as dark, small spheres with a rough surface, whilst droplets in Figure B.9.b appeared as larger, dark grey smooth spheres; a possible explanation for these darker spheres could be the osmium tetroxide (unsaturated fatty acid stain) stained the droplets more, since the Span® 60 to safflower oil ratio present in the **(NE1)** was smaller than that of the **(NE2)**.

Consequently, it can be said that both the **(NE1)** and the **(NE2)** presented with nano-sized droplets ranging between the acceptable ranges of 20 – 200 nm, hence, nano-emulsions were successfully formulated.



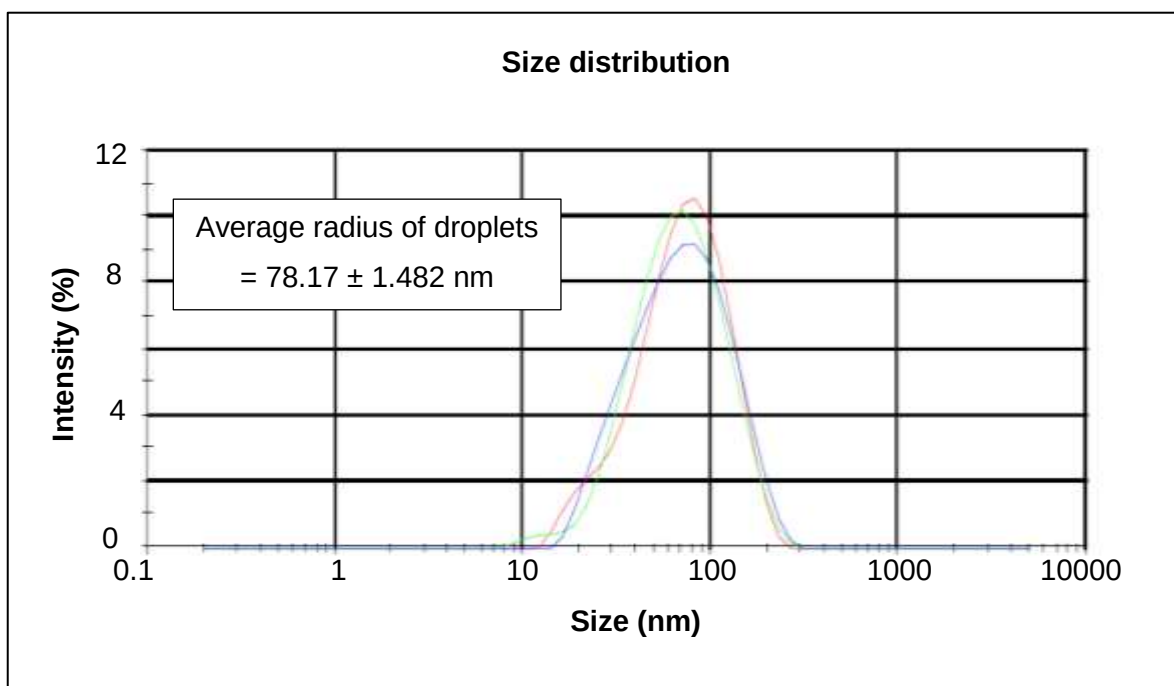
**Figure B.9:** TEM micrographs of the dispersion without any API: a) **(NE1)** sonicated for 3 min and placed in an ultrasonication bath for 15 min and b) **(NE2)** sonicated for 3 min and placed in an ultrasonication bath for 15 min

### B.8.2.2 Droplet size and distribution

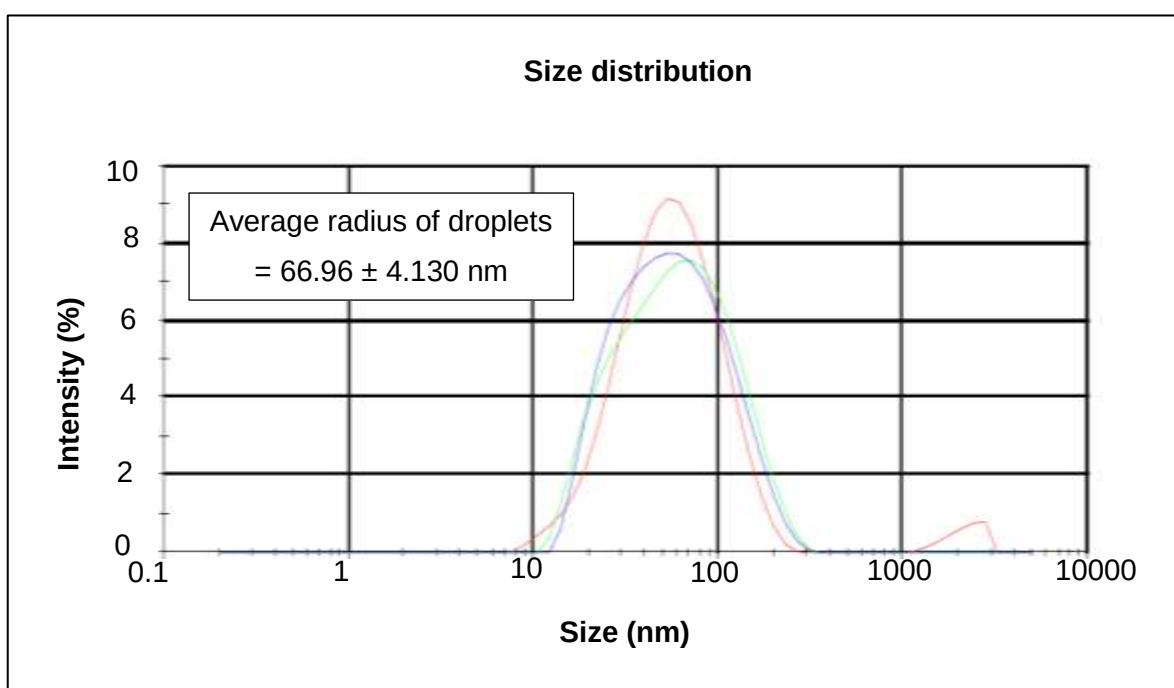
As confirmation, droplet size determination through means of PCS (a Zetasizer) was completed. Since a dispersion can be classified as a nano-emulsion when the dispersed droplets range between 20 – 200 nm (Abolmaali *et al.*, 2011:139), the formulated dispersion required droplets within this range. When the average droplet size of the dispersions (**(NE1)** and **(NE2)**) were compared, it was observed the **(NE1)** presented with an average droplet size of  $156.34 \pm 2.964$  nm and the **(NE2)** with an average droplet size of  $133.92 \pm 8.260$  nm (Table B.4). Hence, the dispersed droplets in the **(NE2)** were smaller than that of the **(NE1)**. The quality report obtained with the Malvern Zetasizer revealed that the results for all the samples were “good”, confirming acceptability. It can therefore be said that the measured droplets of the dispersions (**(NE1)** and **(NE2)**) fell within the acceptable nano-metric range of 20 – 200 nm.

**Table B.4:** Comparison of the average droplet size, as well as polydispersity index of the formulated dispersions ((**NE1**) and (**NE2**))

|              | Average droplet size (nm) | Average polydispersity index (Pdl) |
|--------------|---------------------------|------------------------------------|
| <b>(NE1)</b> | $156.34 \pm 2.964$        | $0.24 \pm 0.005$                   |
| <b>(NE2)</b> | $133.92 \pm 8.260$        | $0.27 \pm 0.014$                   |



**Figure B.10:** Average droplet size (nm) of the (**NE1**) measured per droplet radius



**Figure B.11:** Average droplet size (nm) of the (**NE2**) measured per droplet radius

The average radius of the droplets of the **(NE1)** and the **(NE2)** are respectively depicted in Figures B.10 and B.11, as measured through a Malvern Zetasizer Nano ZS 2000. Figures B.10 and B.11 represent the triplicate readings taken for each dispersion combined into one figure, respectively. It is observed that the radius of the average droplet size, for both dispersions, is relatively in the nano-metric range, averaging at  $< 100$  nm. The **(NE1)** has an average droplet radius of  $78.17 \pm 1.482$  nm, compared to the average droplet radius of the **(NE2)** measuring at  $66.96 \pm 4.130$  nm. All three curves in Figure B.10 have the same relative symmetrical curve, whereas the three curves in Figure B.11 present with different profiles. Figure B.11 may indicate the droplets varied greatly between the three readings.

The dispersity of the droplets throughout the water phase can best be described by the Pdl, which can as such be indicative of the uniformity of the droplets throughout the dispersion (Shakeel *et al.*, 2007:E6). Pdl can be measured on a scale of 0 – 1 (Shaw, 2016). A Pdl value closest to 0 can be viewed as a greatly homogenous dispersion (monodispersed), whereas a Pdl closer to 1 can indicate that the droplet size varies and that the dispersion presents to be polydispersed (Gaumet *et al.*, 2008:5). The Pdl, as measured for the **(NE1)** and the **(NE2)**, is tabulated in Table B.4. The **(NE1)** measured with an average Pdl of  $0.24 \pm 0.005$ , which can be viewed as close to 0 and the same can be said of the **(NE2)**, which has a Pdl of  $0.27 \pm 0.014$ ; both dispersions are therefore relatively monodispersed. It must be noted that a nano-emulsion will never be fully monodispersed, since it is characteristically a heterogeneous dispersion constituted through oil and water (Abolmaali *et al.*, 2011:140; Chime *et al.*, 2014:77). It can be assumed that both dispersions will remain stable against sedimentation and aggregation over time, although the **(NE1)** could remain more stable than the **(NE2)**, since the Pdl of the **(NE1)** measured closest to 0.

### B.8.2.3 pH

The pH of the skin averages at 5 and therefore appears to be slightly acidic (Ng & Lau, 2015:8; Williams, 2013:678). Although the skin can withstand products with a pH range of 3 – 9, a pH outside of these ranges can influence the permeability and integrity of the skin (Barry, 2002:512). From Table B.5, it can be observed that for the **(NE1)**, the average pH was measured as  $6.82 \pm 0.030$  *versus* the pH of the **(NE2)** averaging at  $6.30 \pm 0.025$ . The **(NE1)** presented with a slightly higher pH than the **(NE2)**, yet both these dispersions' pH values were in the accepted pH ranges of the skin, consequently, the dispersions should not cause any irritation or sensitivity when applied topically.

Although the difference in pH values between the **(NE1)** and the **(NE2)** was relatively small, it is worth mentioning that it could possibly be ascribed to the different amounts of surfactants used within the dispersions. It can be proposed that the acid value (acidity) of the surfactants used

can influence the pH measured for each dispersion. When the acid value of the two surfactants (Tween® 80 and Span® 60) were investigated, it was detected that Tween® 80 and Span® 60 have an acid value of 2 and 10, respectively (Zhang, 2009a:551; Zhang, 2009b:678). Therefore, the **(NE2)** presented with a lower pH value (more acidic) since it contained more Span® 60 than the **(NE1)**, which is the more acidic surfactant.

**Table B.5:** The measured average pH for **(NE1)** and **(NE2)**, both containing artemether

|              | pH (reading 1) | pH (reading 2) | pH (reading 3) | Average pH   |
|--------------|----------------|----------------|----------------|--------------|
| <b>(NE1)</b> | 6.80           | 6.79           | 6.82           | 6.82 ± 0.030 |
| <b>(NE2)</b> | 6.33           | 6.30           | 6.28           | 6.30 ± 0.025 |

#### B.8.2.4 Viscosity

In the case of an o/w nano-emulsion, the largest component is the water phase; since the oil droplets are dispersed within an aqueous medium. Due to this large amount of aqueous medium, the dispersions are not very viscous hence measuring a very low viscosity (Chime *et al.*, 2014:97). Low viscosity is therefore a characteristic of nano-emulsions (Shakeel *et al.*, 2007:E6). Both the dispersions' viscosity was measured at 200.00 rpm, a torque of ± 10.48% and at a temperature of 25.24 ± 0.000 °C (Table B.6). The **(NE1)** and the **(NE2)** presented with a viscosity of 1.64 ± 0.016 cP and 1.50 ± 0.016 cP, respectively; although minor, the **(NE2)** was less viscous than the **(NE1)**. A study conducted by Kennedy and Kennedy (2007:E4), found that Tween® 80 can lead to an increase in viscosity. It can therefore be proposed that the higher viscosity of the **(NE1)** was because it contained 0.3 ml more Tween® 80 than the **(NE2)**. It is known that water possesses a viscosity of 1 cP, therefore it can be said that the viscosity of the **(NE1)** and the **(NE2)** measured close to that of water (V&P Scientific Inc., 2010).

**Table B.6:** Viscosity readings of **(NE1)** and **(NE2)**

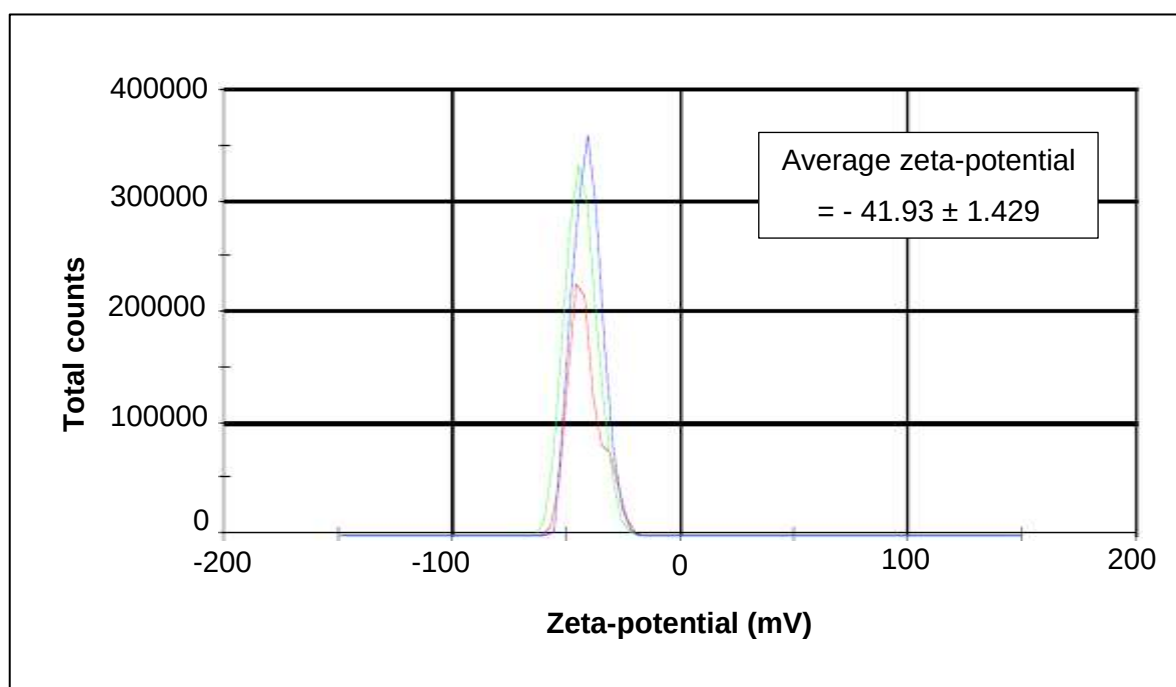
|              | Reading         | Viscosity (cP)      | Torque (%)           | Temperature (°C)     | Speed (RPM)           |
|--------------|-----------------|---------------------|----------------------|----------------------|-----------------------|
| <b>(NE1)</b> | <b>1</b>        | 1.66                | 11.02                | 25.20                | 200.00                |
|              | <b>2</b>        | 1.63                | 10.87                | 25.20                | 200.00                |
|              | <b>3</b>        | 1.60                | 10.83                | 25.20                | 200.00                |
|              | <b>Averages</b> | <b>1.64 ± 0.016</b> | <b>10.91 ± 0.100</b> | <b>25.20 ± 0.000</b> | <b>200.00 ± 0.000</b> |
| <b>(NE2)</b> | <b>1</b>        | 1.51                | 10.05                | 25.40                | 200.00                |
|              | <b>2</b>        | 1.52                | 10.08                | 25.10                | 200.00                |
|              | <b>3</b>        | 1.49                | 10.00                | 25.40                | 200.00                |
|              | <b>Averages</b> | <b>1.50 ± 0.016</b> | <b>10.04 ± 0.042</b> | <b>25.28 ± 0.203</b> | <b>200.00 ± 0.000</b> |

### B.8.2.5 Zeta-potential

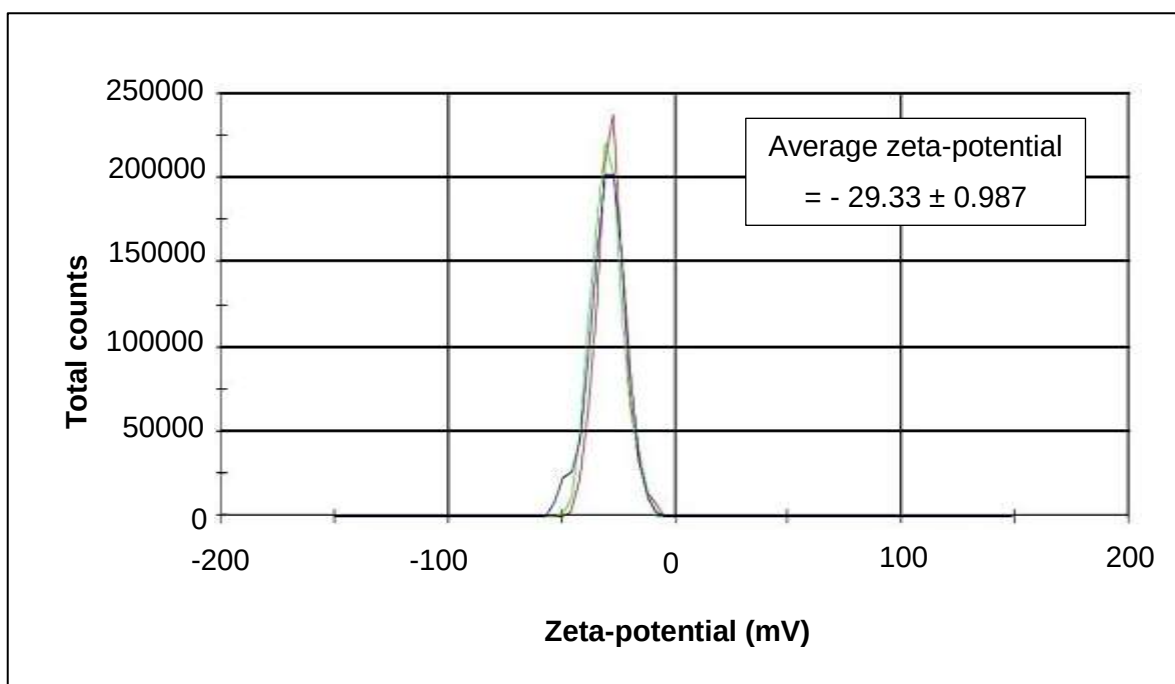
Literature states that a zeta-potential equal to or lower than - 30 mV can be an indication that the dispersion could remain stable (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860). It is also said that highly negative zeta-potential values are required for successful skin permeation (Duangjit *et al.*, 2011:6). It is proposed that the skin generally presents with a surface that is negatively charged and that negatively charged molecules could lead to better diffusion and consequently, increase the flux of the APIs (Sinico *et al.*, 2005:129). The average zeta-potential for the two dispersions ((**NE1**) and (**NE2**)) are represented in Table B.7. It was observed that both dispersions presented with negative values. The (**NE2**) had a zeta-potential value ( $- 29.33 \pm 0.987$  mV) in the range between 0 and - 30 mV, therefore appearing with less than favourable skin permeation (Silva *et al.*, 2015:860). The zeta-potential value of the (**NE1**) ( $- 41.93 \pm 1.429$  mV) indicated that the attraction forces between the droplets were greater than that of the repulsion, consequently forming a stable dispersion without any aggregation and flocculation (Silva *et al.*, 2012:860).

**Table B.7:** Comparison of the average zeta-potential (mV) of the two formulated dispersions

|                | Zeta-potential<br>(reading 1) | Zeta-potential<br>(reading 2) | Zeta-potential<br>(reading 3) | Average<br>zeta-potential (mV) |
|----------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|
| ( <b>NE1</b> ) | - 41.60                       | - 43.50                       | - 40.70                       | $- 41.93 \pm 1.429$            |
| ( <b>NE2</b> ) | - 28.20                       | - 29.80                       | - 30.00                       | $- 29.33 \pm 0.987$            |



**Figure B.12:** Average zeta-potential (mV) of the (**NE1**)



**Figure B.13:** Average zeta-potential (mV) of the **(NE2)**

From Figures B.12 and B.13, it is observed that both dispersions (**(NE1)** and **(NE2)**) presented with negative zeta-potential values. When the zeta-potential is zero (zero representing the isoelectric point at 0 mV), it can be said that a dispersion is unstable (Paderborn University, 2016) therefore, when a curve is further away from zero, either positive or negative, the zeta-potential can be viewed as ideal. The cumulative graphs of Figure B.12 are closer to - 50 mV than the cumulative graphs of Figure B.13, which leans more towards zero. The shape of the curves in Figures B.12 and B.13 present with symmetrical and pointed curves, hence a narrow distribution of data is indicated. With an ideal negative zeta-potential, proposing stability and skin permeation, it can be said that the **(NE1)** presented more ideal than the **(NE2)**.

## **B.9 Conclusion and decision of final formula used**

The process of pre-formulation of an o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil, with two different surfactant ratios, was investigated. In order to allocate the final and optimal nano-emulsion, the characteristics of the formulated dispersions (**(NE1)** and **(NE2)**) were evaluated and a summary of which is represented in Table B.8.

Drug flux, as well as topical drug delivery can be influenced by a variety of properties, one being the delivery system selected to bring the API in contact with the skin (Barry, 2002:508; Weiss, 2014:472). Thus, certain characteristics need to meet the requirements of an ideal topical delivery system. The following characteristics of an o/w nano-emulsion (**(NE1)** and **(NE2)**) containing 0.8% (w/v) artemether and 5% (w/v) safflower oil were investigated: morphology (i.e.

light microscopy and TEM), droplet size, distribution of droplets, pH, zeta-potential and viscosity.

**Table B.8:** Summary of the characteristics of **(NE1)** and **(NE2)**

| Characteristics determined | (NE1)           | (NE2)           |
|----------------------------|-----------------|-----------------|
| TEM size average (nm)      | 60.10 – 119.30  | 114.68 – 179.86 |
| Droplet size (nm)          | 156.34 ± 2.964  | 133.92 ± 8.260  |
| Pdl                        | 0.24 ± 0.005    | 0.27 ± 0.014    |
| pH                         | 6.82 ± 0.030    | 6.30 ± 0.025    |
| Viscosity (cP)             | 1.64 ± 0.016    | 1.50 ± 0.016    |
| Zeta-potential (mV)        | - 41.93 ± 1.429 | - 29.33 ± 0.987 |

Following light microscopic investigations, TEM was used to determine the droplet size for the **(NE1)** and the **(NE2)**; it measured in the nano-metric range of 20 – 200 nm. Using the PCS based apparatus (Zetasizer), the dispersion's average droplet size was measured and the TEM findings were confirmed. The experiments found that both dispersions (**(NE1)** and **(NE2)**) produced nano-sized droplets, successfully forming nano-emulsions. A dispersion with a Pdl of 0, or closest to 0, can be viewed as a relatively monodispersed dispersion, hence droplets spread homogenous throughout the aqueous phase. Although both the **(NE1)** and the **(NE2)** measured with low Pdl values, the **(NE1)** measured closest to 0, proposing a more stable dispersion over time. This could also propose that the **(NE1)** would be more effective, since a more homogenous dispersion would be applied to the skin.

Since the nano-emulsion would be applied to the skin surface to treat the topical condition CTB, the dispersions were required to present with a safe pH. Both dispersions, the **(NE1)** and the **(NE2)**, presented with adequate pH levels, since both fell within the accepted levels (pH range between 3 and 9 (Barry, 2002:512)). The viscosity for the **(NE1)** and the **(NE2)** measured close to 1 (that of water), hence low viscosity was recorded for both dispersions and although characteristically similar to nano-emulsions, this presented a difficulty during the application to the skin. For ideal and effective skin permeation, a highly negative zeta-potential was needed. The **(NE1)** had a higher negative zeta-potential value than the **(NE2)**; consequently the **(NE1)** offered effective permeation, as well as stability.

Considering all these characteristics, it can be said the **(NE1)** presented with the most optimal characteristics, hence it presented with nano-sized droplets, a low Pdl value and with a highly negative zeta-potential. In conclusion, the **(NE1)** was chosen as an optimal o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil for further studies and the full characterisation will be presented in Appendix C.

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# APPENDIX C:

## STABILITY AND CHARACTERISATION OF AN OPTIMISED O/W NANO-EMULSION CONTAINING ARTEMETHER AND SAFFLOWER OIL

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### C.1 Introduction

The physicochemical properties of the optimised nano-emulsion were determined to establish whether it adhered to the limits for topical drug delivery. The evaluation of certain characteristics provided an overview of the physical and chemical state of the optimised formulation (Chime *et al.*, 2014:95). The physical and chemical state was therefore characterised and described in terms of morphology and structure, droplet size, droplet distribution, pH, zeta-potential, viscosity, as well as the entrapment efficiency (%EE) (Chime *et al.*, 2014:95). The compatibility of the excipients used in the optimised formulation, was also determined (Chime *et al.*, 2014:95).

Literature states that an optimised nano-emulsion reflects a dispersion with small droplets, low Pdl measurement and great stability (Gutiérrez *et al.*, 2008:247). It can also be proposed that an optimised nano-emulsion should successfully achieve drug delivery (Gutiérrez *et al.*, 2008:247). It was therefore important to determine whether the incorporation of the API within the optimised nano-emulsion formula would influence any of the characteristics. Thus, this appendix provides an overview of the:

- \* characteristics of the optimised nano-emulsion (**NE1**), with and without 0.8% (w/v) artemether, both containing 5% (w/v) safflower oil;
- \* compatibility and stability between the two main excipients, i.e. artemether and safflower oil, was also determined when used in combination.

### C.2 Optimised o/w nano-emulsion containing artemether and safflower oil

#### C.2.1 Optimised o/w nano-emulsion formulation

Through pre-formulation an optimised o/w nano-emulsion formula and method was determined (as discussed in Appendix B). The formula of an optimised o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil is outlined in Table C.1. From here on, the optimised o/w nano-emulsion containing artemether is referred to as (**NE1**) and the optimised o/w nano-emulsion placebo (containing no artemether) is referred to as (**PNE1**).

**Table C.1:** Formula of **(NE1)** and **(PNE1)** (50 ml)

| Phase           | Ingredient     | Function                             | Amount      |
|-----------------|----------------|--------------------------------------|-------------|
| Oil phase (A)   | Artemether*    | API                                  | 0.4 g       |
|                 | Safflower oil  | Natural oil and penetration enhancer | 5% (2.3 ml) |
|                 | Span® 60       | Lipophilic surfactant                | 0.5 g       |
| Water phase (B) | Tween® 80      | Hydrophilic surfactant               | 1.8 ml      |
|                 | Milli-Q® water | Solvent                              | 45.0 ml     |

\*not included within **(PNE1)**

A systematic representation of the formulation process of the **(NE1)** is illustrated in Figure C.1 (full method described in Section B.8.1.1). The method was employed for the formulation of **(NE1)** and **(PNE1)**.



**Figure C.1:** Formulation method of the **(NE1)**: a) water and Tween® 80 preheated (phase B); b) safflower oil, Span® 60 and artemether preheated (phase A); c) phase A was added to phase B by means of a syringe; d) mixing of phases A and B together; e) sonication through 3 min ultrasonic probe and f) dispersion was placed in the ultrasonication bath

### C.3 Excipients used to formulate the optimised o/w nano-emulsion

The function of each excipient used to formulate the **(NE1)** and the **(PNE1)** is described in Section B.4. The **(NE1)** and the **(PNE1)** consisted of a two-phase dispersion formed by an oil phase and a water phase. The oil phase constituted Span® 60 and safflower oil together with artemether (it must be noted that artemether was not included when the **(PNE1)** was formulated), and Tween® 80 together with Milli-Q® water formed the water phase.

### C.4 Characterisation methods

Characteristics such as the morphology, droplet size, droplet distribution, pH, viscosity and zeta-potential, including, visual examination of the optimised nano-emulsions (**(NE1)** and **(PNE1)**) were investigated. The %EE of the nano-emulsion **(NE1)** was also determined. Prior to the characterisation of the optimised nano-emulsions, artemether and safflower oil stability testing was conducted. All these characteristics were evaluated through various techniques and equipment, as described in the following section.

#### C.4.1 Artemether and safflower oil stability

It is very important to investigate the compatibility concerning the API and excipients (Schmitt *et al.*, 2001:176). Once the formula for the optimised nano-emulsion **(NE1)** was determined, through pre-formulation investigations, the compatibility between artemether and safflower oil in a combination in the **(NE1)** had to be determined in order to evaluate stability.

##### C.4.1.1 Compatibility studies of artemether and safflower oil

It has been stated that there is a direct correlation between the stability of a formulation and the interactions between the API and excipients used (Narang *et al.*, 2012:2661). Artemether and safflower oil were used as API and excipient, respectively in the optimised **(NE1)**, as determined and described in Appendix B.

Although compatibility studies can be conducted through a variety of screening methods, such as differential scanning calorimetry, HPLC analysis and hot-stage microscopy, microcalorimetry was used during this study as a compatibility screening aid (Schmitt *et al.*, 2001:176). Isothermal microcalorimetry is based on the monitoring of any physical and chemical process that may occur between the API and the excipient that is detected through heat exchange (Phipps & Mackin, 2000:9). Dissolution, evaporation, absorption and other processes can be the cause of these heat exchanges (Schmitt *et al.*, 2001:177). Selzer *et al.* (1998:228) explain heat flow signals can be a result of any physicochemical process, which can be identified through heat exchange within the prepared sample. Microcalorimetry therefore measures the heat exchange and reaction as a function of time (Phipps & Mackin, 2000:9). A positive heat

flow value is a result of an exothermic process (decomposition or any change in free energy); whilst a negative heat flow value is a result of an endothermic process (Selzer *et al.*, 1998:228).

#### **C.4.1.2 Method of compatibility analysis**

A 2277 Thermal Activity Monitor (TAMIII) (TA Instruments, USA) was used as microcalorimeter during this compatibility study, equipped with an oil bath with a stability of  $\pm 100 \mu\text{K}$  over 24 h. The compatibility of artemether and safflower oil was investigated at 32, 40 and 60 °C, respectively. The heat flow signals were investigated for the individual excipients, as well as the combinations of the excipients. The calorimetric outputs observed for the individual samples are calculated to give a theoretical and hypothetical response. Generally, no interaction between the excipients is represented by this theoretical expected calorimetric output. When an interaction occurs between the excipients, the measured calorimetric output will be different from the calculated theoretical output.

#### **C.4.2 Morphology**

##### **C.4.2.1 Light microscopy**

Imaging techniques, such as light microscopy, are a useful aid when information about the dispersions' droplets is required (Silva *et al.*, 2012:862). Information such as droplet size, shape and aggregation condition, hence morphologic characteristics, can be given through microscopic evaluation (Silva *et al.*, 2012:862). The morphology of the droplets was identified by means of light microscopy. This was performed by using a Nikon Eclipse E4000 microscope, which was fitted with a Nikon DSFi1 camera (Nikon, Japan Linkam THMS600) and a T95 LinkPad temperature controller (Surrey, ENG). The micrographs were captured through Motic Images Plus software. A microscope slide was prepared by placing a drop of the **(NE1)** and the **(PNE1)** onto the slides, respectively, and covered with a 16 mm cover slip for safe evaluation through microscopy. Microscopy at 40x magnifications was used to investigate the morphology, or the droplet size, of the **(NE1)** and the **(PNE1)**.

##### **C.4.2.2 Transmission electron microscopy**

Through means of TEM, the morphological characteristics, such as droplet size and structural shape, were investigated (Chime *et al.*, 2014:96; Silva *et al.*, 2012:862). At the Electron Microscopy Laboratory (North-West University, Potchefstroom, RSA) the morphological characteristics of the **(PNE1)** were investigated by means of TEM, which was operated by Dr A Jordaan. As an API could possibly cause harm to the TEM, a placebo formulation was required during this investigation.

Diluted samples of the **(PNE1)** were therefore prepared. A drop of the diluted sample was placed on a microscopic carbon-coated 300 mesh copper grid using a micropipette, which was stained using osmium tetroxide in order to obtain high contrast quality images (Chime *et al.*, 2014:96; Nomaki *et al.*, 2015:33). The oil droplets in the **(PNE1)** could therefore be viewed with ease, as the osmium tetroxide was able to preserve any unsaturated fatty acid present within the dispersion (Nomaki *et al.*, 2015:33). The osmium stained carbon-coated grid was left to dry for 10 – 15 min, where after it was investigated at the adequate voltage under the TEM. Using a FEI Tecnai G2 20S-Twin 200 kV HRTEM (Czech Republic, EU), with an Oxford INCA X-Sight EDS System, the droplet size and structure of the **(PNE1)** were investigated.

#### C.4.3 Droplet size and distribution

PCS is a technique which can be employed to also determine the droplet size and the distribution of droplets throughout a dispersion (Chime *et al.*, 2014:96; Patravale *et al.*, 2004:833). This technique is based on the study of the variations in the light scattering of droplets as a result of Brownian motion (Chime *et al.*, 2014:96). Droplet size, as well as how the droplets are dispersed, are very important characteristics of a dispersion as it can provide information about dissolution, solubility, drug performance, such as drug release and absorption and most importantly, about stability (Patravale *et al.*, 2004:833; Reddy *et al.*, 2013:88). The droplet size can also have an effect on the clearance and bio-distribution of the droplets (Gaumet *et al.*, 2008:2). The stability of a formulation can be greatly influenced by the size and charge of the droplet size (Kumar & Rajeshwarrao, 2011:213). The Pdl can be used to describe the size distribution (homogeneity) range of the droplets within the dispersion (Chime *et al.*, 2014:97; Patravale *et al.*, 2004:833). This Pdl therefore determines whether the droplets of the dispersion are monodispersed (narrow size distribution) or polydispersed (broad size distribution) (Gaumet *et al.*, 2008:3-4; Gaur *et al.*, 2014:36).

The average droplet size and the Pdl of the **(NE1)** and the **(PNE1)** were measured by means of a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK). Gaumet *et al.* (2008:7) stated that diluted samples of the dispersion should be used during an experiment so that the amount of interaction between the droplets were reduced. Two drops of each, **(NE1)** and **(PNE1)**, were placed, respectively, in a polytop containing 10 ml Milli-Q® water resulting in a dilution. A clear disposable zeta cell (DTS1070 folded capillary cell) was used during analysis, which was filled with 2 ml of the diluted sample by means of a syringe. Readings of the **(NE1)** and the **(PNE1)**, on the Malvern Zetasizer Nano ZS 2000, were done in triplicate and the average was determined.

#### C.4.4 pH

It is of great importance that topical formulations should refrain from causing any irritation to the skin, as it should treat a cutaneous condition not create one (Basera *et al.*, 2015:1881; Paudel *et al.*, 2010:118). Since the topical formulation is applied directly to the skin, it has the ability to change the pH of the skin surface (Paudel *et al.*, 2010:118).

Consequently, the pH of the **(NE1)** and the **(PNE1)** were measured using a digital Mettler Toledo® pH meter (Mettler Toledo, CU), which was fitted with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU). The pH of the **(NE1)** and the **(PNE1)** was measured by placing the fitted electrode into a sufficient amount of each of the dispersions, respectively. Readings were taken in triplicate where after the average pH was calculated for each dispersion. The average pH could provide a clear indication whether the formulation could possibly cause changes in the skin's pH.

#### C.4.5 Viscosity

Chime *et al.* (2014:94) stated that the combination of excipients, such as surfactant, oils and aqueous mediums, used within the dispersion can be described in terms of viscosity. The viscosity of the **(NE1)** and the **(PNE1)** was determined by means of a Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA). This viscometer was connected to a thermostatic water bath, maintained at  $\pm 25\text{ }^{\circ}\text{C}$  throughout the experiment. A T-bar SC4-18 spindle was fitted to the viscometer in order to determine the viscosity of each dispersion. One hour prior to the conduction of the experiment, samples of the **(NE1)** and the **(PNE1)** were placed into the water bath (set to  $\pm 25\text{ }^{\circ}\text{C}$ ) to acclimatise to the required temperature of  $\pm 25\text{ }^{\circ}\text{C}$ . The sample chamber was filled with 6.7 ml of each of the dispersions, respectively and was consequently fitted to the small sample adapter of the viscometer. To determine the viscosity of the **(NE1)** and the **(PNE1)**, the cylindrical T-bar spindle was rotated at 200 rpm. The viscosity calculations were conducted by means of Rheocalc T 1.2.19 software and were read in terms of centipoise (cP). Multipoint readings at 10 s intervals were taken for 1 min and the average viscosity was determined.

#### C.4.6 Zeta-potential

Both the API, as well as the surfactant(s) used can control the zeta-potential results (Patravale *et al.*, 2004:833). Zeta-potential can be defined as the electrokinetic potential, i.e. the surface charge, within the colloidal system – the dispersion (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860; Thakur *et al.*, 2012:223). This surface charge is determined through PCS, which evaluates light fluctuations between the dispersed droplets (Gaur *et al.*, 2014:40). Not only can the stability of

a dispersion be described by the zeta-potential, but its entrapment ability and interaction with the skin as well (Bhatt & Madhav, 2011:2296; Klang *et al.*, 2015:262; Silva *et al.*, 2012:860).

The zeta-potential of the **(NE1)** and the **(PNE1)** was determined using a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK). A Malvern Zetasizer is based on the PCS technique, which evaluates the droplet light fluctuations caused by Brownian motion (Gaur *et al.*, 2014:40). Using a syringe, 2 ml of the diluted sample (prepared through adding two drops of the **(NE1)** and the **(PNE1)** to a polytop containing 10 ml Milli-Q<sup>®</sup> water, respectively) was injected into a clear disposable zeta cell (DTS1070 folded capillary cell) making sure no bubbles were present and that the cell was filled with an adequate amount of dispersion. The zeta-potential of the **(NE1)** and the **(PNE1)** was measured at  $\pm 25$  °C and at an angle of 90°, in triplicate, on the same day of sample preparation.

#### C.4.7 Entrapment efficiency and drug release

The %EE can be defined as the amount of API which has been entrapped within the dispersion (Kumar & Rajeshwarrao, 2011:213). The amount of API entrapped can therefore be calculated as the difference between the total concentration API and the unentrapped API concentration (Kumar & Rajeshwarrao, 2011:213). Consequently, it can be an indication of the drug loading capacity of the dispersion.

The %EE of artemether in the optimised nano-emulsion **(NE1)** was therefore determined and quantified by means of HPLC. Samples of the **(NE1)** were centrifuged with the help of Me S Lowe at the Laboratory for Applied Molecular Biology (LAMB) of the North-West University (NWU), Potchefstroom Campus, RSA. Samples were centrifuged using an Optima L-100XP Ultra-centrifuge (Beckman Coulter, RSA) at 25 000 rpm for 30 min at a temperature of 23 °C, presenting a supernatant. Of the supernatant,  $\pm 1$  g was withdrawn by means of a needle and syringe where after it was transferred to a 50 ml volumetric flask and made up to volume with methanol. The amount of artemether in the supernatant was quantified by means of HPLC.

Equation C.1, adapted from Kurakula *et al.* (2012:37), was used to calculate the EE% of the **(NE1)**, where  $C_t$  is the total API concentration and  $C_f$  is the concentration of the free unentrapped drug:

$$\%EE = [(C_t - C_f) / C_t] \times 100$$

**Equation C.1**

#### C.4.8 Visual and physical examination

The optimised dispersions (**(NE1)** and **(PNE1)**), were visually evaluated in terms of the colour of the dispersion, consistency, distribution of droplets (homogeneity), spreadability and whether any instabilities such as sedimentation or separation had occurred (Mohamed, 2004:2). The

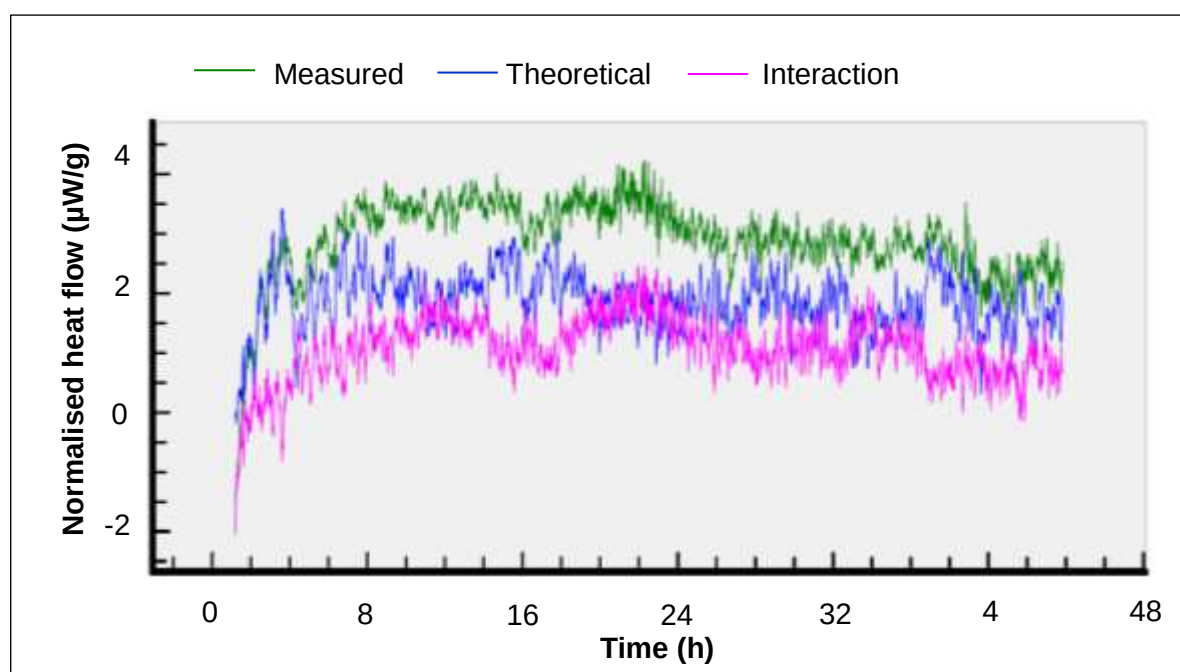
visual examination thus gave a better idea about the homogeneity and stability of the final optimised dispersion directly after formulation.

## C.5 Results and discussion for the characterisation of the optimised dispersion

### C.5.1 Artemether and safflower oil stability

#### C.5.1.1 Artemether in combination with safflower oil at 32 °C

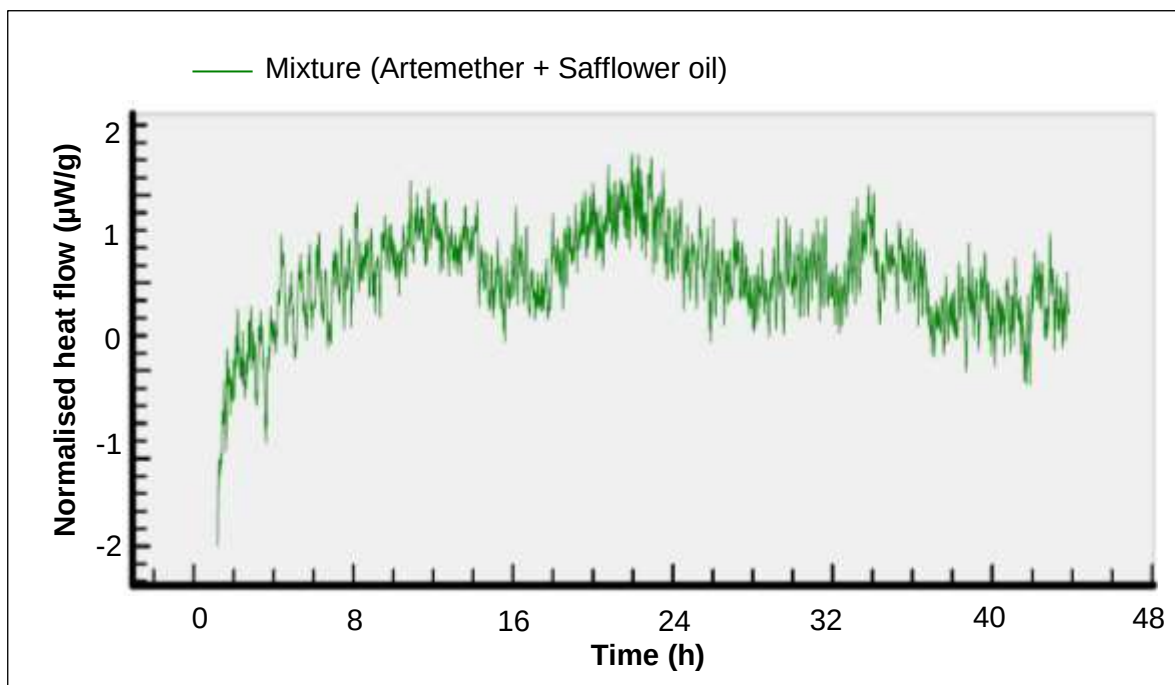
During the *in vitro* diffusion studies, dispersions were preheated to 32 °C. This temperature represented the *in vivo* conditions of the skin that were also mimicked and maintained during the *in vitro* diffusion studies (discussed in Appendix F) (Williams, 2013:685). Consequently, as artemether and safflower oil formed the core components of the topical formulations, it was important to evaluate their compatibility at 32 °C in order to determine the stability thereof during *in vitro* experimental conditions.



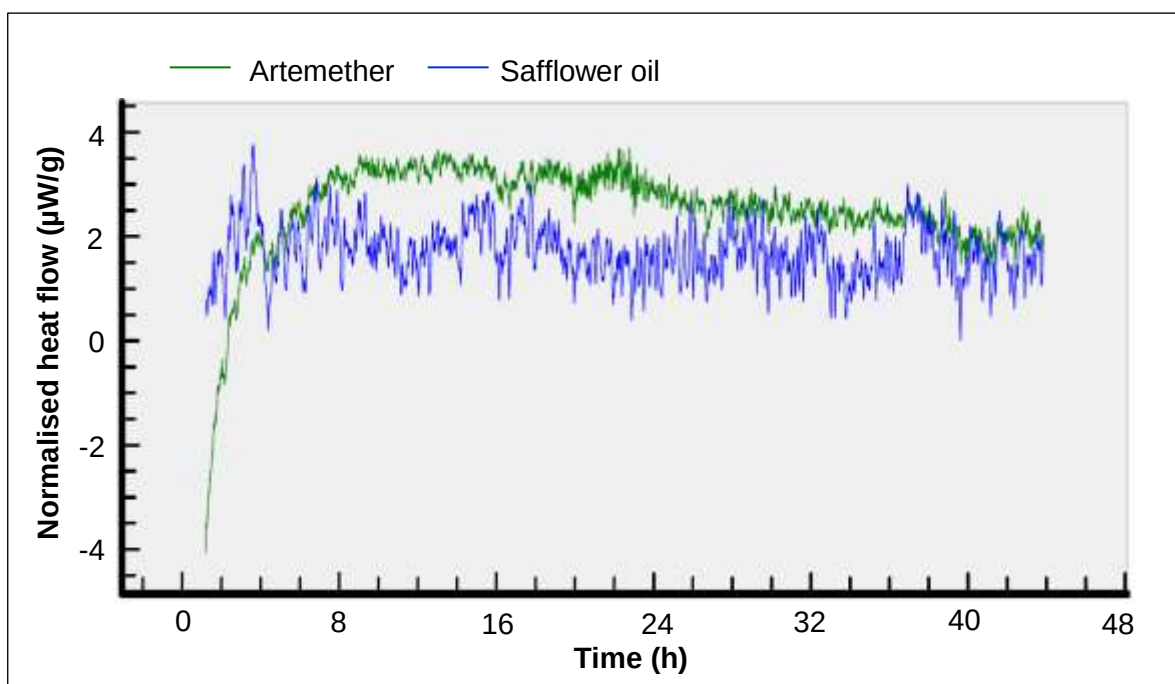
**Figure C.2:** Combined graph depicting the measured, theoretical and interaction heat flow obtained for a mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 32 °C

Figure C.2 represents the heat flow (μW/g) against time (h) for a mixture of artemether and safflower oil. The artemether and safflower oil were mixed in a 0.17:1.00 (%w/w) ratio (as determined through previous solubility investigations in Section B.4.2.1) and was also the ratio used throughout the complete study. At 32 °C, no incompatibility was observed between artemether and safflower oil. The interaction heat flow was calculated to be  $1.07 \pm 1.19$  μW/g and is represented by the magenta curve, which formed a relative straight line (Figure C.2).

The magenta curve would have shown a sudden increase or decrease in the presence of an incompatibility or interaction. If there were any incompatibilities or interactions, the magenta curve would also not have been so close to 0  $\mu\text{W/g}$ . Therefore, it can be concluded that artemether in combination with safflower oil at 32 °C would remain stable.



**Figure C.3:** Graph representing the heat flow *versus* time data for the mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 32 °C

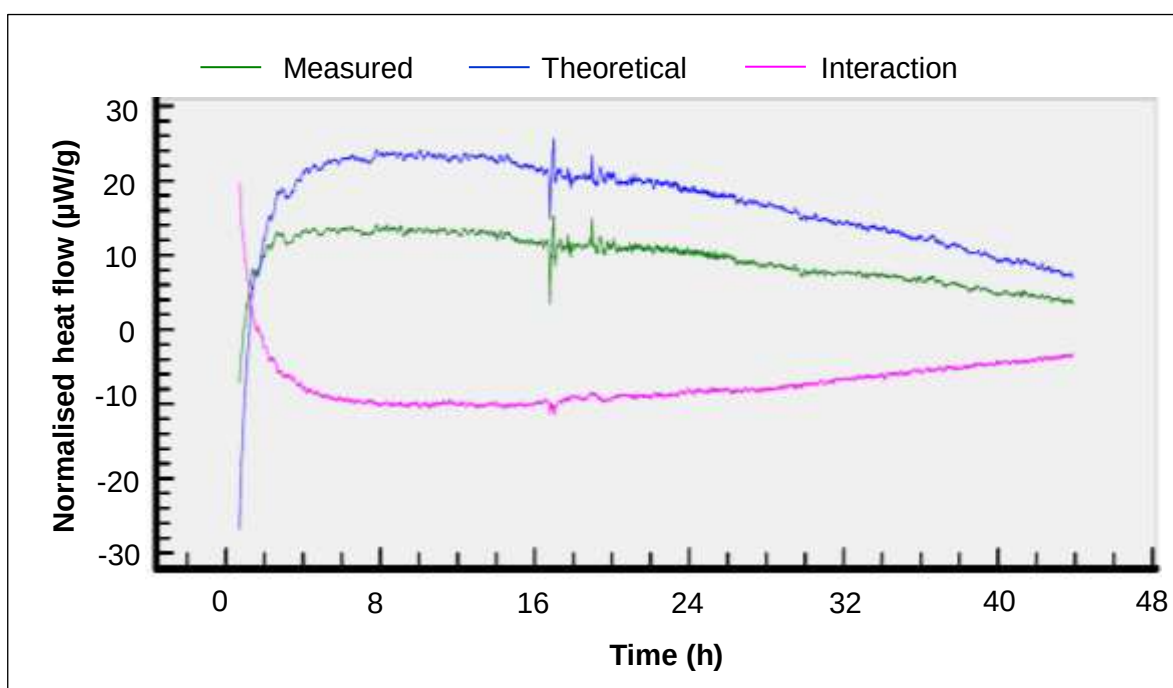


**Figure C.4:** Graph representing the heat flow *versus* time data of the separate compounds (artemether and safflower oil) at 32 °C

Figure C.3 reflects the heat flow *versus* time graph for the mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 32 °C, and Figure C.4 represents the heat flow *versus* time data of the separate excipients, namely artemether and safflower oil at 32 °C. These figures confirmed no incompatibility was measured at 32 °C.

#### C.5.1.2 Artemether in combination with safflower oil at 40 °C

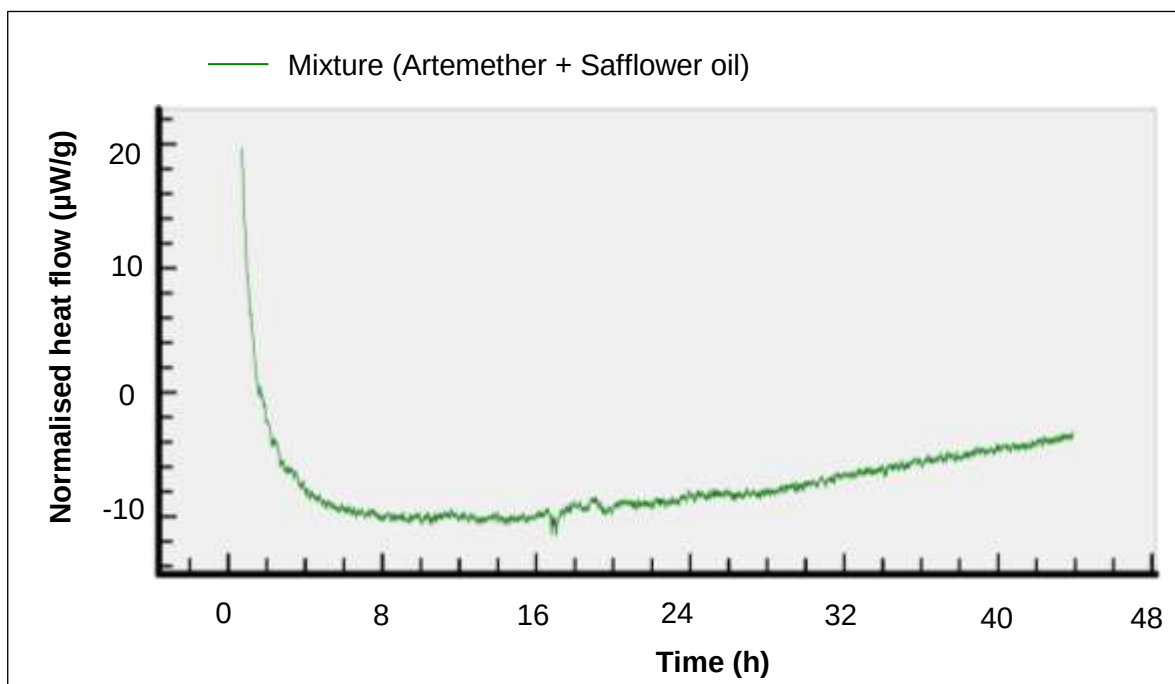
Artemether, in combination with safflower oil in a ratio of 0.17:1.00 (%w/w), was investigated at 40 °C. Figure C.5 represents the heat flow data obtained over a period of 48 h for the mixture of artemether and safflower oil. A heat flow difference of 15  $\mu\text{W/g}$  was determined between the physical measurement and the theoretical heat flow as the average interaction heat flow (represented by the magenta curve) was calculated to be  $-7.39 \pm 8.03 \mu\text{W/g}$ . Since there was a general deviation concerning the magenta curve and that of the measured and theoretical curves, it could therefore be an indication that an incompatibility possibly existed. However, the heat flow difference was too small to be clearly identified as an incompatibility. To make a conclusive decision on the compatibility of artemether and safflower oil, compatibility at higher temperatures was investigated.



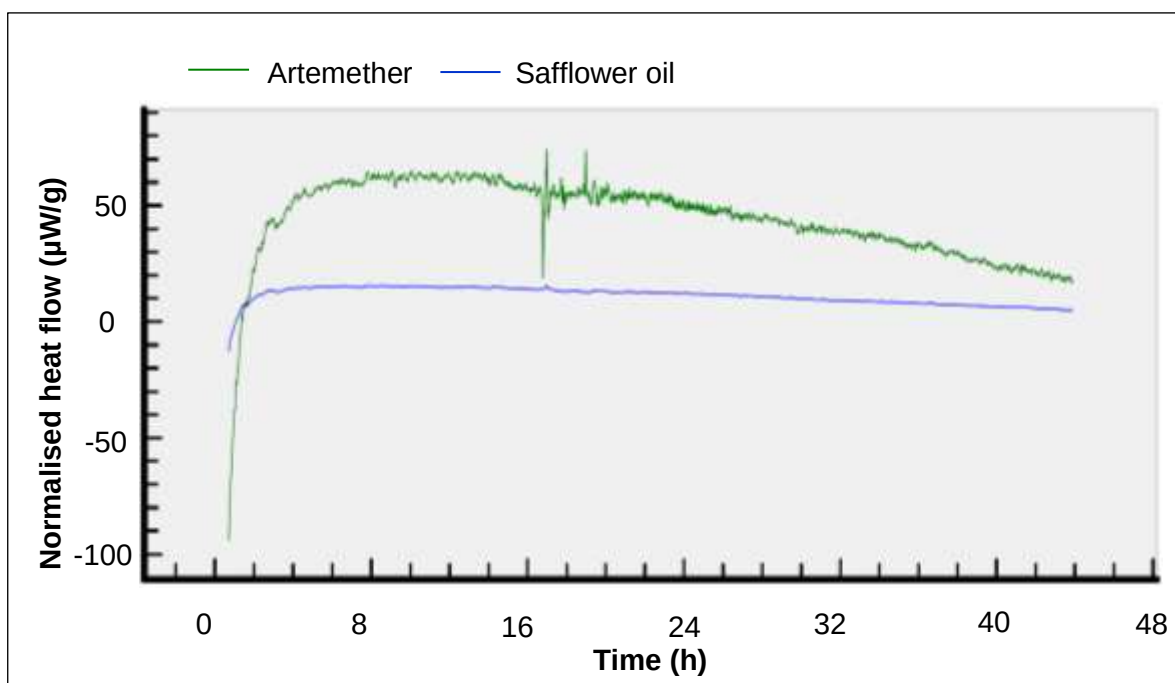
**Figure C.5:** Combined graph depicting the measured, theoretical and interaction heat flow obtained for a mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 40 °C

Figure C.6 reflects the heat flow *versus* time graph for the mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 40 °C, and Figure C.7 represents the heat flow *versus* time data of the separate excipients, namely artemether and safflower oil at 40 °C. The graphs

presented with two different heat flow behaviours, which could possibly indicate an incompatibility at 40 °C, when combined.



**Figure C.6:** Graph representing the heat flow *versus* time data for a mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 40 °C

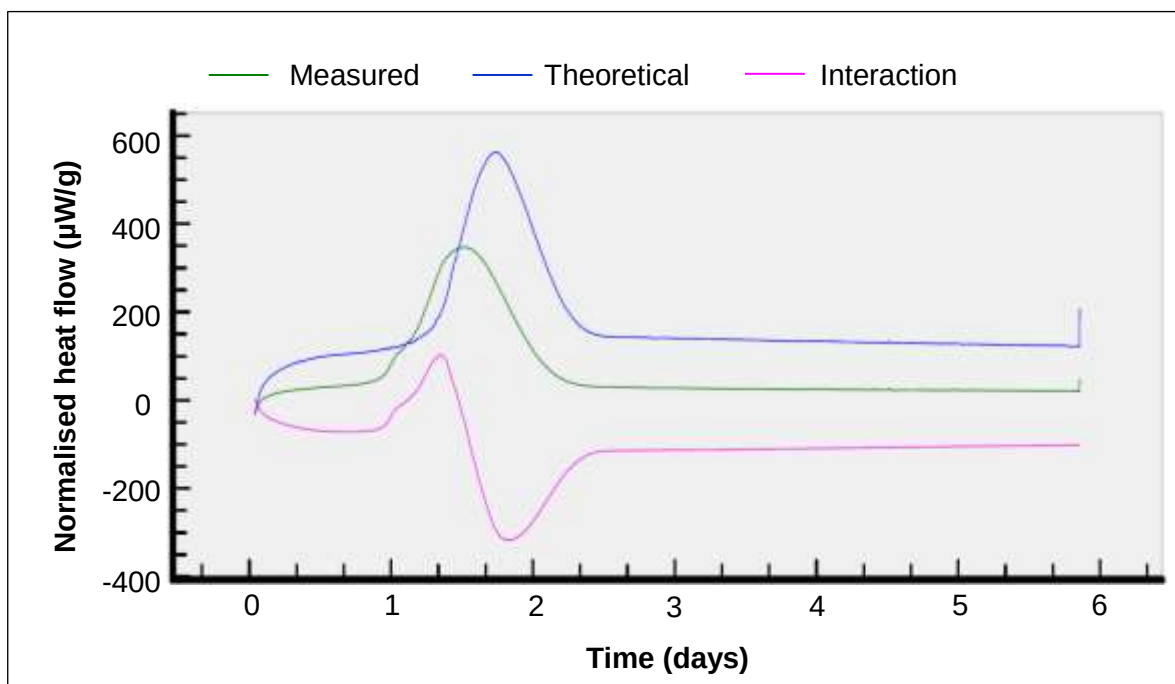


**Figure C.7:** Graph representing the heat flow *versus* time data for the separate excipients (artemether and safflower oil) at 40 °C

### C.5.1.3 Artemether in combination with safflower oil at 60 °C

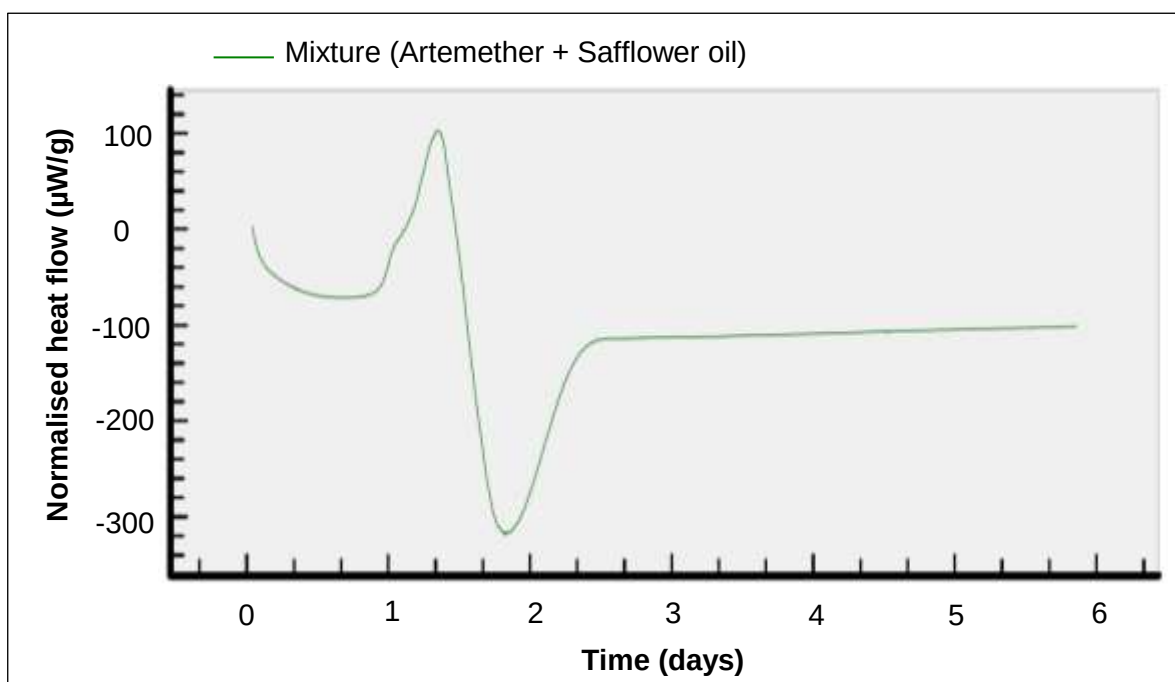
Due to the possibility of an incompatibility, further investigation was required, therefore a much higher temperature was used to enhance or accelerate any reaction due to this possible incompatibility detected at 40 °C. Consequently, an artemether and safflower oil mixture in a ratio of 0.17:1.00 (%w/w) was tested at 60 °C.

From Figure C.8 it is evident there was an interaction between artemether and safflower oil used in combination at 60 °C.

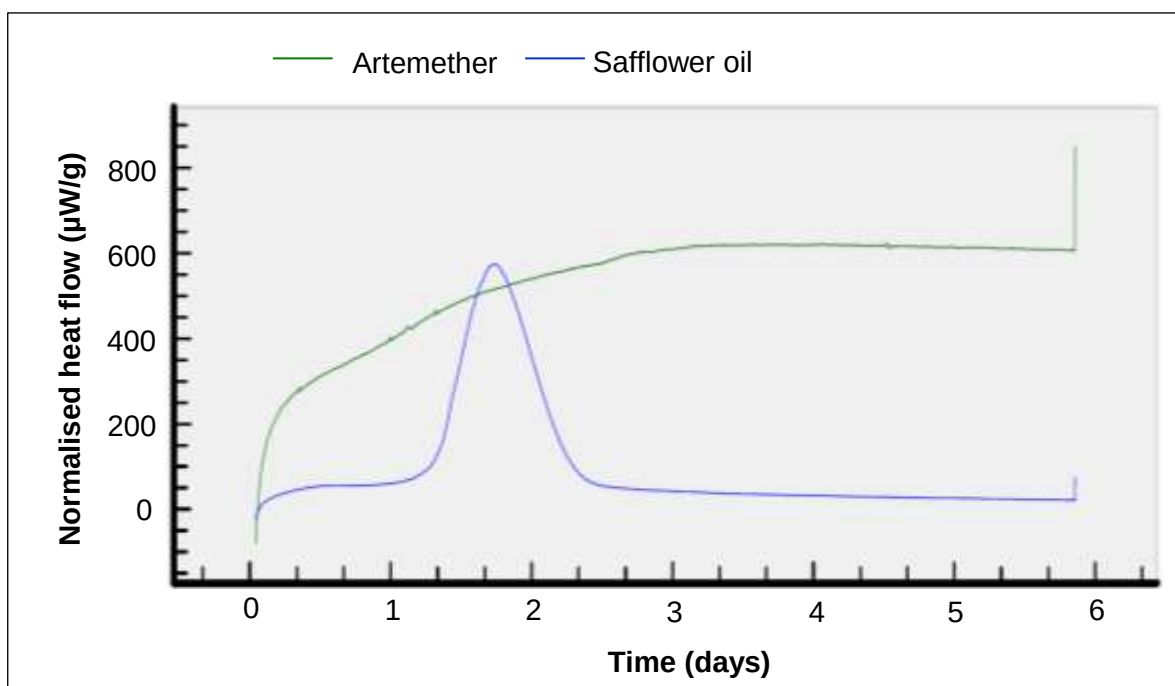


**Figure C.8:** Combined graph depicting the measured, theoretical and interaction heat flow obtained for a mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 60 °C

Figure C.9 reflects the heat flow *versus* time graph for the mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 60 °C, and Figure C.10 represents the heat flow *versus* time data of the separate excipients, namely artemether and safflower oil at 60 °C. Figure C.10 indicates that an interaction or incompatibility had occurred within the safflower oil sample. As seen in Figure C.9, the graph for the mixture of artemether and safflower oil indicates that the reaction which occurred was also present in the mixture and not only for the separate safflower oil sample. Therefore, taking both Figures C.9 and C.10 into consideration, it was evident that the reaction of the safflower oil was also responsible for the interaction or incompatibility when combined with artemether.



**Figure C.9:** Graph representing the heat flow *versus* time data for a mixture of artemether and safflower oil in a ratio of 0.17:1.00 (%w/w) at 60 °C



**Figure C.10:** Graph representing the heat flow *versus* time data for the separate excipients (artemether and safflower oil) at 60 °C

#### C.5.1.4 Conclusion of compatibility studies

In conclusion, when artemether in combination with safflower oil was tested at 32, 40 and 60 °C, respectively, no incompatibility was visible at 32 °C. A possible incompatibility or interaction

was present when the temperature increased to 40 °C, and at 60 °C a definite incompatibility or interaction was identified. During these studies, the exact mechanisms or interactions that can explain the incompatibility were not identified and therefore further studies would be required to clarify the results obtained. Information found in literature could provide possible explanations.

Two possibilities for this incompatibility and instability could exist: the first possibility being the increase in temperature and the second could be the oxidative stress caused by artemisinin and its derivatives. Literature has shown that safflower oil is susceptible to oxidation. It should be kept in mind that oxidative stability can increase with an increase in temperature and in the presence of water or acid (Khatoon & Krishna, 1998:247; Lee *et al.*, 2004:5; Noria Corporation, 2016). As the compatibility studies were conducted at increased temperatures (32, 40 and 60 °C, respectively) this could also aid in oxidation. In addition to this, the incompatibility could be ascribed to the fact that artemether is rather sensitive to heat, therefore an increase in temperature could lead to easy decomposition of artemether and hence an instability (Gao *et al.*, 2013:134).

It could also be proposed that artemether could possibly oxidise the safflower oil. Artemisinin and its derivate, artemether, contains an endoperoxide bridge which leads to the production of free radicals as well as ROS (Nneji *et al.*, 2013:2619; Shrivastava *et al.*, 2010:79). A known effect of ROS is that it can donate oxygen molecules (Lobo *et al.*, 2010:1). The effects of oxidative stability are important, as it is a reaction occurring among the natural oil and oxygen molecules (Noria Corporation, 2016). Free radicals have the ability to target macromolecules, such as lipids and proteins (Lobo *et al.*, 2010:1-2). When the production of free radicals overwhelms defences posed by antioxidants, oxidative stress is caused (Lobo *et al.*, 2010:2). Oxidative stress is also responsible for the damage of lipids and proteins (Lobo *et al.*, 2010:2). It can therefore be speculated that this production of free radical and ROS by artemisinin leads to oxidative stress, which can oxidise the natural fatty acid, i.e. safflower oil, leading to instability.

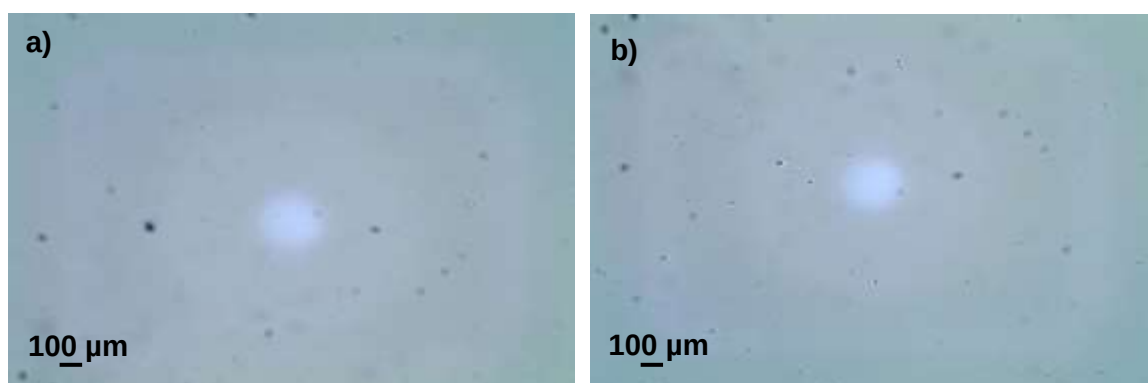
It cannot be said with certainty that one of these possibilities was the root of the instability and therefore further experimental studies are required.

## **C.5.2 Morphology**

### **C.5.2.1 Light microscopy**

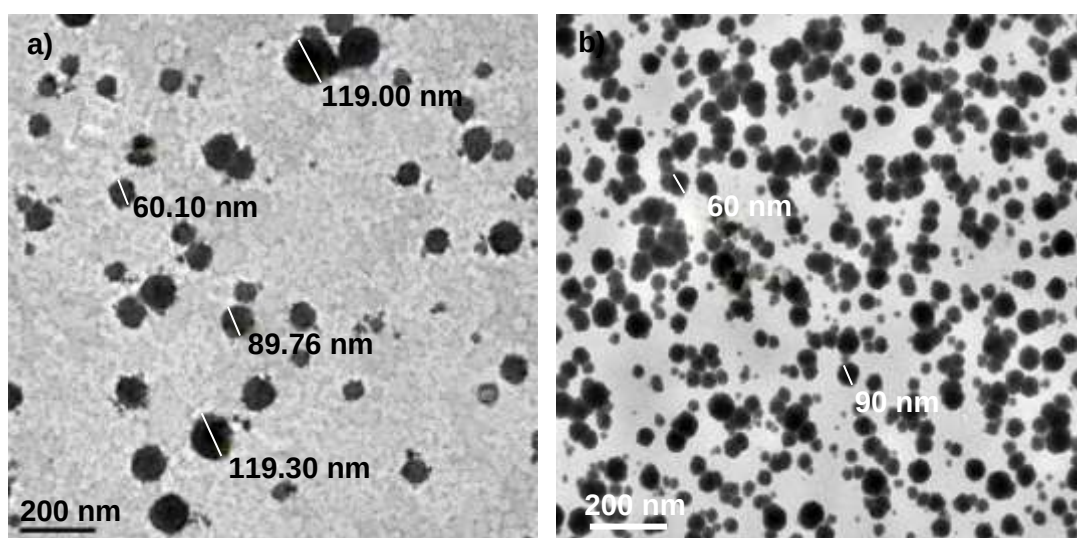
The small droplets of nano-emulsions are viewed with difficulty through normal microscopy, yet it was used as confirmation that nano-sized droplets were formed during the formulation process (Graves, 2008:44). Consequently, micrographs were captured of the **(NE1)** and the **(PNE1)**, as depicted in Figures C.11.a and C.11.b respectively. Very few droplets surrounded

by a large aqueous medium were visible on the micrographs. As the droplets were too small to determine the size through normal light microscopy accurately, it was possible that nano-sized droplets were created for both dispersions ((**NE1**) and (**PNE1**)). Hence, further microscopic investigation of these small droplets, through TEM, was necessary.



**Figure C.11:** Micrographs as captured through light microscopy: a) (**NE1**) and b) (**PNE1**)

#### C.5.2.2 Transmission electron microscopy



**Figure C.12:** TEM images of the (**PNE1**): a) sample one sonicated for 3 min and placed in an ultrasonication bath for 15 min and b) sample two sonicated for 3 min and placed in an ultrasonication bath for 15 min

Two samples of the (**PNE1**) were prepared to be investigated through TEM. Nano-emulsions normally present with droplets in the nanometric range of 20 – 200 nm (Abolmaali *et al.*, 2011:139). From Figures C.12.a and C.12.b, the HRTEM micrographs for two samples of the (**PNE1**) are shown. Figure C.12.a exhibited droplets ranging from 60.10 – 119.30 nm, whereas in Figure C.12.b the droplets ranged from 60.00 – 90.00 nm for the second sample. Both the dilutions' droplets presented as small spheres. The droplets in Figures C.12.a and C.12.b appeared as dark, small spheres with a rough surface. A possible explanation for these dark

spheres could be ascribed to the osmium tetroxide (unsaturated fatty acid stain), which stained the droplets greatly. Figure C.12.b (representing a second sample) indicated a more dense solution of oil droplets than Figure C.12.a. This could be due to more sample being present on the carbon-coated mesh copper grid and therefore more droplets were stained in Figure C.12.b than in Figure C.12.a. With the presence of small droplets, no coagulation of the droplets was visible on either micrographs (Figures C.12.a or C.12.b), which confirmed that **(PNE1)** was a successful dispersion. Therefore, both micrographs illustrated that **(PNE1)** successfully resulted in the formulation of nano-sized droplets. Micrographs obtained through light microscopy, as well as through TEM observations confirmed the formation of nano-emulsions.

### C.5.3 Droplet size and distribution

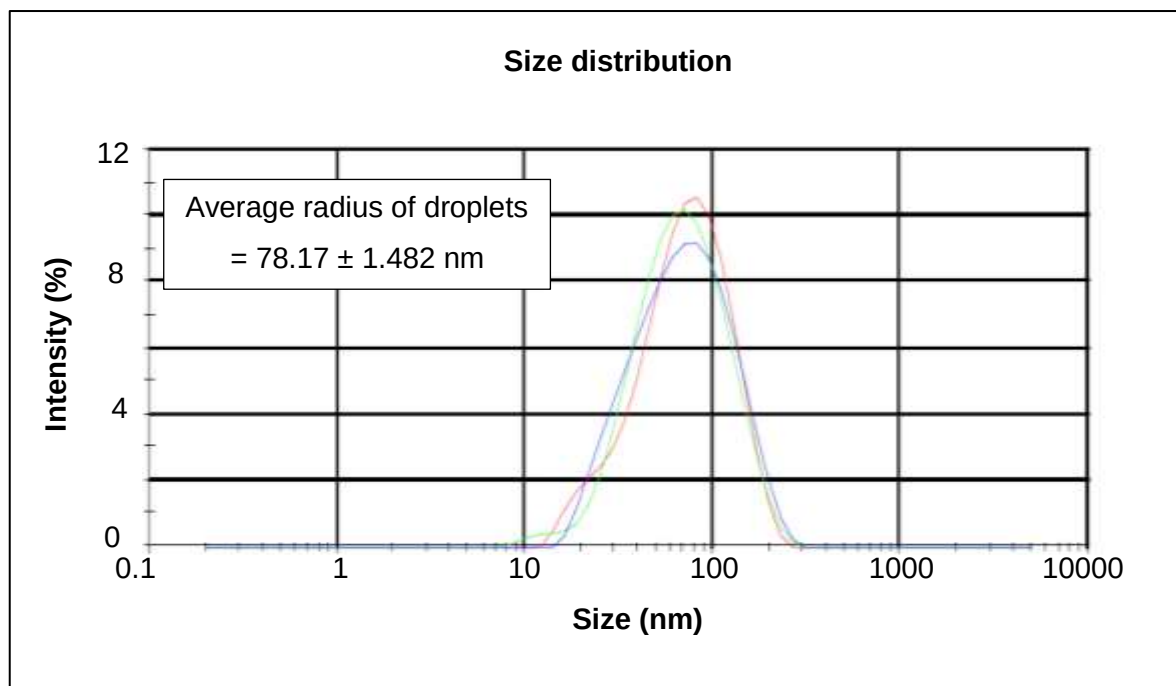
Literature states that dispersions with droplets ranging from 20 – 200 nm can be viewed as nano-emulsions (Abolmaali *et al.*, 2011:139). Table C.2 represents the average droplet size of the **(NE1)** and the **(PNE1)**; the **(NE1)** presented with an average droplet size of  $156.34 \pm 2.964$  nm whilst the **(PNE1)** presented with an average droplet size of  $134.13 \pm 4.530$  nm, which was smaller than that of **(NE1)**. The quality report obtained with the Malvern Zetasizer revealed that the results for both samples were “good”. Consequently, both the dispersions (**(NE1)** and **(PNE1)**) can be classified as nano-emulsions, as their droplets fell within in this nano-metric range.

The uniformity of the droplets spread throughout the dispersion can best be described by the Pdl (Shakeel *et al.*, 2007:E6). Literature provides that Pdl can be measured on a scale of 0 – 1 (Shaw, 2016). A Pdl value closest to 0 can be viewed as monodispersed (a homogenous dispersion), whereas a Pdl closer to 1 can indicate the droplet size varies and that the dispersion presents to be polydispersed (Gaumet *et al.*, 2008:5). The Pdl, as measured for the **(NE1)** and the **(PNE1)**, is also tabulated in Table C.2. The **(NE1)** measured with an average Pdl of  $0.24 \pm 0.005$ , which can be viewed as close to 0. The same can be said of the **(PNE1)**, which measured an average Pdl of  $0.27 \pm 0.006$ . Both dispersions, **(NE1)** and **(PNE1)**, are therefore relatively monodispersed. Abolmaali *et al.* (2011:140) and Chime *et al.* (2014:77) stated that a nano-emulsion is characteristically a heterogeneous dispersion constituted through oil and water. Hence, a nano-emulsion will never be fully homogenous. It can be proposed that both of the dispersions will also remain relatively stable against aggregation and sedimentation over time.

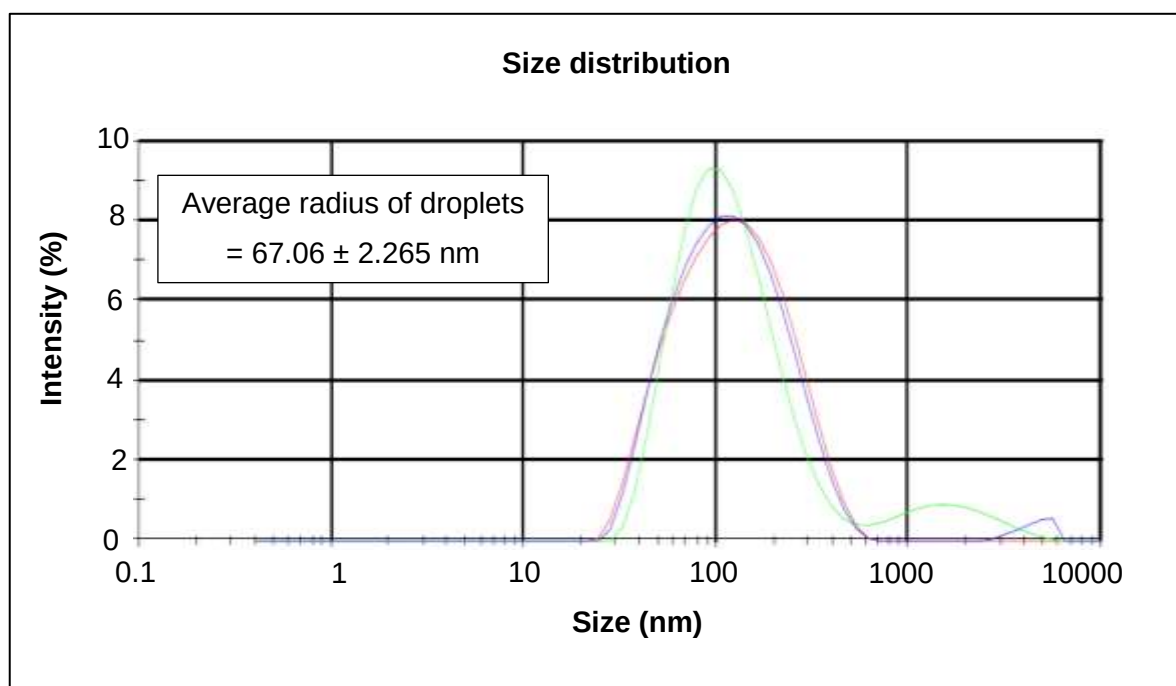
It might be said there was a correlation between the incorporation of artemether and the droplet size and Pdl. When artemether was incorporated within a dispersion (**(NE1)**) the average droplet size was large with a smaller Pdl compared to when the artemether was not incorporated (**(PNE1)**). With the API incorporated, the average droplet size was small with a larger Pdl.

**Table C.2:** Comparison of the average droplet size as well as polydispersity index of **(NE1)** and **(PNE1)**

|               | Average droplet size (nm) | Average polydispersity index (Pdl) |
|---------------|---------------------------|------------------------------------|
| <b>(NE1)</b>  | $156.34 \pm 2.964$        | $0.24 \pm 0.005$                   |
| <b>(PNE1)</b> | $134.13 \pm 4.530$        | $0.27 \pm 0.006$                   |



**Figure C.13:** Average droplet size (nm) of the **(NE1)** measured per droplet radius



**Figure C.14:** Average droplet size (nm) of the **(PNE1)** measured per droplet radius

Figures C.13 and C.14 are a representation of the average radius of the droplet size (nm), as measured through the Malvern Zetasizer, for the **(NE1)** and the **(PNE1)**. Figures C.13 and C.14 represent the triplicate readings taken for each dispersion combined in one figure, respectively. It is observed that the droplet size for both dispersions is relatively in the range of nano-emulsion droplets as they average below 200 nm. All the curves in both Figures presented with the same relative symmetrical shape.

#### C.5.4 pH

The stratum corneum has a wide pH value, ranging from 3 – 9 (Barry, 2002:512). From Table C.3 it is observed that the average pH of the **(NE1)** and the **(PNE1)** was  $6.82 \pm 0.03$  and  $7.02 \pm 0.02$ , respectively. Consequently, the pH of the **(NE1)** and the **(PNE1)** fell within this acceptable range. It is therefore believed that the pH of both dispersions, **(NE1)** and **(PNE1)**, would not influence the integrity of the skin. Although there is not a large difference between the pH of the two dispersions, it is noticed that when artemether was incorporated within the dispersion, the pH was lowered and therefore made more acidic.

**Table C.3:** Average pH of **(NE1)** and **(PNE1)**

|               | pH (reading 1) | pH (reading 2) | pH (reading 3) | Average pH      |
|---------------|----------------|----------------|----------------|-----------------|
| <b>(NE1)</b>  | 6.85           | 6.79           | 6.82           | $6.82 \pm 0.03$ |
| <b>(PNE1)</b> | 7.00           | 7.02           | 7.04           | $7.02 \pm 0.02$ |

#### C.5.5 Viscosity

**Table C.4:** Viscosity readings of **(NE1)** and **(PNE1)**

|               | Reading         | Viscosity (cP)   | Torque (%)        | Temperature (°C)  | Speed (RPM)        |
|---------------|-----------------|------------------|-------------------|-------------------|--------------------|
| <b>(NE1)</b>  | <b>1</b>        | 1.66             | 11.02             | 25.20             | 200.00             |
|               | <b>2</b>        | 1.63             | 10.87             | 25.20             | 200.00             |
|               | <b>3</b>        | 1.63             | 10.83             | 25.20             | 200.00             |
|               | <b>Averages</b> | $1.64 \pm 0.016$ | $10.91 \pm 0.100$ | $25.20 \pm 0.000$ | $200.00 \pm 0.000$ |
| <b>(PNE1)</b> | <b>1</b>        | 1.59             | 10.65             | 25.10             | 200.00             |
|               | <b>2</b>        | 1.63             | 10.75             | 25.40             | 200.00             |
|               | <b>3</b>        | 1.60             | 10.61             | 25.20             | 200.00             |
|               | <b>Averages</b> | $1.61 \pm 0.020$ | $10.67 \pm 0.070$ | $25.20 \pm 0.153$ | $200.00 \pm 0.000$ |

A viscosity reading of a formulation could be directly ascribed to the delivery system. Normally nano-emulsions present with very low viscosity. Since an o/w nano-emulsion was formulated, the largest component was the water phase, which contributes to a lower viscosity (Chime et

*al.*, 2014:97). Therefore, a low viscosity reading was expected for the **(NE1)** and the **(PNE1)**. The average viscosity of the **(NE1)** ( $1.64 \pm 0.016$  cP) was not really affected by the addition of artemether when compared to the average viscosity of the **(PNE1)** ( $1.61 \pm 0.020$  cP). Both dispersions were not very viscous and measured close to that of water (viscosity of 1 cP) (V&P Scientific Inc., 2010). It can also be said that the amounts of surfactants in relation to the amounts of water and oil used, lead to an adequate interfacial tension, which resulted in a low viscosity (Chime *et al.*, 2014:97). The experimental findings therefore correlated with that found in literature.

### C.5.6 Zeta-potential

Literature states that a highly negative zeta-potential (lower than - 30 mV), especially for a dispersion with small droplets can present with great stability over time (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860). It has been proposed that the surface of the skin is primarily negatively charged and that highly negative charged molecules (a highly negative zeta-potential value) could lead to better diffusion and consequently, to the increased flux of the API (Duangjit *et al.*, 2011:6; Sinico *et al.*, 2005:129).

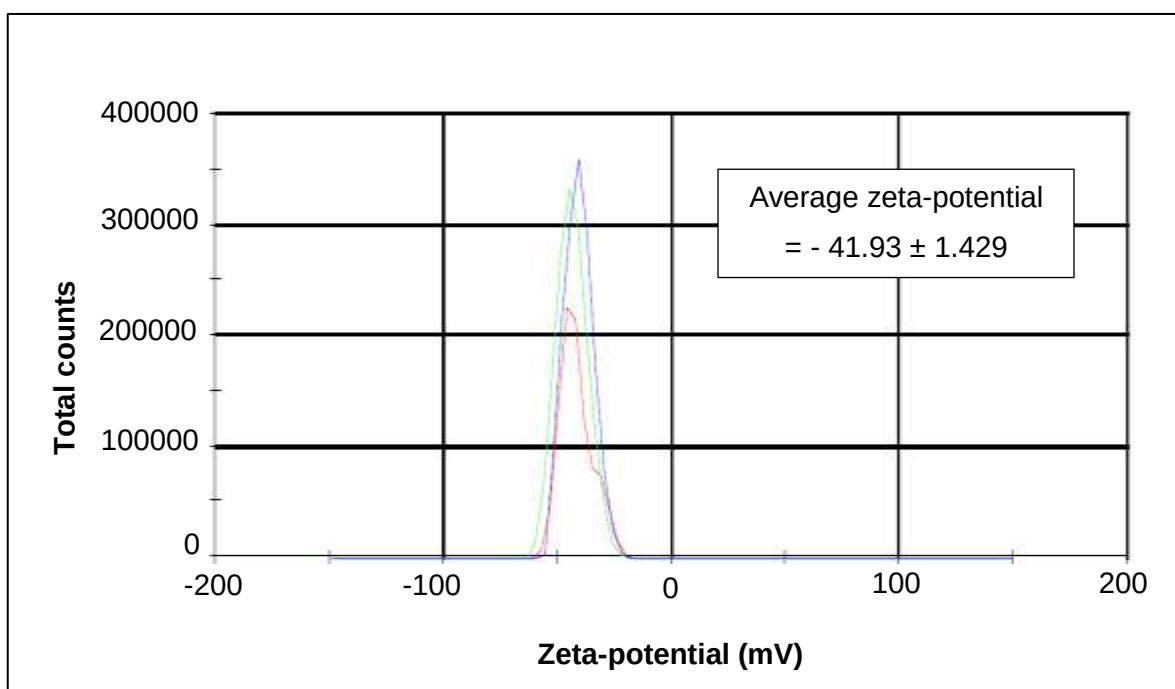
In Table C.5, the average zeta-potential values of the **(NE1)** and the **(PNE1)**, as measured at room temperature (25 °C), was illustrated as  $- 41.93 \pm 1.429$  mV and  $- 44.73 \pm 2.950$  mV, respectively. The average zeta-potential for both dispersions, **(NE1)** and **(PNE1)**, were highly negative and could therefore be ideal for topical delivery and may result in the successful permeation through the skin (Duangjit *et al.*, 2011:6). It could also be proposed that the dispersions (**(NE1)** and **(PNE1)**) would be more stable over time and present with less aggregation and coagulation.

**Table C.5:** Average zeta-potential of **(NE1)** and **(PNE1)**

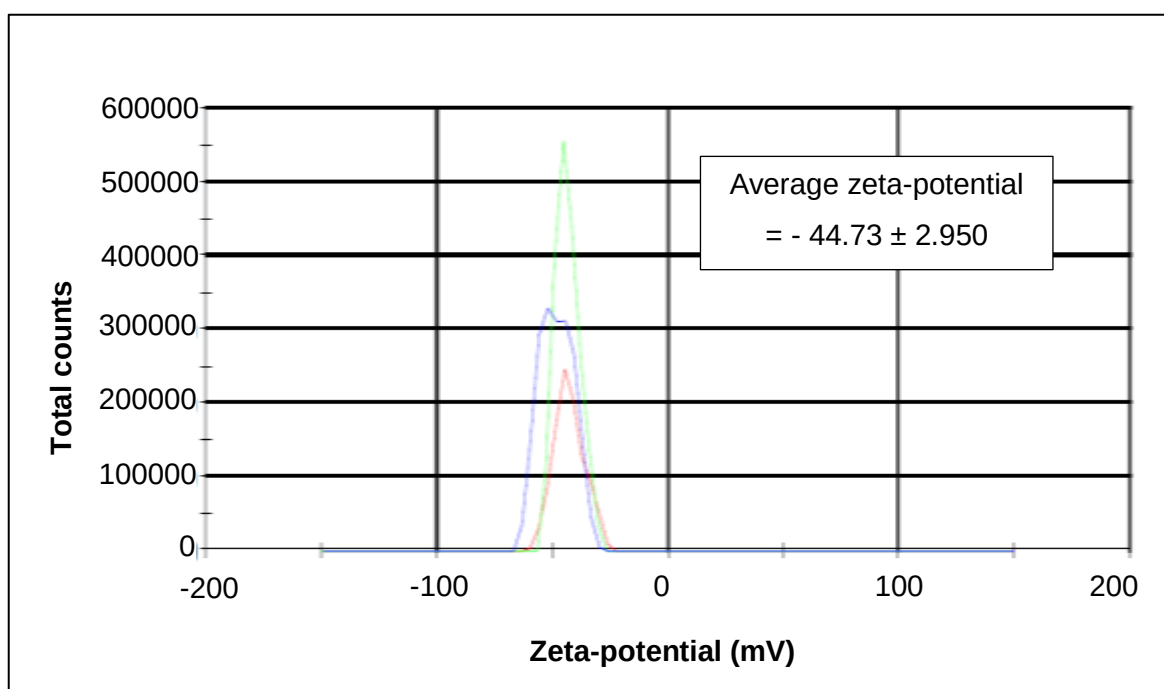
|               | Zeta-potential<br>(reading 1) | Zeta-potential<br>(reading 2) | Zeta-potential<br>(reading 3) | Average<br>zeta-potential (mV) |
|---------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|
| <b>(NE1)</b>  | - 41.60                       | - 43.50                       | - 40.70                       | $- 41.93 \pm 1.429$            |
| <b>(PNE1)</b> | - 42.60                       | - 48.10                       | - 43.50                       | $- 44.73 \pm 2.950$            |

Figures C.15 and C.16 are a representation of the average zeta-potential (mV) measured with the Malvern Zetasizer for the **(NE1)** and the **(PNE1)**. The isoelectric point on the curve is represented by zero (0 mV) and this point indicates that a dispersion is unstable (Paderborn University, 2016). Hence, when a curve is further away from zero, either positive or negative, the zeta-potential can be viewed as ideal. Figures C.15 and C.16 are also a representation of the results shown in Table C.5, as they depict the zeta-potential values of the three readings taken graphically. The graphs in both Figures C.15 and C.16 are leaning toward the negative

zeta-potential values, as all the curves average close to the - 50 mV. The highly negative zeta-potential value could therefore lead to better skin diffusion, as well as improved bioavailability of the artemether (Sinico *et al.*, 2005:129). The shape of the curves in Figure C.15 indicates a narrow distribution of data, since the curves are symmetrical and pointed. The curves on Figure C.16 present with a variation in shape and size, consequently indicating a wider distribution of data points.



**Figure C.15:** Average zeta-potential (mV) of (NE1)

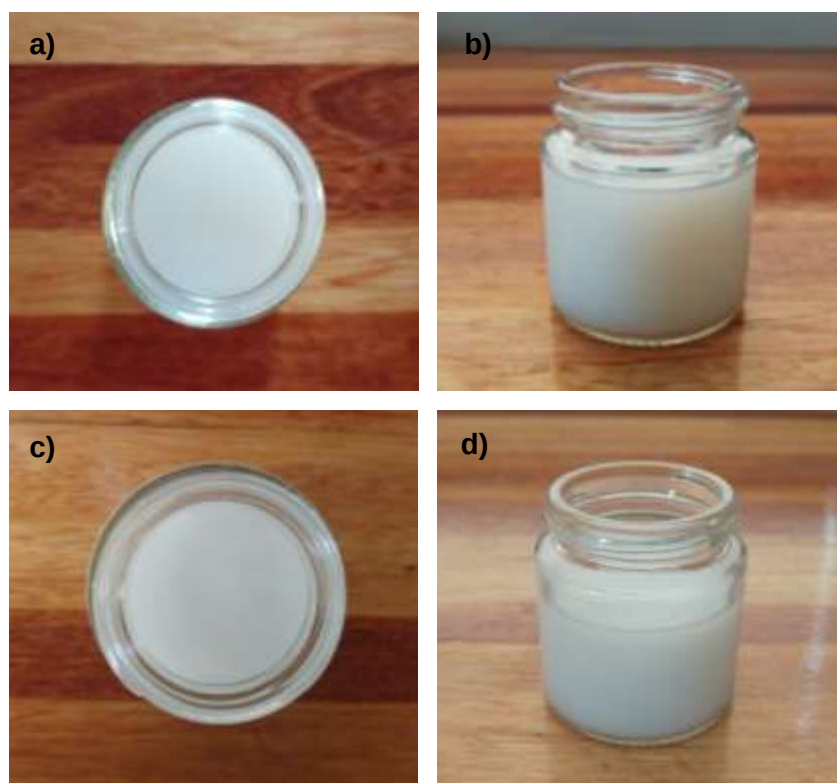


**Figure C.16:** Average zeta-potential (mV) of (PNE1)

### C.5.7 Entrapment efficiency

A standard curve with a linear line was plotted through the preparation of a standard solution and optimised chromatographic conditions (as discussed in Appendix A). It has been proposed that 100%, or near to 100%, %EE can be achieved with a lipophilic API incorporated within a nano-emulsion (Loureiro *et al.*, 2015:96). The %EE was calculated according to a linear line obtained through HPLC investigations together with Equation C.1. Consequently, after calculation, it was determined that the **(NE1)** presented with a %EE of 99.578%. This was considered a high entrapment; high entrapment of an API ensures a larger amount of the API can be delivered to the target site (Kurakula *et al.*, 2012:37). This could also possibly result in sustained drug release (Kurakula *et al.*, 2012:37). This experimental %EE value of 99.578% could propose that the lipophilic API, artemether, was almost fully incorporated within the oil phase of the **(NE1)** as it neared 100% entrapment (Loureiro *et al.*, 2015:96).

### C.5.8 Visual and physical examination



**Figure C.17:** Images of the **(NE1)** and the **(PNE1)**: a) top view of **(NE1)**; b) a side view of **(NE1)**; c) top view of the **(PNE1)** and d) a side view of **(PNE1)**

Both the **(NE1)** (Figures C.17.a and C.17.b) and the **(PNE1)** (Figures C.17.c and C.17.d) presented as a homogenous, milky, translucent liquid. No oil droplets, sedimentation or separations in the oil and water phases, were visible for both dispersions and as a result, the droplets were well distributed throughout. Spreadability of the dispersion to the skin would be

difficult, since the **(NE1)** and the **(PNE1)** presented with low viscosity, although it did not feel oily or leave a residue on the skin.

## C.6 Discussion and conclusion

It has been proposed that the characterisation can provide an overview of the physical and chemical state of the dispersion (Chime *et al.*, 2014:95). A summary of the characteristics of the **(NE1)** and the **(PNE1)**, as determined and described in Section C.5, is represented in Table C.6.

**Table C.6:** Characteristics of **(NE1)** and **(PNE1)**

| Characteristics determined | <b>(NE1)</b>    | <b>(PNE1)</b>   |
|----------------------------|-----------------|-----------------|
| TEM size (nm)              | NA              | 60.00 – 119.30  |
| Droplet size (nm)          | 156.34 ± 2.964  | 134.13 ± 4.530  |
| Pdl                        | 0.24 ± 0.005    | 0.27 ± 0.006    |
| pH                         | 6.82 ± 0.030    | 7.02 ± 0.020    |
| Viscosity (cP)             | 1.64 ± 0.016    | 1.61 ± 0.020    |
| Zeta-potential (mV)        | - 41.93 ± 1.429 | - 44.73 ± 2.950 |
| Entrapment efficiency (%)  | 99.578          | NA              |

From Table C.6, it is observed the **(NE1)** and the **(PNE1)** presented with characteristics required for effective topical delivery. The following characteristics of the **(NE1)** and the **(PNE1)** were investigated: morphology (i.e. light microscopy and TEM), droplet size, distribution of droplets, pH, zeta-potential, viscosity, as well as the %EE. The droplet size measured with the Zetasizer and the morphology characteristics obtained through TEM, confirmed that nano-sized droplets were achieved through the formulation process. For dispersions, **(NE1)** and **(PNE1)**, relative homogenous and monodispersed dispersions were formulated. This could suggest better stability over time, which in turn could also result in better effectiveness.

A safe and non-irritating, skin acceptable pH was measured for both dispersions (**(NE1)** and the **(PNE1)**). It was also calculated that artemether was greatly entrapped within the oil phase of the **(NE1)**, which could possibly result in effective drug release and sufficient bioavailability in the target, i.e. the stratum corneum. With an ideally negative zeta-potential for both the **(NE1)** and the **(PNE1)**, it can also be concluded that a very stable dispersion was formulated. Less coagulation, creaming and sedimentation over time could therefore be achieved. This negative zeta-potential and the nano-sized droplets of both dispersions, in essence, could result in effective skin permeation (**(NE1)** and **(PNE1)**).

The **(NE1)** and the **(PNE1)** were therefore characterised and remained as optimal for a topical formulation, due to no great influence being found with the incorporation or exclusion of artemether. Since a low viscosity for the **(NE1)** and the **(PNE1)** was measured, close to that of water, application to the skin was seen as a challenge. It was therefore concluded that this optimised **(NE1)** should be formulated within a semi-solid formulation to provide easier application. Consequently, the formulation of two semi-solid dosage forms of the optimised o/w **(NE1)**, i.e. a nano-emulgel and a conventional emulgel, is discussed further in Appendix D.

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# APPENDIX D:

## FORMULATION OF SEMI-SOLID DOSAGE FORMS OF AN O/W NANO-EMULSION CONTAINING ARTEMETHER AND SAFFLOWER OIL

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### D.1 Introduction

An optimised nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil ((**NE1**)) was formulated and characterised as a novel CTB treatment. It is well known that a nano-emulsion, although very advantageous, is very liquid and therefore not very viscous; this could possibly complicate the topical delivery of APIs (Ali *et al.*, 2014:1128). Consequently, to increase API delivery, as well as patient compliance, a more viscous formulation could be achieved through the formulation of a semi-solid dosage form (Williams, 2013:687). This is because a semi-solid dosage form is easier applied to the skin than a liquid, which in turn increases patient compliance (Pund *et al.*, 2015:152).

Currently, there is no topical treatment, only systemic treatment, available for the treatment of CTB. Unfortunately, systemic treatment poses with unfavourable patient compliance due to an extensive combination of drugs used over a period of months (Van Zyl *et al.*, 2015:634-635). To decrease treatment duration, it has been proposed that the concomitant use of a topical formulation together with the systemic treatment could provide effective treatment of CTB, especially because systemic TB treatment can cause many side effects. A semi-solid dosage form can usually be utilised when topical conditions are treated (Mahalingham *et al.*, 2008:267). A semi-solid dosage form is a formulation with a semi-solid (between a solid and a liquid) consistency, which is aimed at localised application to the skin either for a therapeutic or protective function (Kaur *et al.*, 2013:202). Another advantage of a semi-solid as a topical formulation is that it can result in direct contact between the API and the skin; therefore, it can be said that a semi-solid dosage form can behave as a carrier together with aiding in the delivery of APIs (Gupta & Garg, 2002:144).

The objective of this section was to focus on increasing the low viscosity of the (**NE1**) (characterised in Appendix C) by incorporating the (**NE1**) within a semi-solid dosage form, thus ensuring contact between the API and the skin (Mahalingham *et al.*, 2008:288). APIs formulated within a semi-solid dosage form are targeted to either work on the skin surface or to penetrate to the layers of the skin (Mahalingham *et al.*, 2008:268), which could also be ideal for the treatment of CTB.

## **D.2 Intended purpose of the formulation**

Firstly, it was intended to increase the viscosity of the **(NE1)** for easier application and increased effectiveness, through the formulation of a semi-solid dosage form (Chellapa *et al.*, 2015:787; Williams, 2013:689). Secondly, the type of semi-solid dosage form chosen is greatly influenced by the condition and more so, the lesion type (Williams, 2013:687). As CTB can present through a variety of inflamed, caseous, purulent and necrotic lesions, ranging from nodules to abscesses and consequently ulcers, a suitable topical semi-solid dosage form is required (Almaguer-Chávez *et al.*, 2009:564; Bravo & Gotuzzo, 2007:174-177; Van Zyl *et al.*, 2015:3). To date, no topical treatment with a localised rather than systemic effect for CTB exists and presents as an opportunity for the investigation thereof (Van Zyl *et al.*, 2015:3). It was therefore necessary to choose an appropriate semi-solid dosage form, which will not aggravate or irritate the topical condition (Purushottam *et al.*, 2013:239). As CTB presents with ulcer-like lesions, a semi-solid dosage form with an aqueous base, i.e. a gel or an aqueous based cream, was proposed as most suitable (Williams, 2013:687).

### **D.2.1 Semi-solid dosage form selection**

A semi-solid dosage form is normally constituted through two phases, i.e. an oil and water phase. One phase represents a larger portion as it forms the external phase, whereas the other phase is normally a dispersed phase. The API is therefore dissolved either in the oil or water phase (Valentine, 2014:153). A variety of semi-solid dosage forms, such as creams, gels, ointments, foams or pastes can aid the delivery of APIs and each of these forms present with individual advantages (Allen *et al.*, 2011:272; Bora *et al.*, 2014:3594; Gupta & Garg, 2011:144; Valentine, 2014:153). These individual advantages aid the choice of the most suitable dosage form.

For the purpose of this study, a gel and versions of gels were investigated for the topical delivery of artemether, to aid in the possible treatment of CTB.

### **D.2.2 Semi-solid dosage form: gel**

A gel can be defined as a semi-solid dosage form composed through a combination of a dispersion, (either having small or large droplets) and a jellylike medium (consisting of an aqueous base thickened through a gelling agent), consequently presenting with properties of both a solid and liquid (Allen *et al.*, 2011:278; Kute & Saudagar, 2013:368). Direct contact between the API and the skin, or site of absorption, can be achieved through gels (Mahalingam *et al.*, 2008:288).

Gels provide advantages such as (Gaur *et al.*, 2014:37):

- \* non greasy feel,
- \* spreads with ease across the skin surface,
- \* removed without trouble,
- \* presents with emollient properties and
- \* presents as compatible with various excipients.

When compared to other semi-solid dosage forms, such as ointments and creams, gels present more favourably. This could be ascribed to the fact that gels contain a larger aqueous phase, resulting in better dissolution of the API and so facilitates easier movement of the API from the formulation to the skin (Khullar *et al.*, 2012:63). Although the use of gels as a semi-solid dosage form is beneficial, it does pose a challenge for the incorporation of lipophilic APIs (Gaur *et al.*, 2014:38; Hyma *et al.*, 2014:2).

#### **D.2.2.1 Emulgel**

In this study, artemether is a lipophilic API and therefore the formulation of a conventional gel would not suffice. To incorporate lipophilic APIs successfully within a semi-solid dosage form, emulgels exist (Gaur *et al.*, 2014:38; Hyma *et al.*, 2014:2; Kute & Saudagar, 2013:368). An emulgel can be defined as the combination of a coarse emulsion and a gel base, hence an o/w or w/o emulsion mixed with a gelled liquid base (hydrogel) creating an emulgel (Khullar *et al.*, 2011:117; Kute & Saudagar, 2013:368). Furthermore, emulgels can offer the many advantages of gels together with various other advantages, which are listed in Section 2.10.1.

Unfortunately, permeation through the skin is challenging when emulgels are used. This can be ascribed to the fact that they present with large droplets limiting the permeation and delivery process (Baibhav *et al.*, 2011:66).

#### **D.2.2.2 Nano-emulgel**

To overcome this permeation challenge, nano-emulgels are formulated providing a better skin permeability response (Eid *et al.*, 2014:1). Nano-emulgels are formulated similar to emulgels, except a nano-emulsion (instead of a coarse emulsion) is combined with a gelling agent (Eid *et al.*, 2014:1; Hyma *et al.*, 2014:4). Increased permeation can be ascribed to the fact that the nano-emulsion combined with the gelling agent consists of nano-sized droplets ranging between 20 – 200 nm (Abolmaali *et al.*, 2011:140).

It is also known that nano-emulsions are very advantageous, yet through the formulation of a nano-emulgel these advantages can further be increased (Basera *et al.*, 2015:1872; Eid *et al.*,

2014:1). Through this combination, advances can be made in better stability, increased viscosity, easy spreadability and removal, patient acceptability and most importantly, increased penetration of the skin can be achieved (Eid *et al.*, 2014:1). It has been said that a nano-emulgel, when in contact with the skin, works by releasing the API oil droplets from the gel network (Chellapa *et al.*, 2013:44). Controlled release of APIs, especially those with a short half-life, can also be achieved (Panwar *et al.*, 2011:337).

### **D.2.3 Suitable semi-solid dosage form**

Following the investigation of the different types of semi-solid dosage forms and whether they would be suitable to treat CTB lesions, the final semi-solid was chosen. It must be noted that the final formulation represents a delivery system with an adequate pH, API solubility, skin permeability effects, as well as being functional as a semi-solid form; it should also help to increase the viscosity (Woodruff, 2010:56). Since artemether can be viewed as lipophilic and CTB presents with ulcer-like lesions, a nano-emulgel appears to be the most appropriate semi-solid dosage form for the topical delivery of artemether. The formulated and optimised **(NE1)** was subsequently formulated as a nano-emulgel, which was compared to a conventional emulgel. Hence, the nano-emulgel and the conventional emulgel will both contain 0.4% (w/v) artemether and 2.5% (w/v) safflower oil. From this point, the nano-emulgel containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil will be referred to as **(NEG)** and the conventional emulgel containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil as **(CEG)**.

## **D.3 Excipients used to formulate a nano-emulgel and conventional emulgel**

### **D.3.1 General excipients used for nano-emulgel and conventional emulgel formulation**

A semi-solid is generally composed of an oil and water phase. The ingredients used therefore form the two phases respectively. Thus, general ingredients used to formulate a nano-emulgel or conventional emulgel can be classified as aqueous material, oils, emulsifiers, gelling agents, penetration enhancers, preservatives and antioxidants (Purushottam *et al.*, 2013:241-243). Some of these ingredients (used to formulate **(NEG)** and **(CEG)**) are discussed in the following section.

### **D.3.2 Excipients used in a nano-emulgel and a conventional emulgel**

Two semi-solids, the **(NEG)** and the **(CEG)**, were formulated using the ingredients as listed in Table D.1.

**Table D.1:** Excipients, function, suppliers and batch numbers as used during the formulation of a **(NEG)** and **(CEG)**

| Excipients                      | Function                           | Supplier                   | Batch number   |
|---------------------------------|------------------------------------|----------------------------|----------------|
| Nano-emulsion / coarse emulsion | Active ingredient                  | NA                         | NA             |
| Liquid paraffin                 | Emollient                          | Merck Chemicals            | 1035428        |
| Milli-Q <sup>®</sup> water      | Solvent                            | In house                   | Direct Pure UP |
| Span <sup>®</sup> 60            | Emulsifier                         | Fluka                      | 423065/1 41002 |
| Tween <sup>®</sup> 80           | Emulsifier                         | Merck                      | 1043695        |
| Xanthan gum                     | Thickening agent/<br>gelling agent | Warren Chem<br>Specialties | 4450902790     |

#### D.3.2.1 Oils: Liquid paraffin

Light liquid paraffin, also referred to as light mineral oil, is primarily acquired from the combination of saturated petroleum hydrocarbons and presents as a clear, odourless and highly viscous oil (Sheng, 2009:445-446). During the formulation of topical preparations, liquid paraffin can mainly be used as an emollient, lubricant or solvent (Sheng, 2009:445). As liquid paraffin also possesses moisturising effects, it can be viewed as a valuable excipient in pharmaceutical and cosmetic topical formulations (Mitsui, 1997:124; Sheng, 2009:446). In this study, liquid paraffin formed part of the oil phase constituents of the **(NEG)** and the **(CEG)**.

#### D.3.2.2 Emulsifiers

Emulsifiers, also referred to as surface acting agents, decrease interfacial tension that exists between two phases of the o/w interface (Setya *et al.*, 2014:2218). The use of emulsifiers can contribute to better stability properties over time (Baibhav *et al.*, 2011:68). Span<sup>®</sup> 60 and Tween<sup>®</sup> 80 were, respectively, the lipophilic and hydrophilic emulsifiers used during the formulation of the **(NEG)** and the **(CEG)** (Magdassi & Garti, 1999:156).

#### D.3.2.3 Gelling agent

Gelling agents can be viewed as a natural sourced compound with large molecular weights (Mahalingam *et al.*, 2008:293). Gelling agents can also be referred to as thickening agents, as they have the ability to enhance/better the viscosity of a formulation, i.e. nano-emulsion, which is achieved through the swelling characteristics that the gelling agent encompasses (Mahalingam *et al.*, 2008:293; Mitsui, 1997:138). They can also help to ensure stability of the product, since they can prevent the emulsified particles separating (Mitsui, 1997:138). Various gelling agents are available, however xanthan gum was utilised in this study.

#### D.3.2.3.1 Xanthan gum

Xanthan gum can be viewed as a polysaccharide gum and functioned as a gelling agent in this study (Shah & Singh, 2009:782). Xanthan gum is the result of the fermentation of glucose with the bacterium, *Xanthomonas campestris* and can be viewed as a microbial natural mucopolysaccharide, with pH stability and low temperature dependence (Mitsui 1997:139-140). Xanthan gum can be utilised in diverse sectors, ranging from oral to topical pharmaceutical formulations, from cosmetics to the food industry (Shah & Singh, 2009:782). It acts as an ideal excipient in formulations as it presents without toxicity and with excipient compatibility (Shah & Singh, 2009:782). It is immensely stable, providing maximum stability over a wide pH range (pH 4 – 10), at temperatures between 10 – 60 °C (Shah & Singh, 2009:783). Besides increasing the viscosity, xanthan gum can result in sustained release and also stabilise the formulation (Shah & Singh, 2009:782).

#### D.3.2.4 Water

A solvent, such as Milli-Q® water, makes up the largest part of a nano-emulgel or conventional emulgel. The solvent was mainly used during the formulation of the hydrogel base, together with the gelling agent, i.e. xanthan gum.

### D.4 Formulation of a nano-emulgel and a conventional emulgel

When designing a topical formulation, the following aspects need to be taken into consideration (Khullar *et al.*, 2011:119):

- \* the aim to be achieved,
- \* the target site or lesion type,
- \* suitable formulation type,
- \* the formulation and target compatibility and
- \* properties of the delivery system.

Once adequate knowledge was gained through the literature study, the formulation could be continued. Information about the API, the type of cutaneous condition and the delivery system intended to treat the condition was gathered. It was proposed that a nano-emulgel would be used to deliver the optimised nano-emulsion (**NE1**) to the skin. To determine the effect of a nano-emulgel, it was compared to a conventional emulgel. A (**NEG**) and a (**CEG**) were formulated and prepared through the method reported and used by Me N Naudé (2010:144). Minor modifications were made to the formulation and preparation method.

## D.5. Characterisation of the semi-solid forms

These two semi-solids, the **(NEG)** and the **(CEG)**, were characterised and subsequently used to perform *in vitro* diffusion studies (discussed in Appendix F). The characteristics of a semi-solid are indicative of its effectiveness in reaching the set aim/s. Therefore, evaluating and comparing these properties would help to determine which semi-solid could be regarded as optimal. The following characteristics of both semi-solids (**(NEG)** and **(CEG)**) were determined (discussed further in Appendix E):

- \* morphology,
- \* droplet size and distribution,
- \* pH,
- \* zeta potential and
- \* viscosity.

## D.6 Formulation method

### D.6.1 General method of formulation

The general method of formulating a nano-emulgel and a conventional emulgel is based on the following three steps (Baseri *et al.*, 2015:1877-1878):

1. Nano-emulsion or coarse emulsion, either as o/w or w/o, formulation;
2. Hydrogel base preparation by a thickening agent and an aqueous medium; and
3. Nano-emulsion or coarse emulsion incorporation into the gel base through continuous stirring.

### D.6.2 Formulation of the **(NEG)** and the **(CEG)**

The **(NEG)** was formulated as the main focus, while the **(CEG)** was formulated for comparison purposes of the two semi-solids. The general formula used for the formulation of the **(NEG)** and the **(CEG)** is outlined in Tables D.2 and D.3, respectively.

**Table D.2:** Formula of a **(NEG)** (100 ml)

| Phase              | Ingredient      | Function                       | Amount        |
|--------------------|-----------------|--------------------------------|---------------|
| <b>Oil phase</b>   | Liquid Paraffin | Emollient                      | 20.00 g       |
|                    | Span® 60        | Lipophilic surfactant          | 0.5 g         |
|                    | Tween® 80       | Hydrophilic surfactant         | 4.5 g         |
| <b>Water phase</b> | <b>(NE1)</b>    | Active ingredient              | 50 ml         |
|                    | Xanthan gum     | Thickening agent/gelling agent | 1.50 g        |
|                    | Milli-Q® water  | Solvent                        | Add to 100 ml |

**Table D.3:** Formula of a **(CEG)** (100 ml)

| Phase              | Ingredient                      | Function                       | Amount        |
|--------------------|---------------------------------|--------------------------------|---------------|
| <b>Oil phase</b>   | Liquid Paraffin                 | Emollient                      | 20.00 g       |
|                    | Span® 60                        | Lipophilic surfactant          | 0.5 g         |
|                    | Tween® 80                       | Hydrophilic surfactant         | 4.5 g         |
| <b>Water phase</b> | Coarse emulsion of <b>(NE1)</b> | Active ingredient              | 50 ml         |
|                    | Xanthan gum                     | Thickening agent/gelling agent | 1.50 g        |
|                    | Milli-Q® water                  | Solvent                        | Add to 100 ml |

### D.6.3 Formulation method of a **(NEG)** and a **(CEG)**

The three-step formulation method as used to formulate the **(NEG)** and the **(CEG)** in this study is listed in this section. The **(NEG)** and the **(CEG)** were formulated in the same way, the only difference was the type of emulsion that was incorporated (either a nano-emulsion or a coarse emulsion of the optimised **(NE1)**) (as seen in Table D.2 and D.3, respectively).

#### D.6.3.1 Formulation of the nano-emulsion or coarse emulsion

The first step in the formulation of the **(NEG)** and the **(CEG)** was to formulate the nano-emulsion or coarse emulsion. These dispersions contained the API, artemether, which was needed for topical delivery through a semi-solid. As artemether presented without an ideal solubility, it was decided (during pre-formulation) that an o/w dispersion would be formulated with the hope that the natural oil used would have sufficient solubility effects.

As discussed in Appendix B, an optimised nano-emulsion **(NE1)** was formulated. The method used to formulate the optimal nano-emulsion **(NE1)** was fully described in Section B.8.1.1 and the same method was applied to formulate the coarse emulsion (the emulsion prior to

ultrasonication is referred to as the coarse emulsion). Table D.4 represents the optimised formula used to formulate the optimised nano-emulsion **(NE1)**.

**Table D.4:** Formula used to formulate an optimised o/w nano-emulsion **(NE1)**

| Phase                        | Ingredient     | Function                             | Amount      |
|------------------------------|----------------|--------------------------------------|-------------|
| <b>Oil phase (phase A)</b>   | Artemether     | API                                  | 0.8 g       |
|                              | Safflower oil  | Natural oil and penetration enhancer | 5% (2.3 ml) |
|                              | Span® 60       | Lipophilic surfactant                | 0.5 g       |
| <b>Water phase (phase B)</b> | Tween® 80      | Hydrophilic surfactant               | 1.8 ml      |
|                              | Milli-Q® water | Solvent                              | 45.0 ml     |

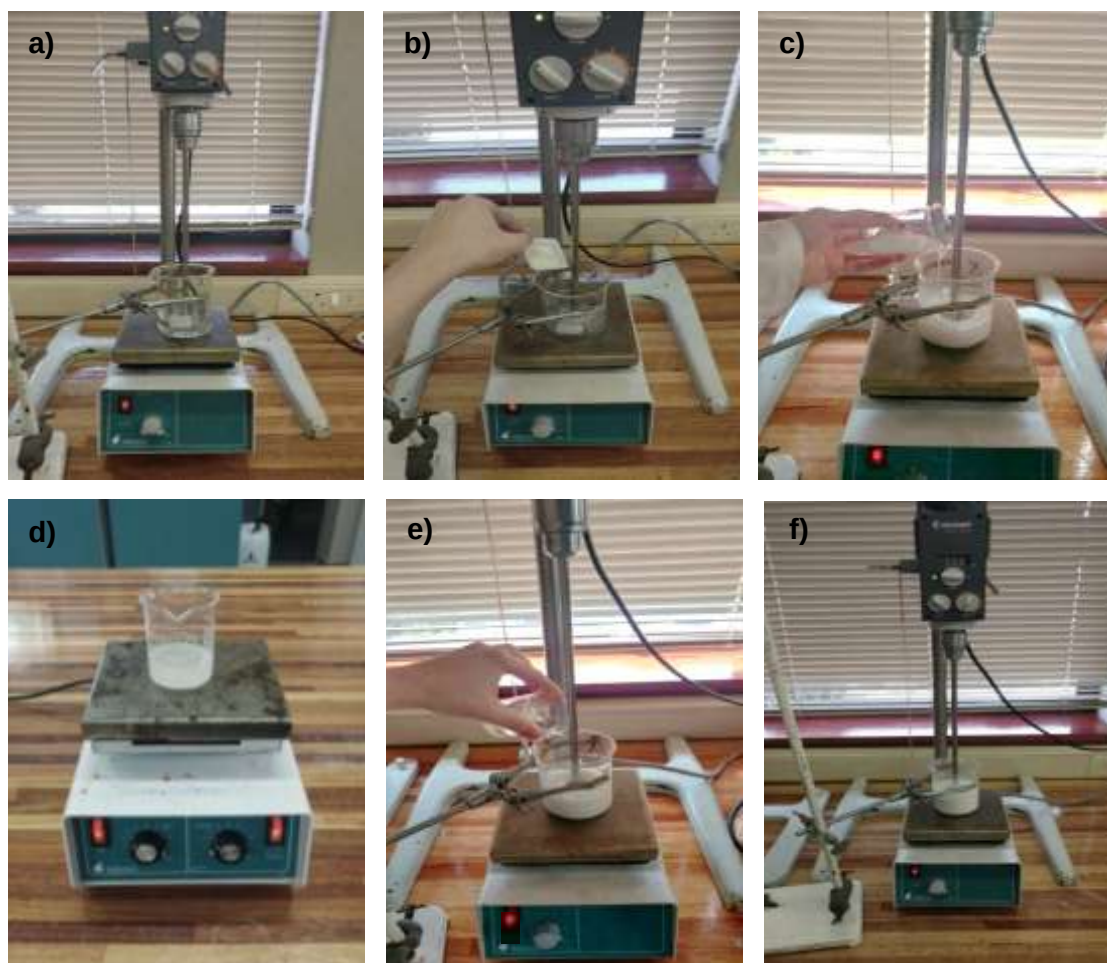
The nano-emulsion, or coarse emulsion, was formulated by weighing and measuring all the ingredients (as listed in Table D.4). The water phase (phase B) was prepared by placing the Milli-Q® water and Tween® 80 together in a glass beaker and placing it on a hot plate. A magnetic stirring rod was added to this phase to ensure continuous stirring until the Tween® 80 was completely dissolved. Once adequately mixed, the oil phase (phase A) was prepared by heating the Span® 60 and safflower oil together in a glass beaker, on a hot plate. A magnetic stirring rod was also added to the mixture to ensure continuous stirring. Once the Span® 60 was dissolved, the artemether was added and mixed until it was dissolved. Phase A was added to phase B by means of a syringe, resulting in a drop wise addition; the two phases were left to mix for 5 min. At this point, the mixture is referred to as the coarse emulsion. To produce the nano-emulsion, ultrasonication (a high-energy emulsification method) was applied to the coarse emulsion resulting in small droplets. In this study, ultrasonication was achieved through three 1 min interval sessions with an ultrasonic probe, where after it was placed in the ultrasonication bath for 15 min.

#### **D.6.3.2 Formulation of the (NEG) and the (CEG)**

The second step in the formulation process of the **(NEG)** and the **(CEG)** was to formulate the hydrogel base. The hydrogel base formed the water phase of the semi-solid. This phase was prepared by combining the Milli-Q® water, gelling agent (xanthan gum) and the nano-emulsion or the coarse emulsion. The amount and ingredients used can be found in Tables D.2 and D.3, respectively, for the **(NEG)** and the **(CEG)**.

The hydrogel base was formed by heating the required amount of Milli-Q® water to approximately 40 °C on a heating plate. The xanthan gum was added slowly to the heated water, whilst being homogenised at  $\pm 777$  rpm. Homogenisation was applied continuously to ensure the xanthan gum was completely dissolved and that the water and xanthan gum mixed

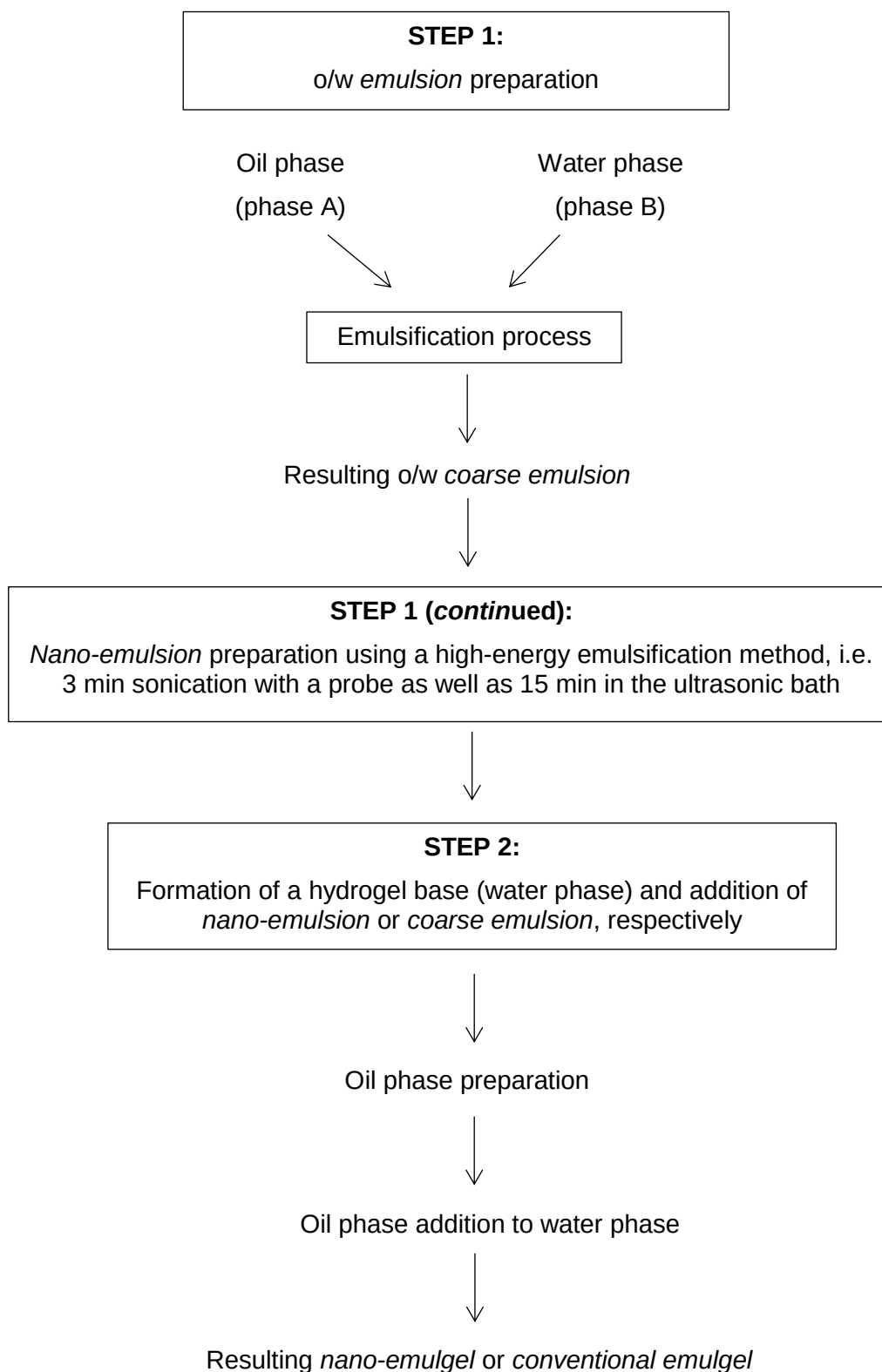
uniformly. Once a clear hydrogel base was formed, the nano-emulsion (or coarse emulsion) was added, respectively. The water, xanthan gum and nano-emulsion (or coarse emulsion) was homogenised for a further 5 min until adequately mixed.



**Figure D.1:** Method of formulation of the **(NEG)** and the **(CEG)**: a) water phase preparation; preheating of the water; b) addition of the gelling agent, xanthan gum, to the preheated water under homogenisation; c) the nano-emulsion or coarse emulsion was added to the hydrogel base; d) oil phase preparation: excipients mixed; e) addition of oil phase to water phase and f) final mixture of the two phases

The final step in the formulation process was to add the oil phase to the hydrogel base. The oil phase was prepared by combining the liquid paraffin, Span® 60 and Tween® 80. All the ingredients needed to form the oil phase were placed, with a magnetic stirring rod, in a glass beaker and then placed on a separate hot plate with magnetic stirring. This mixture was heated to approximately 80 °C. Once completely mixed and all of the excipients dissolved, the oil phase was added to the hydrogel, whilst still homogenising at  $\pm 777$  rpm. With the addition of the oil phase, the mixture becomes white in colour. The hot plate was then turned off and homogenisation continued until a temperature of approximately 40 °C was reached. The

mixture was left to cool at room temperature. Figures D.1 and D.2 serve as representation of the method of formulation of the **(NEG)** and the **(CEG)**.



**Figure D.2:** Diagrammatic representation of the formulation of the **(NEG)** and the **(CEG)**

### D.6.3.3 Outcome

Both semi-solids, i.e. the **(NEG)** and the **(CEG)**, presented as a white gel-like substance with a homogenous and smooth texture. It could be applied with ease to the skin, had a pleasant feel when applied and no oily residue was noted.

## D.7 Discussion and conclusion

When compared to other delivery systems, nano-emulgels and conventional emulgels present as some of the best semi-solid approaches for the topical delivery of lipophilic APIs due to the many advantages they present, e.g. to be non-greasy, easy spreadable, non-staining, patient acceptable, etc. (Kumar *et al.*, 2015:1182). As these semi-solids present with characteristics of a nano-emulsion or coarse emulsion, respectively, as well as a gel, the resulting characteristics needed to be determined.

This appendix described the preparation and formulation of two final semi-solid dosage forms of the optimised **(NE1)**. The **(NEG)** and the **(CEG)**, both containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil, were formulated during this study. Both semi-solids, the **(NEG)** and the **(CEG)**, were white of colour with a gel like consistency, which presented as not oily and with easy spreadability. These semi-solids therefore increased the low viscosity of the optimised o/w nano-emulsion **(NE1)**. Visually, the two formulated semi-solids presented ideal, yet it was important that their characteristics be determined. The characterisation of the two formulated semi-solids (**(NEG)** and **(CEG)**) are investigated and discussed further in Appendix E, which could aid in the determination of the most ideal semi-solid.

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# APPENDIX E:

## STABILITY AND CHARACTERISATION OF A NANO-EMULGEL AND EMULGEL CONTAINING ARTEMETHER AND SAFFLOWER OIL

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### E.1 Introduction

It is proposed that a more viscous topical formulation would be greatly advantageous and result in better patient compliance (Pund *et al.*, 2015:152; Williams, 2013:687). Appendix D indicated the thought process for choosing the ideal semi-solid dosage form to pose as novel treatment of CTB. Following this process, a gel type semi-solid, a nano-emulgel, was chosen as the most suitable semi-solid dosage form for the delivery of the optimised o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil (**NE1**) (as discussed in Appendix B and Appendix C).

This was to achieve the aim of formulating a novel CTB treatment through the topical delivery of artemether. Consequently, two semi-solids, i.e. the (**NEG**) and the (**CEG**), both containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil was formulated and characterised. The (**NEG**) was now the focus and was compared to a (**CEG**) in order to determine the success of the nano-emulgel as a topical drug delivery system.

Characterisation of a delivery system is of grave importance, as it provides information about whether a therapeutic effect would be achieved, whether the intended target, i.e. the skin (epidermal layer), can be reached, as well as whether the final semi-solid would be safe and suitable for the treatment of CTB lesions.

### E.2 Characterisation of (**NEG**) and (**CEG**)

The formulated semi-solid dosage forms of the optimised nano-emulsion (**NE1**), i.e. the (**NEG**) and the (**CEG**) were investigated. This was done to determine the characteristics of each semi-solid dosage form as a potential delivery system of (**NE1**). These characteristics included light microscopy, droplet size and distribution, pH, viscosity and zeta-potential. Visual examination was also done to determine stability of the semi-solids ((**NEG**) and (**CEG**)).

The characteristics of the (**NEG**) and the (**CEG**) depend largely on the dispersed droplets. Other factors influencing the characteristics include the interfacial tension between the two phases, the API's partition coefficient, as well as the viscosity of the formulation (Valentine, 2014:153). The release of the API can be greatly influenced by these characteristics (Valentine, 2014:153).

### E.2.1 Light microscopy

Light microscopy is an imaging technique used to investigate the droplets formed within a formulation, such as a semi-solid dosage form (Silva *et al.*, 2012:862). Morphology information provided by light microscopy can include aggregation condition, droplet size, shape etc. (Silva *et al.*, 2012:862). Light microscopy of the **(NEG)** and the **(CEG)** were performed using a Nikon Eclipse E4000 microscope, which was fitted with a Nikon DSFi1 camera (Nikon, Japan Linkam THMS600), equipped with a T95 LinkPad temperature controller (Surrey, ENG). Motic Images Plus software was used to capture the micrographs of the droplets as identified through light microscopy. A drop of each semi-solid (**(NEG)** and **(CEG)**) was placed on a microscope slide, respectively. It was covered with a 16 mm cover slip for safe evaluation through microscopy. Microscopy at 40x magnifications was used to investigate the morphology, more so the droplet size, of the semi-solid dosage forms.

### E.2.2 Droplet size and distribution

Droplet size and how the droplets are dispersed are very important characteristics of a formulation, since it can provide information about solubility, dissolution, performance (such as drug release and absorption) and most importantly, stability (Patravale *et al.*, 2004:833; Reddy *et al.*, 2013:88). The droplet size and the distribution of droplets throughout a formulation can be determined through a technique called PCS (Chime *et al.*, 2014:96; Patravale *et al.*, 2004:833). PCS is based on the measurement of light scattering variations caused by Brownian motion between the droplets (Chime *et al.*, 2014:96). The droplet size and charge of the droplet can influence the stability of the semi-solid greatly (Kumar & Rajeshwarrao, 2011:213). Measuring Pdl can provide information about the size distribution (homogeneity) of the droplets within the semi-solid (Chime *et al.*, 2014:97; Patravale *et al.*, 2004:833). Pdl therefore determines whether the droplets are monodispersed (narrow size distribution) or polydispersed (broad size distribution) throughout the semi-solid (Gaumet *et al.*, 2008:3-4; Gaur *et al.*, 2014:36).

Both, the average droplet size and the average Pdl, was measured by means of a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK). In order to reduce the interaction between droplets, diluted samples were prepared (Gaumet *et al.*, 2008:7). One drop of each of the semi-solid dosage forms was placed in a polytop containing 10 ml Milli-Q® water resulting in a dilution. This was done for the **(NEG)** and the **(CEG)**, respectively. Readings on the Malvern Zetasizer Nano ZS 2000 were done in triplicate and the average determined.

### E.2.3 pH

As the semi-solid dosage form is aimed at localised application of an API to the skin, hence resulting in direct contact, it is of great importance that the semi-solid does not cause any irritation (Kaur *et al.*, 2013:202). Paudel *et al.* (2010:118) stated that a topical formulation, i.e. semi-solid, can present with the ability to change the pH of the skin.

The pH of the semi-solid dosage forms, i.e. the **(NEG)** and the **(CEG)**, was measured using a digital Mettler Toledo<sup>®</sup> pH meter (Mettler Toledo, CU). The pH meter was fitted with a Mettler Toledo<sup>®</sup> InLab<sup>®</sup> 410 electrode (Mettler Toledo, CU). The pH was consequently measured by placing the electrode within the semi-solids, respectively; readings were done in triplicate. The average pH of each semi-solid (**(NEG)** and **(CEG)**) was evaluated.

### E.2.4 Viscosity

The viscosity of the semi-solids, i.e. the **(NEG)** and the **(CEG)**, was measured by means of a Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA), which was fitted to a thermostatic water bath (controlled at  $\pm 25$  °C). A T-bar SC4-25 spindle was fitted to the viscometer to determine the viscosity of the viscous semi-solids. One hour prior to the conducting the experiment, samples of the two semi-solids (**(NEG)** and **(CEG)**) were placed into the  $\pm 25$  °C water bath to acclimatise to the required temperature. The sample chamber was filled with approximately 16.1 ml of the semi-solid, respectively, and was consequently fitted to the small sample adapter of the viscometer. To determine the viscosity of the semi-solids, the cylindrical T-bar spindle was rotated at 200 rpm. This was done for the **(NEG)** and for the **(CEG)**, respectively. The viscosity calculations were conducted by means of Rheocalc T 1.2.19 software and were read in terms of centipoise (cP). Multipoint readings at 10 s intervals were taken for 1 min and the average viscosity was determined.

### E.2.5 Zeta-potential

Zeta-potential can be defined as the electrokinetic potential, i.e. the surface charge within a colloidal system (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860; Thakur *et al.*, 2012:223). This surface charge can also be determined through PCS (Gaur *et al.*, 2014:40). As mentioned PCS evaluates light fluctuations between the dispersed droplets (Gaur *et al.*, 2014:40). Properties such as stability, drug entrapment, as well as the manner of skin interaction can be described by measuring the zeta-potential (Bhatt & Madhav, 2011:2296; Klang *et al.*, 2015:262; Silva *et al.*, 2012:860).

The zeta-potential of the **(NEG)** and the **(CEG)** were measured using a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments, Worcestershire, UK). A Malvern Zetasizer is based on PCS (Gaur *et al.*, 2014:40). Dilutions were made of the respective semi-solid by placing one drop of

the **(NEG)** or the **(CEG)** into a corresponding polytop containing 10 ml Milli-Q® water. The dilution was stirred to disperse the more viscous semi-solids throughout the water. Using a syringe, 2 ml of the diluted sample was injected into a clear disposable zeta cell (DTS1070 folded capillary cell), making sure no bubbles were present and that the cell was filled with an adequate amount of dispersion. On the day of sample preparation, the zeta-potential of the **(NEG)** and the **(CEG)** were measured at  $\pm 25$  °C and at an angle of 90°, in triplicate.

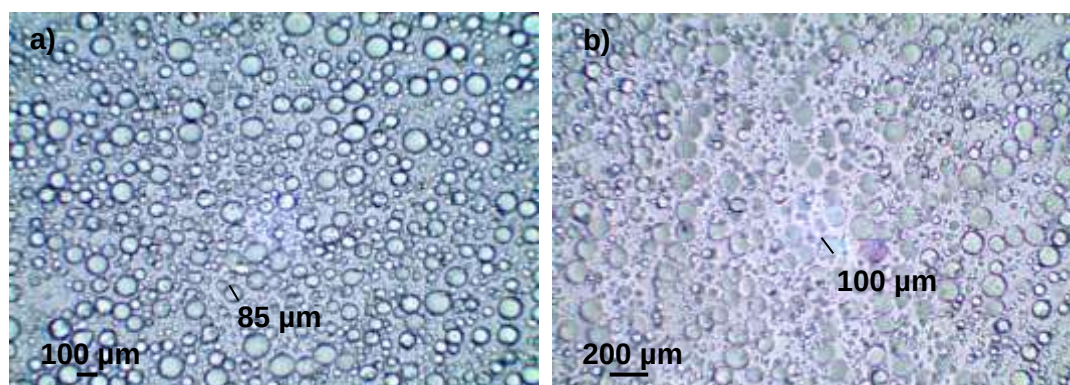
## E.2.6 Visual examination

A visual examination of the semi-solid dosage forms, i.e. the **(NEG)** and the **(CEG)**, was conducted to evaluate the colour, homogeneity, spreadability, consistency and stability of the semi-solid dosage forms.

## E.3 Results and discussion

### E.3.1 Light microscopy

Figure E.1 represents micrographs of the **(NEG)** (Figure E.1.a) and the **(CEG)** (Figure E.1.b) as captured. Large droplets were visible for both micrographs (Figures E.1.a and E.1.b). The light microscopy results correlate with the droplet size findings as measured through the Malvern Zetasizer (discussed in Section E.3.2). A droplet identified in Figure E.1.a was measured as  $\pm 85$   $\mu\text{m}$ , compared to a droplet in Figure E.1.b, which was measured as  $\pm 100$   $\mu\text{m}$ . This confirms that the **(NEG)** presents with smaller droplets than those of the **(CEG)** (Baibhav *et al.*, 2013:369).



**Figure E.1:** Micrographs of the formulated semi-solids: a) the **(NEG)** and b) the **(CEG)**, as captured through light microscopy

### E.3.2 Droplet size and distribution

Literature proposed that emulgels are composed of large droplets, which provide a challenge during skin permeation (Baibhav *et al.*, 2011:66). Hence, since skin permeation is needed, the

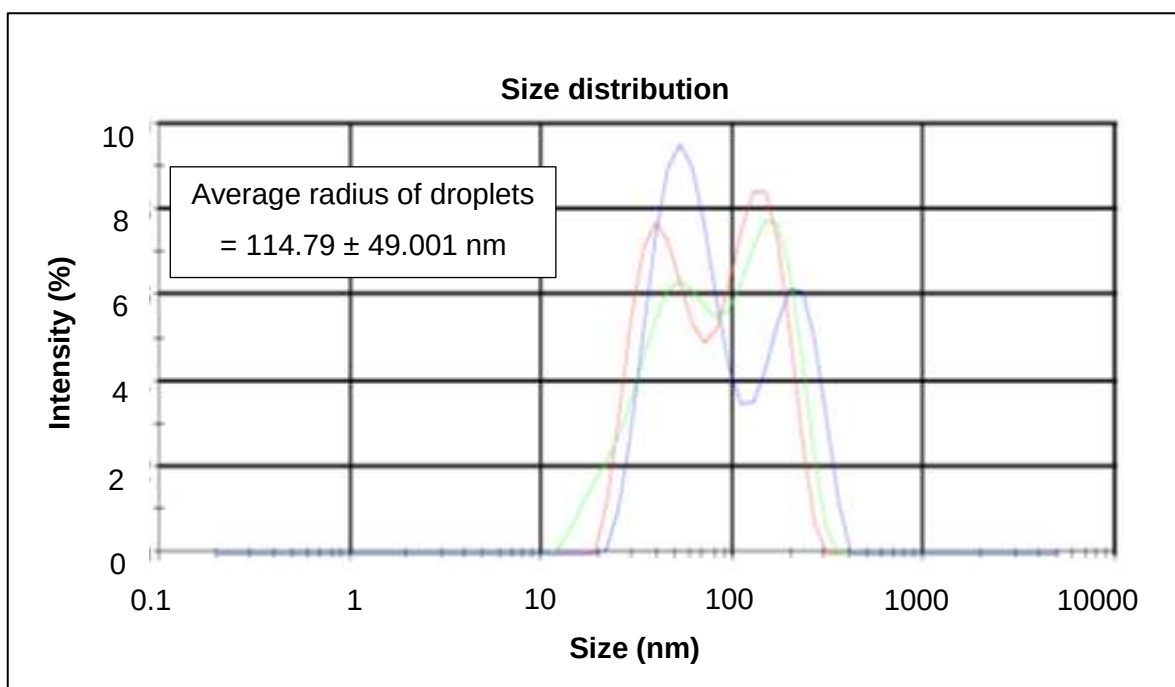
smaller droplets of the nano-emulgel would be ideal. The average droplet size of the **(NEG)** and the **(CEG)** is tabulated in Table E.1. The **(NEG)** presented with an average droplet size of  $229.587 \pm 98.002$  nm and the **(CEG)** presented with an average droplet size of  $401.320 \pm 13.262$  nm. It can therefore be said that the average droplet size of the **(CEG)** was almost double the size of the **(NEG)**. This was a clear indication that the **(NEG)** presented with much smaller droplets than the **(CEG)**. These measurements correlate with that found in literature.

The dispersity of the droplets throughout the aqueous phase of the semi-solid is just as important to determine and can be best described by measuring the Pdl (Shakeel *et al.*, 2007:E6). Criteria for an acceptable Pdl is measured on a scale of 0 – 1, where 0 indicates a monodispersed and ideal formulation and 1 indicates a polydispersed formulation (Gaumet *et al.*, 2008:5; Shaw, 2016). The Pdl as measured for the **(NEG)** and the **(CEG)** is tabulated in Table E.1. The average Pdl of the **(NEG)** and **(CEG)** was  $0.44 \pm 0.08$  and  $1.00 \pm 0.00$ , respectively. According to the acceptable criteria, the Pdl of the **(CEG)** can be viewed as not ideal, as it indicated a largely polydispersed formulation. This Pdl of the **(CEG)**, together with its large average droplet size, proposed a problem during the skin permeation process. The Pdl of the **(NEG)** was also not ideal, according to acceptable criteria, but was more acceptable than the **(CEG)**. As the Pdl measurement can also provide information about the stability of the semi-solid dosage form, it can therefore be said that the **(CEG)** would become relatively unstable much quicker than the **(NEG)**. This is ascribed to the **(CEG)** measuring with a Pdl of  $1.00 \pm 0.00$  compared to the lower Pdl of the **(NEG)**, consequently, results indicate the **(NEG)** presented more ideal than the **(CEG)**.

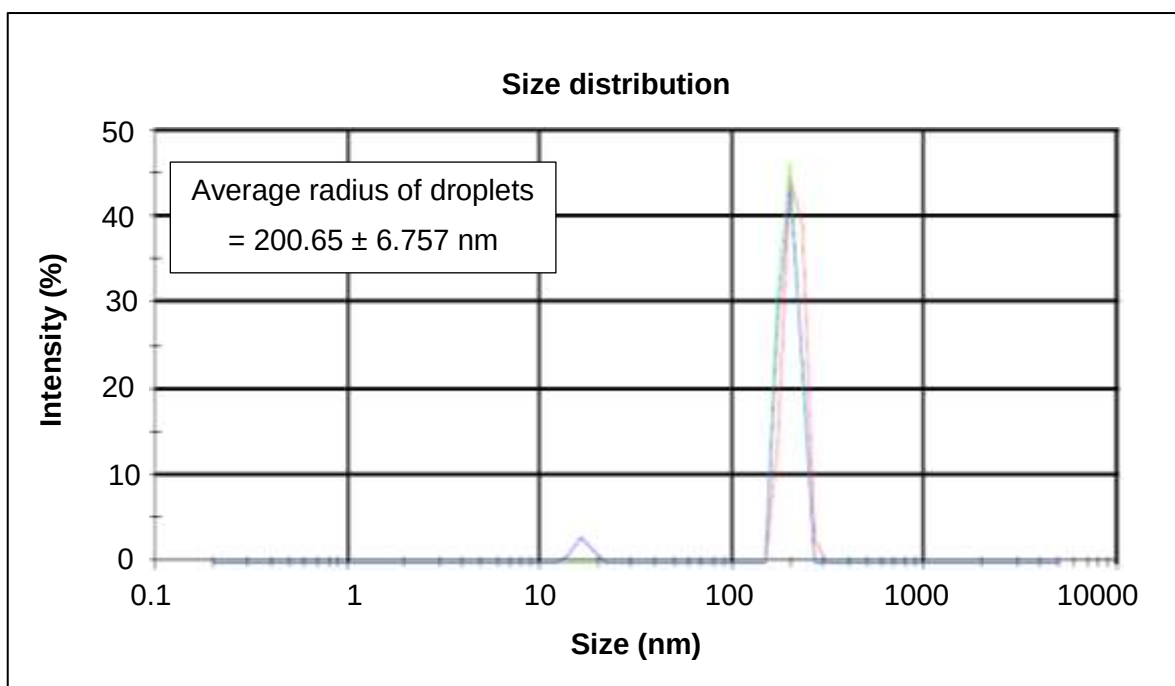
**Table E.1:** Comparison of the average droplet size, as well as the polydispersity index of the **(NEG)** and the **(CEG)**

|              | Average diameter (nm) | Average polydispersity index (Pdl) |
|--------------|-----------------------|------------------------------------|
| <b>(NEG)</b> | $229.587 \pm 98.002$  | $0.44 \pm 0.08$                    |
| <b>(CEG)</b> | $401.320 \pm 13.262$  | $1.00 \pm 0.00$                    |

Figures E.2 and E.3 are representative of the cumulative readings taken of the average droplet size of the **(NEG)** and the **(CEG)**, respectively. These figures indicate the measured radius of the average droplet size, as recorded in Table E.3, for both the semi-solids, respectively.



**Figure E.2:** Average droplet size (nm) of the **(NEG)** measured per droplet radius



**Figure E.3:** Average droplet size (nm) of the **(CEG)** measured per droplet radius

### E.3.3 pH

Acceptable pH for a topical formulation can be viewed as a pH ranging between 3 and 9 (anything below or above this range can irritate the skin) (Barry, 2002:512). From Table E.2, the average pH of the **(NEG)** and the **(CEG)** is given. The average pH of the **(NEG)** and **(CEG)** was  $5.14 \pm 0.02$  and  $5.86 \pm 0.02$ , respectively. It can therefore be said that both of the semi-

solids ((**NEG**) and (**CEG**)) fell within these acceptable pH ranges, consequently not posing any irritation when applied to the skin. It can be noted from Table E.2 that the (**NEG**) appeared to be more acidic than the (**CEG**).

**Table E.2:** Average pH reading of the (**NEG**) and the (**CEG**)

|              | pH (reading 1) | pH (reading 2) | pH (reading 3) | Average pH  |
|--------------|----------------|----------------|----------------|-------------|
| <b>(NEG)</b> | 5.16           | 5.13           | 5.12           | 5.14 ± 0.02 |
| <b>(CEG)</b> | 5.87           | 5.84           | 5.86           | 5.86 ± 0.02 |

### E.3.4 Viscosity

The average viscosity of the (**NEG**) and the (**CEG**) containing 0.4% (w/v) artemether and 2.5% (w/v) safflower oil can be seen from Table E.3. The viscosity of both of the semi-solids was measured at a torque of  $35.01 \pm 0.113\%$ , a temperature of  $25.35 \pm 0.000$  °C and a speed of 200.00 rpm. The average viscosity of the (**NEG**) and (**CEG**) was  $795.87 \pm 3.402$  cP and  $884.53 \pm 2.013$  cP, respectively. The increased viscosity could be ascribed to the delivery system. It has been proposed that droplet size has an enormous effect on the viscosity of a formulation (Malkin *et al.*, 2004:585). Therefore, the higher viscosity of the (**CEG**) could be due to it containing larger droplets compared to the (**NEG**). Primarily the aim was reached, as the viscosity of the optimised o/w nano-emulsion ((**NE1**)), as described in Appendix C, was increased.

**Table E.3:** Viscosity readings of the (**NEG**) and the (**CEG**)

|              | Reading         | Viscosity (cP)        | Torque (%)           | Temperature (°C)     | Speed (RPM)           |
|--------------|-----------------|-----------------------|----------------------|----------------------|-----------------------|
| <b>(NEG)</b> | <b>1</b>        | 792.400               | 33.020               | 25.400               | 200.000               |
|              | <b>2</b>        | 796.000               | 33.170               | 25.400               | 200.000               |
|              | <b>3</b>        | 798.950               | 33.300               | 25.400               | 200.000               |
|              | <b>Averages</b> | <b>795.87 ± 3.402</b> | <b>33.16 ± 0.142</b> | <b>25.40 ± 0.000</b> | <b>200.00 ± 0.000</b> |
| <b>(CEG)</b> | <b>1</b>        | 886.400               | 36.930               | 25.300               | 200.000               |
|              | <b>2</b>        | 884.800               | 36.870               | 25.300               | 200.000               |
|              | <b>3</b>        | 882.400               | 36.770               | 25.300               | 200.000               |
|              | <b>Averages</b> | <b>884.53 ± 2.013</b> | <b>36.86 ± 0.084</b> | <b>25.30 ± 0.000</b> | <b>200.00 ± 0.000</b> |

### E.3.5 Zeta-potential

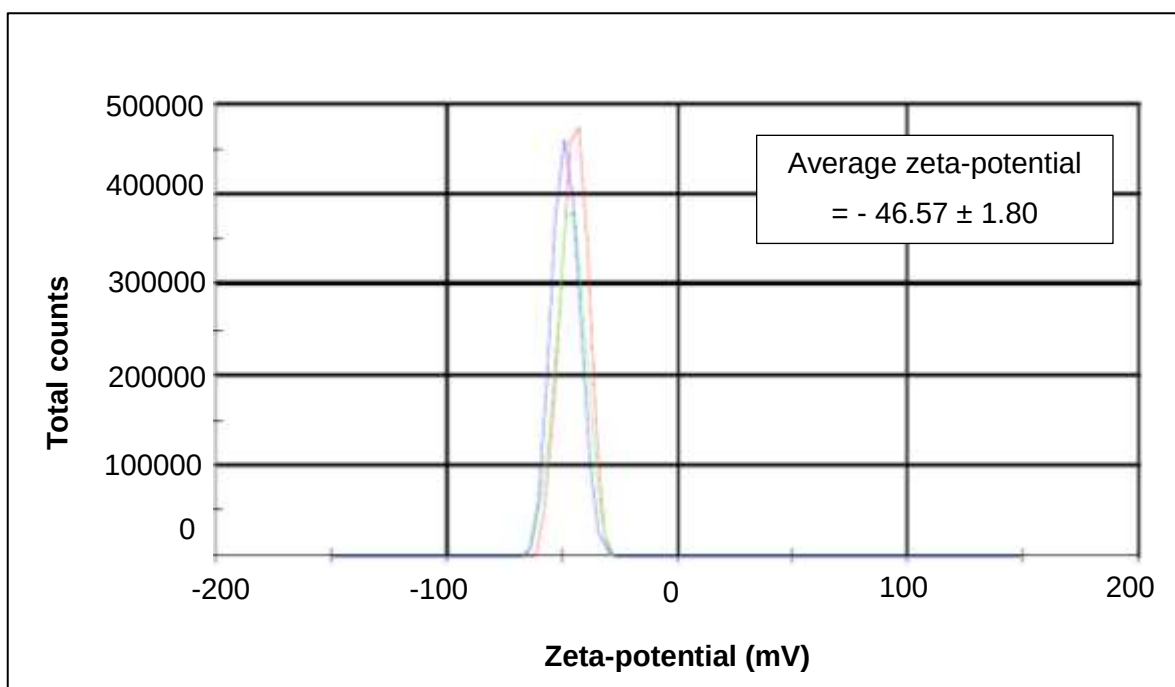
An acceptable zeta-potential for skin diffusion can be viewed as a zeta-potential lower than - 30 mV (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860). It is proposed that the skin generally presents with a surface which is negatively charged and that negatively charged molecules

could lead to better diffusion and consequently, increased flux of the APIs (Duangjit *et al.*, 2011:6; Sinico *et al.*, 2005:129). Eid *et al.* (2014:2) stated that highly negative charges can also indicate good stability over time. Table E.4 represents the average zeta-potential readings of the **(NEG)** and the **(CEG)**, at room temperature ( $\pm 25^{\circ}\text{C}$ ). The average zeta-potential of the **(NEG)** and the **(CEG)** was  $-46.57 \pm 1.80$  mV and  $-43.90 \pm 1.20$  mV, respectively. The average measured zeta-potential for both semi-solids (**(NEG)** and **(CEG)**) were highly negative and as a result, the readings fell within the acceptable ranges. Both semi-solids could therefore be ideal for topical delivery and in turn, result in successful permeation through the skin. Hence, it can be proposed that both semi-solids (**(NEG)** and **(CEG)**) would remain relatively stable over time and present with less aggregation and coagulation.

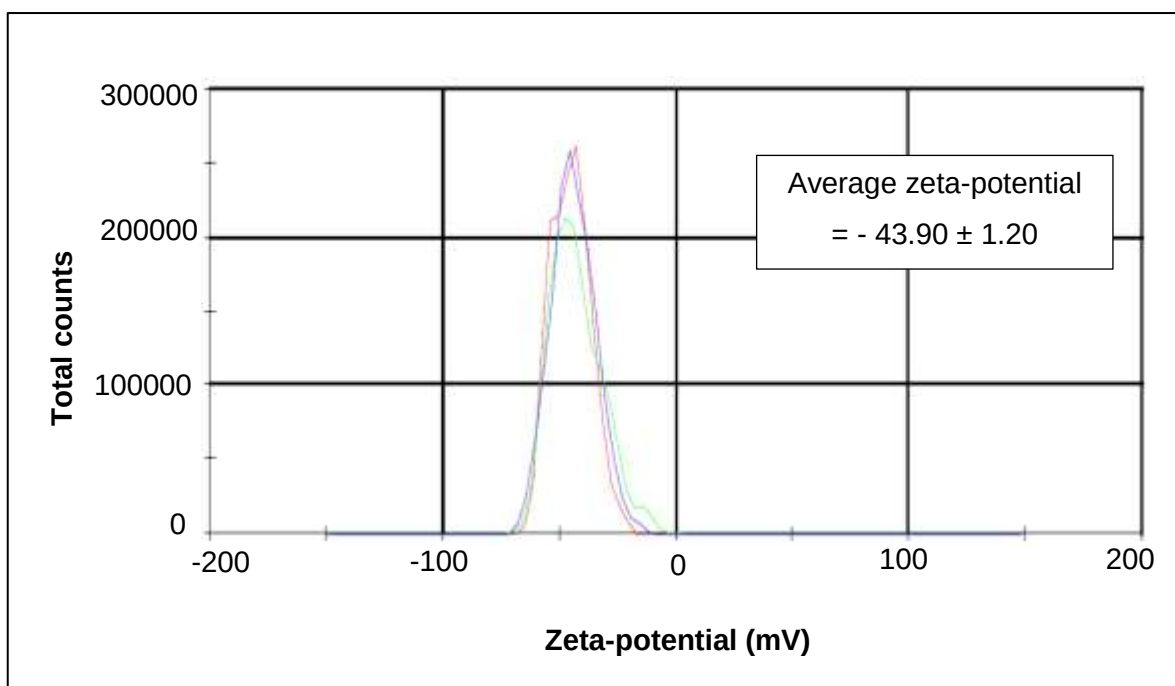
**Table E.4:** Average zeta-potential of the **(NEG)** and the **(CEG)**

|              | Zeta-potential<br>(reading 1) | Zeta-potential<br>(reading 2) | Zeta-potential<br>(reading 3) | Average zeta-<br>potential (mV) |
|--------------|-------------------------------|-------------------------------|-------------------------------|---------------------------------|
| <b>(NEG)</b> | - 44.80                       | - 46.50                       | - 48.40                       | $-46.57 \pm 1.80$               |
| <b>(CEG)</b> | - 45.10                       | - 42.70                       | - 43.90                       | $-43.90 \pm 1.20$               |

Figures E.4 and E.5 represent Table E.4 graphically and from these, it can be noted that both semi-solids, the **(NEG)** and the **(CEG)**, presented with negative zeta-potential values. An isoelectric point, zero (at 0 mV), indicates the most unstable formulation thus, when a curve is further away from zero, either positive or negative, the zeta-potential can be viewed as ideal (Paderborn University, 2016). When the graphs on Figures E.4 and E.5 are compared, it can be seen that both figures present with similar profiles, all the curves are symmetrical and follow the same profile; these similar curve profiles could indicate a narrow distribution of readings. The three readings represented in both figures average close to - 50 mV.



**Figure E.4:** Average zeta potential (mV) of the **(NEG)**



**Figure E.5:** Average zeta-potential (mV) of the **(CEG)**

### E.3.6 Visual examination

The freshly formulated **(NEG)** and **(CEG)** were examined visually. The **(NEG)** is represented in Figures E.6.a and E.6.b, whilst the **(CEG)** is represented in Figures E.6.c and E.6.d. From these images, it can be seen that both the **(NEG)** and the **(CEG)** presented as a white, smooth semi-solid, gel-like formulation. Both presented homogenous with no visible oil droplets, phase

separation, sedimentation or undissolved excipients. Semi-solid dosage forms are generally viscous delivery systems and these semi-solid forms presented rather viscous, consequently a state between a solid and a liquid was achieved.



**Figure E.6:** Images of the semi-solids: a) top view of the **(NEG)**; b) side view of the **(NEG)**; c) top view of the **(CEG)** and d) side view of the **(CEG)**

### E.3 Conclusion

Through the investigation and evaluation of the characteristics of the semi-solid dosage forms, i.e. the **(NEG)** in comparison to the **(CEG)**, valuable information was gained and helped draw conclusions about the more advantageous semi-solid dosage form. The characteristics investigated of the **(NEG)** and the **(CEG)** are summarised in Table E.5.

**Table E.5:** Summary of the characteristics of the **(NEG)** in comparison to the **(CEG)**

| Characteristics determined | (NEG)                | (CEG)                |
|----------------------------|----------------------|----------------------|
| Droplet size (nm)          | $229.587 \pm 98.002$ | $401.320 \pm 13.262$ |
| Pdl                        | $0.440 \pm 0.080$    | $1.000 \pm 0.000$    |
| pH                         | $5.140 \pm 0.020$    | $5.860 \pm 0.020$    |
| Viscosity (cP)             | $795.870 \pm 3.402$  | $884.530 \pm 2.013$  |
| Zeta potential (mV)        | $-46.570 \pm 1.800$  | $-43.900 \pm 1.200$  |

The two formulated semi-solids needed to meet certain characteristics to result in successful drug delivery (Panwar *et al.*, 2011:334). Characteristics such as the morphology, droplet size, Pdl, pH, viscosity and the zeta-potential of the **(NEG)** and the **(CEG)** were investigated.

The average droplet size of the semi-solid dosage forms was measured through PCS. This confirmed both semi-solids, **(NEG)** and **(CEG)**, produced nano-sized droplets, yet the larger droplet size of **(CEG)** presented great difficulties during skin permeation. An acceptable Pdl is measured close to 0, indicating a monodispersed formulation which could remain stable (Gaumet *et al.*, 2008:5; Shaw, 2016). The Pdl of the two semi-solids (**(NEG)** and **(CEG)**) were measured as relatively polydispersed, yet the **(NEG)** posed less polydispersed. Consequently, the **(NEG)** presented as ideal compared to **(CEG)**.

Both semi-solids, i.e. the **(NEG)** and the **(CEG)**, had adequate pH values as both fell within the accepted pH levels (pH of 3 – 9). This is important as the semi-solid is mainly formulated to be applied to the skin and to treat a localised skin disorder, hence the two semi-solid dosage forms should present a pH that would not influence the integrity of the skin. A clear effect of the droplet size was also seen on the measured viscosity of each semi-solid. The **(NEG)** and the **(CEG)** measured as having a large average viscosity, which indicated a completely viscous formulation. Consequently, this met the aim of this section, since the aim was to provide an easier application of the optimised o/w nano-emulsion **(NE1)**.

For ideal and effective skin permeation and stability over time, a negative zeta-potential was needed. The **(NEG)** and the **(CEG)** measured with highly negative zeta-potential values, yet the **(NEG)** presented with a more negative value, making it the ideal formulation. Hence, it can be proposed that both semi-solid dosage forms could result in effective permeation and remain relatively stable over time.

Following the comparison of the characteristics of the two semi-solids, i.e. the **(NEG)** and the **(CEG)**, it was concluded as to which semi-solid presented ideal for topical delivery. In literature, it has been stated that nano-emulgels are more advantageous than conventional emulgels, especially in particle size. The experimentally determined zeta-potential, droplet size and viscosity of the two semi-solids correlate with that found in literature. Consequently, successful skin permeation and therefore drug delivery could possibly be achieved easier through the **(NEG)** rather than the **(CEG)**, due to the aforementioned dispersion's characteristics.

Following this characterisation, both semi-solids were investigated in terms of topical delivery through *in vitro* diffusion studies (Appendix F).

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# APPENDIX F:

## FRANZ CELL DIFFUSION STUDIES OF AN O/W NANO-EMULSION AS WELL AS SEMI-SOLID DOSAGE FORMS CONTAINING ARTEMETHER AND SAFFLOWER OIL

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### F.1 Introduction

The main purpose of formulating a topical formulation is to result in the containment of the API within the skin (Williams, 2013:676). Although the topical route provides many benefits, the skin itself presents as an important barrier. Therefore, the skin's intricate structure creates a great disadvantage during the API penetration and during the release process of topical drug delivery (Williams, 2013:677). When studying the composition of the skin, it is noticed that this multi-layered structure consists of three main layers (Bouwstra & Poncet, 2006:2081; Foldvari, 2000:417; Menon, 2002:S3-S4; Ng & Lau, 2015:4; Williams, 2013:677-678). These layers can influence and potentially limit the permeation of APIs, since a sequence of barriers is produced, especially by the outermost layer, namely the stratum corneum (Kute & Saudagar, 2013:368; Williams, 2013:677). The barrier posed by the stratum corneum can be ascribed to it being the least permeable layer, arranged as a scaffold-like matrix surrounded by unique, intercellular lipid bilayers (El Maghraby *et al.*, 2008:2004; Jepps *et al.*, 2013:154; Venus *et al.*, 2011:471; Williams, 2013:677). It is the stratum corneum lipids, found in these lipid bilayers, which are responsible for the control and regulation of the movement of APIs through the skin (Williams, 2003:10; Williams, 2013:677). As a result, the stratum corneum acts as drug flux regulator and a rate-limiting barrier since it influences the amount of API that can cross the skin layers (El Maghraby *et al.*, 2008:204; Williams, 2003:28). For the most part, APIs encounter the stratum corneum first during the topical permeation process, followed by various partitioning and permeation steps (Jepps *et al.*, 2013:153; Wiechers, 2008:7).

APIs, with ideal physicochemical properties, are required to achieve skin permeation and absorption, since the skin limits the extent of permeation of APIs (Williams, 2013:680). Not only are the physicochemical properties of the selected API important, but the delivery system selected to deliver the API is just as important (Weiss, 2011:471). Taking both the properties of the skin and the API into consideration, a nano-emulsion was chosen as an appropriate delivery system. A two-phase dispersed nano-emulsion creates both hydrophilic and lipophilic characteristics, which can be used to deliver APIs either to the stratum corneum or to the underlying layers (Gaur *et al.*, 2014:37). To improve the application of the nano-emulsion to the skin, two semi-solid dosage forms were also formulated.

Forming part of topical and transdermal delivery evaluation are *in vitro* diffusion studies (Wiechers, 2008:23; Williams, 2013:683). During this study, *in vitro* membrane release and skin diffusion studies, i.e. the vertical Franz cell method, were conducted (Wiechers, 2008:23; Williams, 2013:683). Membrane studies were performed prior to skin diffusion studies to evaluate if artemether was released from the **(NE1)**, the **(NEG)** and the **(CEG)**, respectively. Skin diffusion studies and tape stripping followed to determine whether any transdermal and/or topical delivery have been achieved, respectively, for all three test formulations.

## F.2 Methods

### F.2.1 HPLC analysis of artemether samples

To quantify artemether present in the samples (collected during *in vitro* diffusion studies) an HPLC analytical method was validated under the supervision and with the guidance of Prof JL du Preez, at the Analytical Technology Laboratory of the North-West University (NWU), Potchefstroom, RSA. Consequently, this HPLC method for artemether was successfully validated (as fully described in Appendix A) and found to be sensitive, responsive and reliable for the quantification of the artemether concentration in the **(NE1)**, the **(NEG)** and the **(CEG)**, as investigated during *in vitro* diffusion studies.

The HPLC analysis of the samples, gathered from the Franz cell receptor compartment, was performed using an Agilent® 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with an Agilent® 1100 gradient pump and with a UV detector together with an autosampler injection mechanism fitted to the system. Chromatograms were analysed using ChemStation Rev. A.10.02 acquisition and analysis software. A Venusil® XBP C<sub>18</sub> (2) reverse phase column (100 Å, 150 x 4.6 mm) with a particle size of 5 µm was used (Agela Technologies, Newark, DE). A laboratory environment with a controlled temperature of 25 °C was maintained throughout. Specific chromatographic conditions are shown in Table F.1.

**Table F.1:** HPLC chromatographic conditions as used during the determination of the concentration of artemether in the receptor phase, as well as in the skin layers

|                                  |        |
|----------------------------------|--------|
| <b>Injection volume (µl)</b>     | 50.00  |
| <b>Detection wavelength (nm)</b> | 216.00 |
| <b>Flow rate (ml/min)</b>        | 1.00   |
| <b>Retention time (min)</b>      | ± 9.35 |
| <b>Run time (min)</b>            | 15.00  |

The mobile phase used consisted of 70 volumes (70%) of HPLC grade ACN and 30 volumes (30%) of Milli-Q® water (Milli-Q® Academic water purification system, Merck-Millipore, Midrand,

RSA). Before using the mobile phase, it was filtered through a 0.45 µm nylon membrane filter (Agela Technologies, Newark, DE) and degassed. With each analysis, a standard solution was prepared by dissolving 5.00 mg artemether in 25 ml mobile phase. This standard solution was injected at different injection volumes (2.5, 5.0, 7.5, 10.0, 12.5, 25.0 and 50.0 µl) in order to produce a standard curve for that analysis.

## **F.2.2 Physicochemical properties of artemether**

### **F.2.2.1 Aqueous solubility**

Solubility is a very important physicochemical property of a topical API and it has been proposed that a direct correlation between solubility and permeation exists (Encyclopaedia Britannica, 2016). Consequently, the aqueous solubility of artemether was investigated in order to determine how soluble artemether was in water and in phosphate buffer solution (PBS; pH 7.4). The aqueous solubility of artemether was determined according to the following steps. Prior to sample preparation the water bath was preheated to 32 °C (*in vivo* human conditions) (Williams, 2013:685). Six clean test tubes were taken and three were filled with 3 ml Milli-Q® water by using a micropipette; the other three were filled with 3 ml of the prepared PBS (pH 7.4). A saturated solution was created by adding an excess amount of artemether to each of the test tubes; which was regularly checked to ensure saturation throughout the aqueous solubility evaluation. These test tubes were placed into the preheated water bath (32 °C) for 24 h with continuous shaking. After 24 h, the test tubes were removed and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into an HPLC vial. The samples were analysed in duplicate by HPLC.

A standard solution was prepared by weighing off 5.06 mg artemether that was dissolved in a clean 100 ml volumetric flask containing an amount of mobile phase and was thereafter made up to volume with the same mobile phase. An amount of the standard solution was placed in an HPLC vial and analysed by means of HPLC, in duplicate, at different injection volumes (1.0; 2.5; 5.0; 10.0; 15.0 and 20.0 µl) in order to obtain a standard curve.

### **F.2.2.2 Octanol buffer distribution coefficient**

The octanol buffer distribution coefficient (log D) was attained in order to determine the solubility of artemether in PBS (pH 7.4) and *n*-octanol. This was completed to determine in which phase (either the hydrophilic or the lipophilic phase) artemether would be better soluble. PBS (pH 7.4) and *n*-octanol, in equal volumes, were equilibrated for 24 h to ensure these two phases co-saturated.

A separating funnel was used to separate the two co-saturated solutions. The top layer in the separating funnel represented the octanol phase, whilst the bottom layer was the PBS (pH 7.4).

After separation, 1.00 g of artemether was weighed and placed in a beaker containing 20 ml of the pre-saturated octanol, thereafter, 3 ml of the pre-saturated octanol/artemether solution was placed in three different test tubes and to this an equal amount of pre-saturated PBS (pH 7.4) was added, respectively. The test tubes were placed into a shaker water bath overnight at 32 °C. The test tubes were then removed and 1 ml of the octanol phase (top layer) was taken using a micropipette and placed into a clean 10 ml volumetric flask, which was made up to volume with methanol. This was done for all three test tubes containing the octanol phase in order to dilute the octanol to be safe for HPLC injection. An amount was then taken from this solution and placed into an HPLC vial. The second phase (PBS (pH 7.4)) of each tube was also placed into an HPLC vial, without dilution. All the vials were analysed and the concentration of artemether in each phase determined by means of HPLC.

A standard solution in a 100 ml volumetric flask was also prepared by dissolving 5.10 mg of artemether in 100 ml mobile phase. The standard solution was injected in duplicate onto the HPLC at different injection volumes (5, 10, 20, 30, 40, 50 µl) in order to obtain a standard curve.

The log D was then determined in terms of the ratio of the API concentration in the *n*-octanol (lipophilic) phase to the API concentration in the buffer (PBS) phase. Calculations to determine log D can be done through the application of Equation F.1.

Log D = concentration in *n*-octanol/concentration in PBS

**Equation F.1**

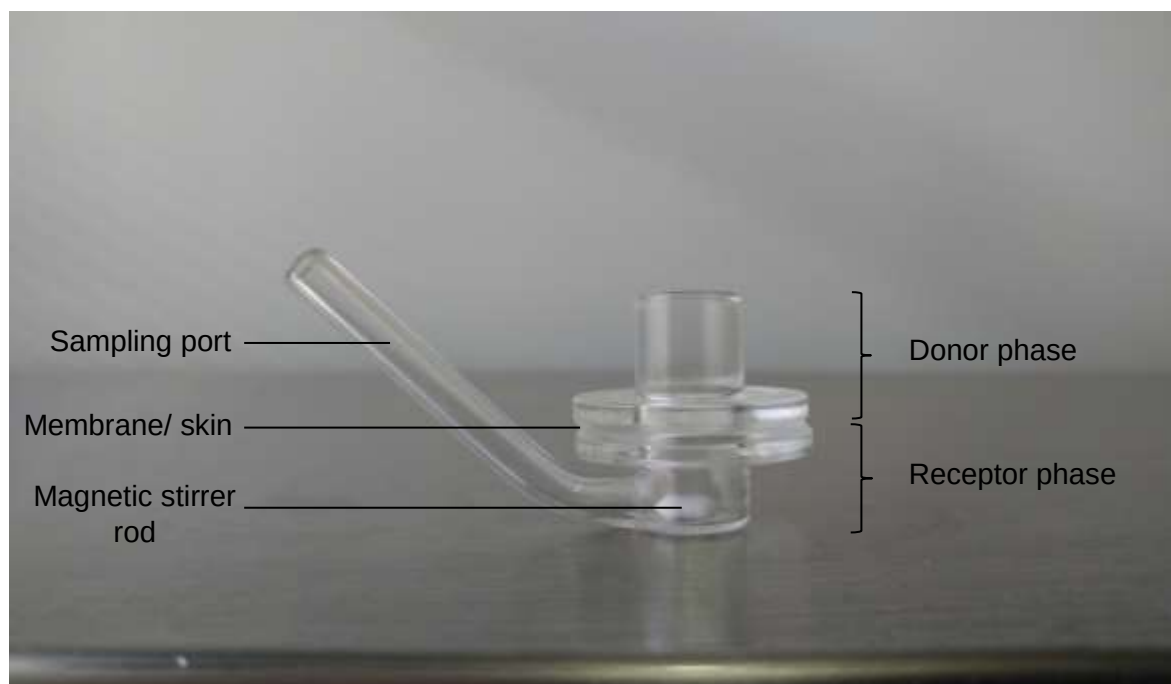
### **F.2.5 *In vitro* diffusion studies: vertical Franz cell method**

Any *in vitro* studies should be a direct imitation of, or as close as possible to, *in vivo* conditions (Modi & Shah, 2015:1). Temperature is an important condition that should be monitored closely. It has been said that the rate of diffusion is greatly dependent on temperature, since temperature variations could influence diffusion and hence the results obtained through *in vitro* studies (Shahzad *et al.*, 2015:2). The receptor phase should be immersed in a water bath regulated at 37 °C, as this can help to achieve normal *in vivo* conditions (Williams, 2013:685). Test formulations were regulated and preheated to a temperature of 32 °C, as this temperature is representative of external skin temperatures (Williams, 2013:685).

#### **F.2.5.1 Vertical Franz cell components**

To determine the release of artemether, and whether it was delivered topically or transdermally from the **(NE1)**, the **(NEG)** and the **(CEG)**, diffusion studies were conducted respectively. A diffusion system consisting of vertical Franz cells is an effective *in vitro* method to determine skin penetration (Wiechers, 2008:24). The vertical Franz cell diffusion method was used during all the diffusion studies and is described in Section F.2.5.4.

This two-phase diffusion method requires that a synthetic membrane or skin sample be placed between the phases, i.e. the donor and receptor phase (Williams, 2013:683). The components of a vertical Franz cell are depicted in Figure F.1.



**Figure F.1:** Components of a vertical Franz cell

#### **F.2.5.2 Preparation of receptor phase**

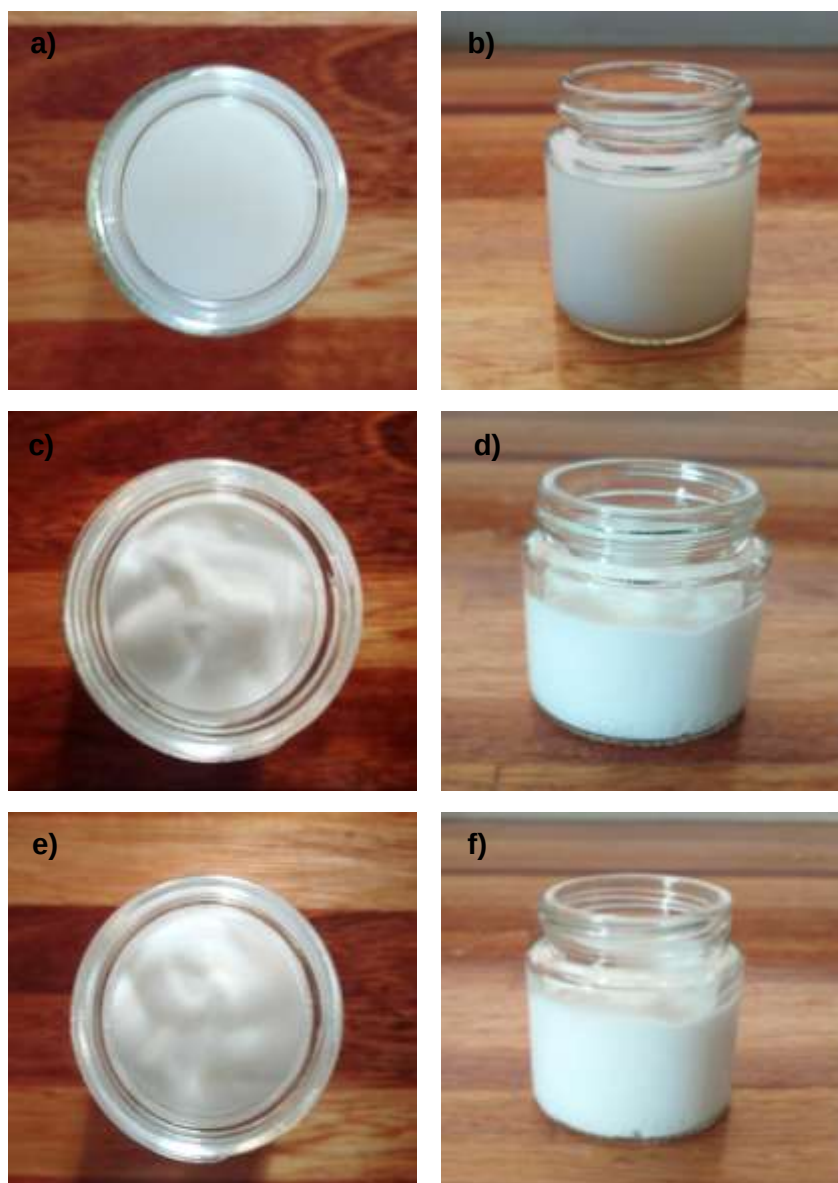
API solubility within the receptor phase solution is very important to ensure it does not influence the dissolution process and consequently the experimental diffusion results (Modi & Shah, 2015:3). Artemether is practically insoluble in water, but presents to be more soluble in dehydrated ethanol; studies have shown artemether's solubility to be 16 mg/ml in ethanol (Cayman Chemical, 2014:4; Sunil *et al.*, 2010:93; USP, 2013).

Since ethanol has the potential to solubilise artemether, absolute ethanol (99%) was used during the *in vitro* membrane release studies to ensure the quantification of artemether within the receptor phase solution. This could give a clearer indication about whether drug release from the formulation occurred.

For *in vitro* skin diffusion studies, PBS at pH 7.4 was used in the receptor phase; since this pH is representative of human blood *in vivo* (OECD, 2011:29). PBS (pH 7.4) was also used instead of ethanol, as the absolute ethanol could influence the integrity of the human skin, which could lead to incorrect results (Williams, 2013:685). The PBS was prepared by dissolving 3.0250 g of sodium hydroxide (NaOH) in 800 ml Milli-Q® water. In 500 ml of Milli-Q® water, 13.6497 g of potassium dihydrogen orthophosphate (KH<sub>2</sub>PO<sub>4</sub>) was dissolved. Both solutions, containing a

magnetic stirring rod respectively, were placed on a magnetic stirrer to ensure adequate mixing and dissolving, where after the NaOH solution was added to the  $\text{KH}_2\text{PO}_4$  solution, whilst being stirred continuously. To this mixture, 700 ml of Milli-Q<sup>®</sup> water was added to ensure a volume of 2 000 ml. The pH of the PBS was measured with a Mettler Toledo<sup>®</sup> pH meter (Mettler Toledo, CU), equipped with a Mettler Toledo<sup>®</sup> InLab<sup>®</sup> 410 electrode (Mettler Toledo, CU). The pH was then set and adjusted to a pH of 7.4 by adding either NaOH or orthophosphoric acid.

#### F.2.5.3 Test formulations and preparation of donor phase



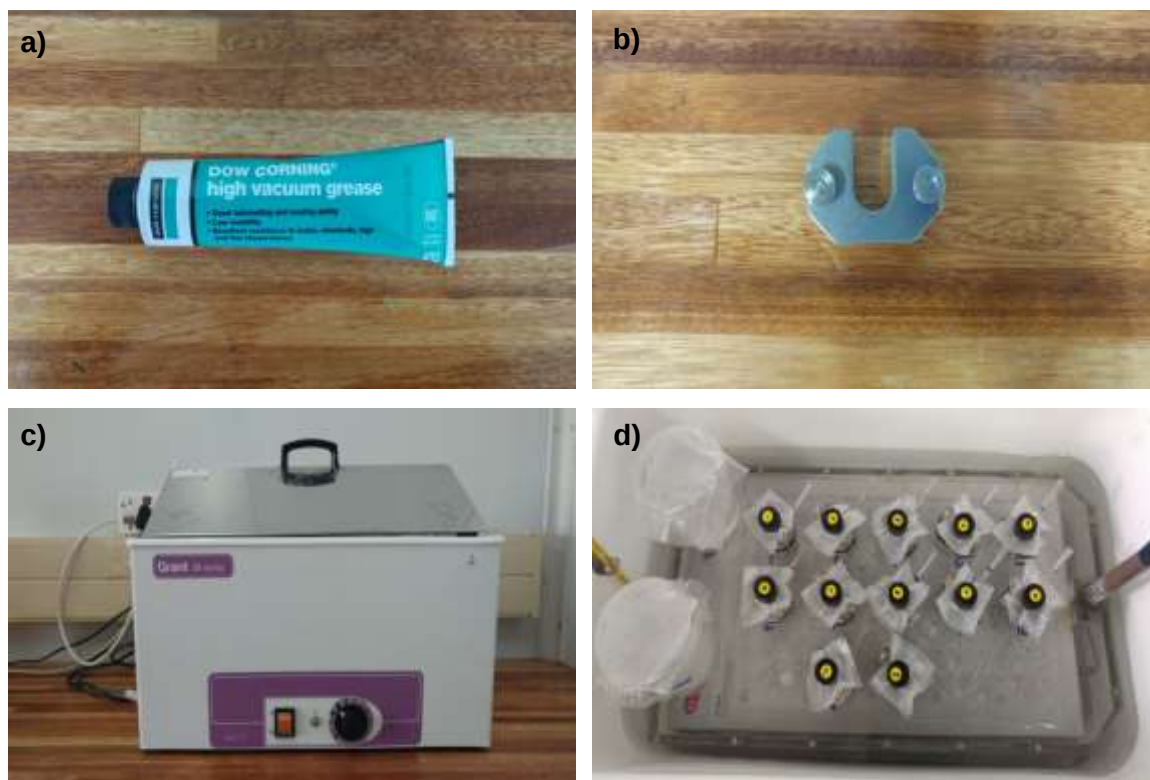
**Figure F.2:** Final test formulations: a) top view of the **(NE1)**; b) side view of the **(NE1)**; c) top view of the **(NEG)**; d) side view of the **(NEG)**; e) top view of the **(CEG)** and f) side view of the **(CEG)**

During the *in vitro* diffusion studies, three formulations were investigated, the **(NE1)** and two semi-solid dosage forms of the **(NE1)** (see Appendix C). The **(NEG)** and the **(CEG)** were formulated as semi-solid dosage forms of **(NE1)** (see Appendix D). All test formulations contained artemether and safflower oil. A placebo (formulation containing no API) of each formulation was also prepared during each diffusion study and used as a control group. Membrane release studies, as well as skin diffusion studies were done on all three test formulations. Figures F.2.a to F.2.f are representative of these test formulations (the **(NE1)**, the **(NEG)** and the **(CEG)**) respectively, which served as the donor phase during the diffusion studies. Consequently, the donor phase of ten Franz cells contained 1 ml of the respective formulation and two Franz cells contained 1 ml of the placebo.

#### **F.2.5.4 Membrane release studies**

Twelve vertical Franz cells were used during each of the membrane release studies, two of which served as the control as their donor phase contained placebo formulations. Prior to conducting the release studies, the test formulations (the **(NE1)**, the **(NEG)** and the **(CEG)**), were formulated and placed into a water bath at 32 °C. The receptor phase (absolute ethanol) was pre-heated to 37 °C in another water bath. Each vertical Franz diffusion cell consisted of a donor and receptor compartment; Dow Corning® high vacuum grease (Figure F.3.a) was applied to both compartments. A magnetic stirring rod was placed into the receptor compartment before placing the membrane on top. Polyvinylidene fluoride (PVDF) (Pall® Life Sciences, Michigan, USA) synthetic membranes, with a pore size of 0.45 µm and 25 mm diameter, was placed between the two vacuum greased compartments. The two compartments were placed together and the sides sealed with vacuum grease to prevent leakage.

A horseshoe clamp (Figure F.3.b) was used to fasten the two compartments (donor compartment on top of the receptor compartment) securely together; this was done for all twelve Franz cells. The receptor compartment, with a diffusion area of 1.075 cm<sup>2</sup>, has a capacity of 2.0 ml and was filled with 2.0 ml (37 °C) absolute ethanol. When filled, the receptor compartment was visually examined to make sure there were no air bubbles present. Each individual donor compartment was filled with 1.0 ml of the preheated test formulation (32 °C) then covered with two layers of Parafilm® and a plastic cap to prevent any loss of the formulation during the experiment. The twelve assembled Franz cells were then placed in a water bath, at a constant temperature of 37 °C, on a Variomag® (Variomag, USA) magnetic stirring plate, which was placed in a Grant® water bath (Grant Instruments, UK) (Figure F.3.c and F.3.d). The entire receptor phase was extracted through the sampling port and refilled with fresh, preheated absolute ethanol (37 °C) hourly, for 6 h. The content of the receptor compartment was then placed into HPLC vials and analysed by means of HPLC, as mentioned in Section F.2.1.



**Figure F.3:** a) Dow Corning® high vacuum grease; b) horseshoe clamp used to fasten Franz cell compartments; c) Grant® water bath and d) vertical Franz cells assembled and placed on a magnetic stirrer plate within the water bath

#### **F.2.5.5 *In vitro* skin diffusion studies**

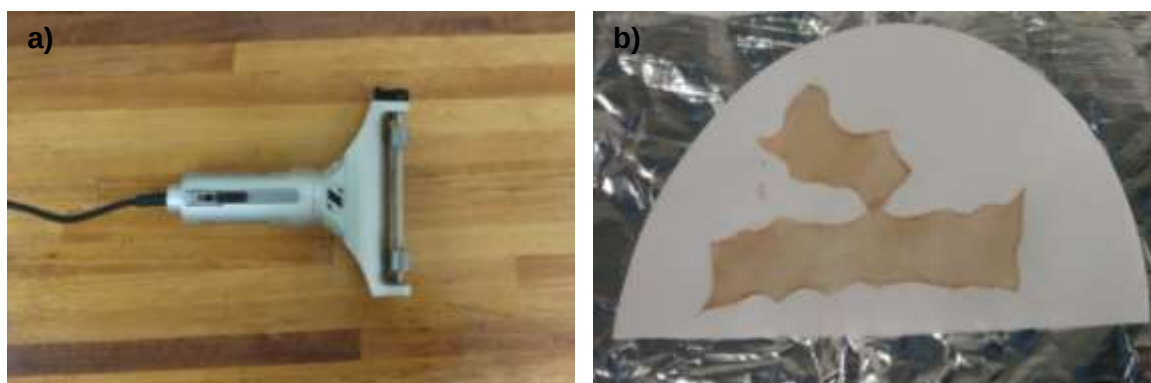
##### **F.2.5.5.1 Skin ethics and collection**

Caucasian female skin, obtained after abdominoplasty surgery, was used during the skin diffusion studies. Ethical approval for the use of biological material (i.e. human skin) during the *in vitro* topical drug delivery experiments was obtained from the Research Ethics Committee of the North-West University (reference number NWU-00114-11-A5). The patients who donated skin were required to complete an informed consent form and remained anonymous at all times. The skin was placed in the freezer at - 20 °C after collection. Before skin diffusion studies were conducted, the skin samples were visually examined for any defects or stretch marks.

##### **F.2.5.5.2 Preparation of dermatomed skin**

In order to prepare the dermatomed skin samples, the skin was defrosted and placed (with the stratum corneum facing upwards) on a paper towel. A dermatome™ (Zimmer TDS, United Kingdom) was used to cut pieces of skin, with a thickness of approximately 400 µm, by pressing on the skin with the dermatome at a 30 – 45° angle and with constant pressure. Figure F.4.a represents the dermatome used to cut 400 µm thick skin. The dermatomed skin was placed on Whatman® filter paper (Figure F.4.b) to dry and thereafter covered with aluminium foil. It was

placed in the freezer at - 20 °C until needed for skin diffusion studies. Before a diffusion study, the dermatomed skin was cut into circles and used between the two compartments of the vertical Franz cells.



**Figure F.4:** a) A dermatome™ (Zimmer TDS, United Kingdom) and b) 400 µm thick dermatomed skin

#### **F.2.5.5.3 Skin diffusion studies**

To determine the correlation between the skin, skin permeation, the API and the delivery system used; skin diffusion studies can be conducted (Ng *et al.*, 2010:210). This is of great importance when the success of the formulation needs to be determined. During the *in vitro* skin diffusion studies, the same technique as for the membrane release studies (see Section F.2.5.4) was employed, the only difference was that the PVDF synthetic membranes, as used in the membrane studies, were replaced with circular dermatomed skin samples, which were placed on top of the receptor compartment with the stratum corneum facing upwards. Instead of absolute ethanol, the receptor compartment was filled with 2.0 ml PBS (pH 7.4), which was preheated to 37 °C.

A pilot study was conducted to determine the extraction intervals during the skin diffusion studies and to validate that the artemether concentration was quantifiable. Following the pilot study, it was concluded that a single, 12 h extraction would suffice for the evaluation of the **(NE1)**. The pilot study indicated that no further investigations of the **(NEG)** and the **(CEG)** were needed as no artemether was quantified for the semi-solid dosage forms.

#### **F.2.5.5.4 Tape stripping**

Tape stripping is a useful technique to evaluate the effectiveness of formulations targeted for localised therapeutic action, as it evaluates whether the formulation resulted in topical drug delivery. After the diffusion studies were completed, the Franz cells' compartments were disconnected and the skin samples carefully removed. The skin samples were pinned to Parafilm®, which was stapled to a wooden board. Tissue paper was used to dab the skin dry to

remove any excess formulation (Walters & Brain, 2008:38). Next, the stratum corneum was removed by making use of adhesive tape (3M Scotch® Magic™ Tape) that was cut into an adequate size to cover the diffusional area, which was approximately 1.075 cm<sup>2</sup> (indicated by the imprint made on the skin by the Franz cells). The tape was cut so that the diffusional area was just covered. The layers of the stratum corneum were removed using sixteen tape strips; the first strip was discarded as it is part of the procedure to remove any excess API and the other fifteen tape strips were used to remove the stratum corneum until the skin glistened. These fifteen strips (with API and the stratum corneum-epidermis (SCE)) were placed inside a polytop containing 5 ml absolute ethanol. The remaining skin (the epidermis-dermis (ED)) was cut into pieces and placed into another polytop containing 5 ml absolute ethanol. Both polytops containing the tape strips (SCE) and the pieces of skin (ED), respectively, were left overnight ( $\pm 8$  h) in the fridge at  $\pm 4$  °C. Thereafter, the samples were filtered and analysed by means of HPLC.

#### **F.2.6 Data analysis**

For each diffusion experiment, samples were analysed by means of HPLC in order to provide a linear line. This linear line was used to determine the concentration of artemether, i.e. drug flux in each of the Franz cells (Ng *et al.*, 2010:213). Outcomes were therefore investigated in terms of the average cumulative amount of artemether per area ( $\mu\text{g}/\text{cm}^2$ ), which had diffused through the membrane, plotted against time (h) (Shakeel *et al.*, 2007:E3). The slope of the linear regression line formed was thus used to determine the average drug flux (Ng *et al.*, 2010:213).

The average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) (the degree at which an API can cross or permeate through the skin) of artemether, which had diffused through the PVDF synthetic membrane, was determined through the linear regression obtained at hourly intervals for 6 h.

To determine the outcome of the skin diffusion studies (topical and/or transdermal delivery), the average cumulative amount of artemether per diffusion area ( $\mu\text{g}/\text{cm}^2$ ) that had diffused through the skin was determined, together with the average concentration of artemether diffused through the skin ( $\mu\text{g}/\text{ml}$ ) after 12 h.

Through both the membrane and skin diffusion studies, a clear indication was given about whether artemether was released from the dispersion or semi-solid dosage forms and whether the target site was reached.

### **F.3 Results and discussion**

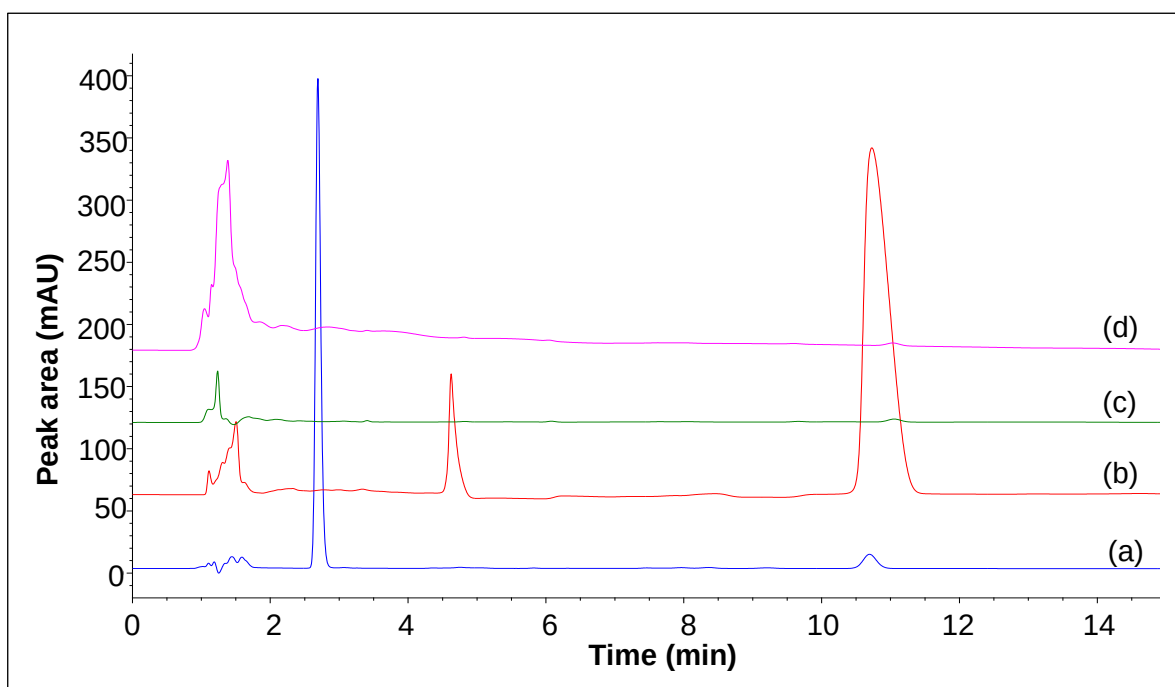
#### **F.3.1 Aqueous solubility**

It is known that an API should ideally possess an aqueous solubility larger than 1 mg/ml for topical delivery. Literature indicates that artemether presents as insoluble, thus with an aqueous solubility of less than 1 mg/ml. During this study it was found that the experimental aqueous solubility of artemether was  $0.1053 \pm 0.0022$  mg/ml in water and  $0.090 \pm 0.0030$  mg/ml in PBS (pH 7.4). Studies conducted by Haynes *et al.* (2006:2136) found that artemether had an aqueous solubility of 0.117 mg/ml (pH 7.2). The experimental aqueous solubility, as found in this study, was close to that found in literature. The minimal difference in aqueous solubility values could be ascribed to the fact that the pH at which the aqueous solubility was determined, differed. It has been stated that the pH, as well as temperature, could influence the solubility of the API in an aqueous medium (Lynch *et al.*, 2001:1549). With an aqueous solubility lower than 1 mg/ml, it can be expected that artemether would not permeate ideally through the skin.

#### **F.3.2 Octanol buffer distribution coefficient (log D)**

Literature indicates that an API ideal for topical drug delivery presents with a log P value between 1 and 3; although Williams (2013:680) stated that a log P between 1 and 4 also appears to be ideal. The log D, instead of the log P, was experimentally determined and used in this study. Calculations determined that artemether presented with an average log D value of  $2.35 \pm 0.1170$  at pH 7.4. This log D value is indicative that artemether was more soluble in the *n*-octanol phase than in the buffer (PBS (pH 7.4) or water); consequently confirming that artemether is a lipophilic API. It can therefore be proposed that at this log D value, artemether would be ideal for topical drug delivery as its value falls between the previously mentioned acceptable ranges. This experimental log D differs from that found in literature, as it has been discovered that artemether presented with a log D value of 3.1 (pH 7.4) (Bergqvist & Lindegårdh, 2008:251). This difference between values can be ascribed to the fact that the composition of the buffer used between studies differed, leading to experimental differences.

The log D value of artemether can clearly be confirmed by the chromatogram, as illustrated in Figure F.5. When compared to an artemether standard solution (Figure F.5.a), it can be seen that artemether presents with great octanol solubility (Figure F.5.b) compared to the limited solubility in water or PBS (pH 7.4) (Figures F.5.c and F.5.d, respectively) and was barely detected within the water or PBS (pH 7.4) samples. Artemether's octanol solubility is approximately 190 times more than that of water or PBS (pH 7.4). Its lipophilic nature is consequently confirmed through these results.



**Figure F.5:** Chromatograph representing the solubility of artemether: a) standard artemether peak serving as reference; b) octanol solubility of artemether; c) water solubility of artemether and d) PBS (pH 7.4) solubility of artemether

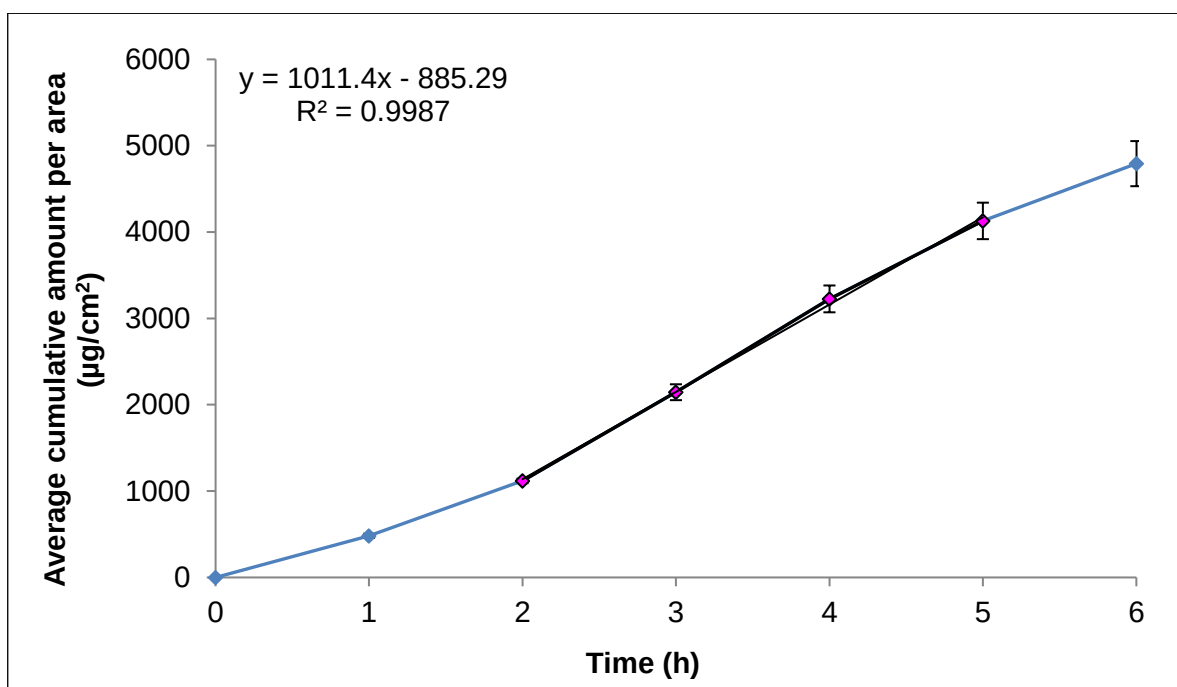
### F.3.3 Membrane release studies results

Membrane drug release results are tabulated in Table F.2 for all three formulations (the **(NE1)**, the **(NEG)** and the **(CEG)**), which aids in the summary of the data gathered and viewed in Figure F.6 to F.11. Table F.2 is therefore indicative of the average percentage of artemether released from each formulation, through the PVDF synthetic membranes, over a period of 6 h. It is also indicative of the average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ).

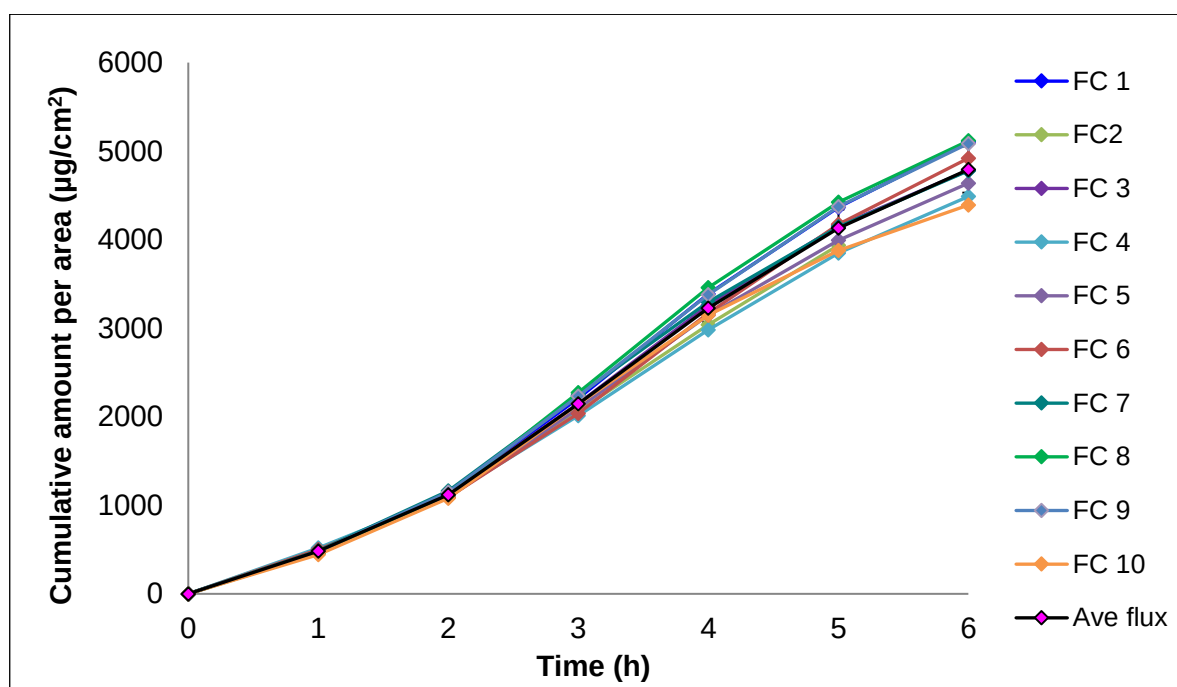
**Table F.2:** Average percentage (%) artemether released through the membranes, as well as the average drug flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) for each formulation after 6 h (n represents the number of Franz cells)

| Formulation  | n  | Average %released (%) | Average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) |
|--------------|----|-----------------------|---------------------------------------------------------|
| <b>(NE1)</b> | 10 | $32.22 \pm 1.760$     | $1\,011.40 \pm 66.968$                                  |
| <b>(NEG)</b> | 10 | $11.36 \pm 0.600$     | $127.57 \pm 12.178$                                     |
| <b>(CEG)</b> | 10 | $9.47 \pm 0.570$      | $100.39 \pm 9.294$                                      |

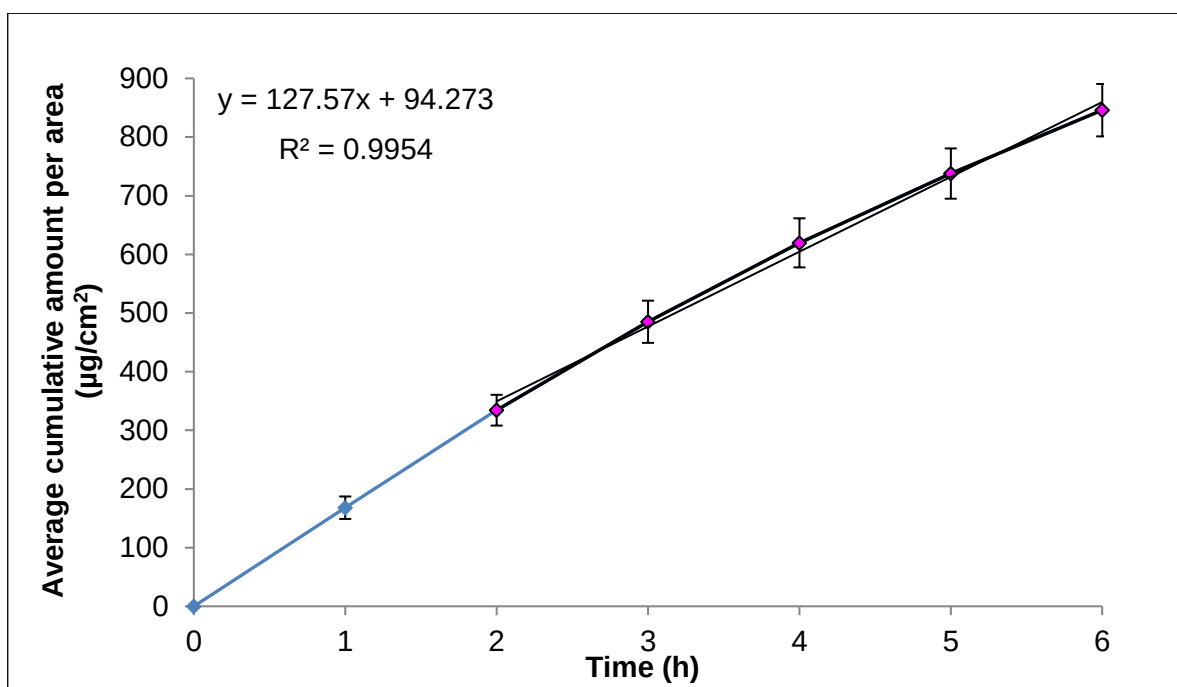
From Figures F.7, F.9 and F.11, it is observed that artemether concentrations were detected in all ten Franz cells during all three diffusion studies, respectively. This indicated that drug release through the PVDF synthetic membranes had occurred for the **(NE1)**, the **(NEG)** and the **(CEG)**.



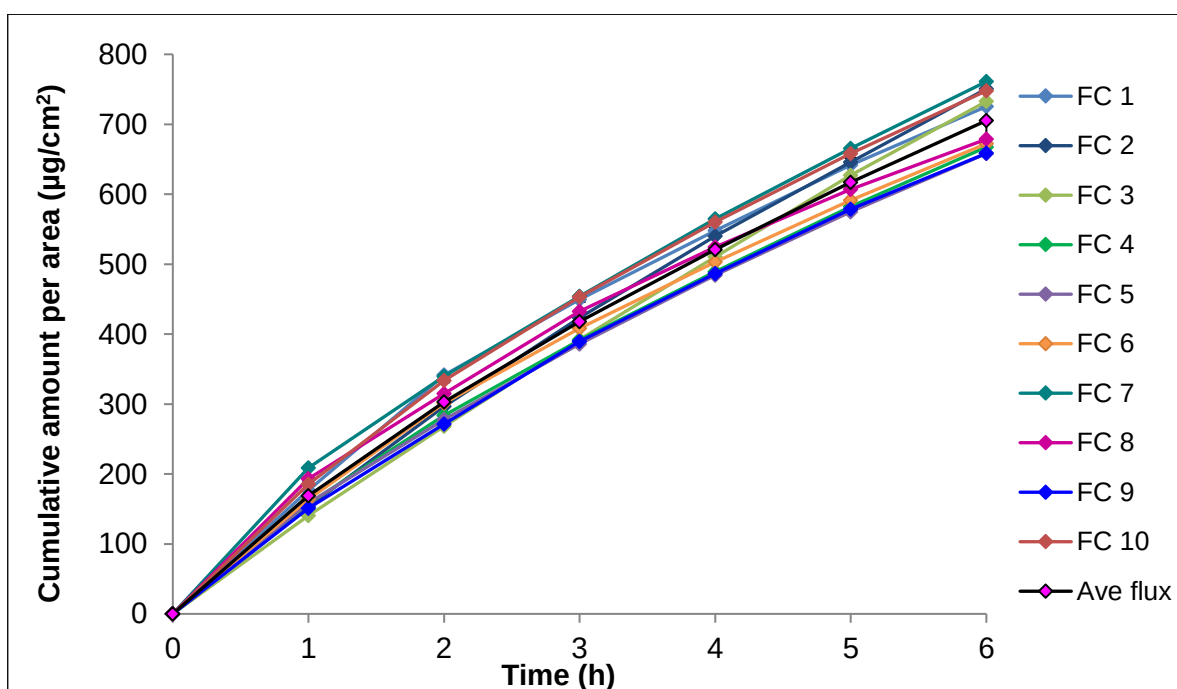
**Figure F.6:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of artemether permeated from the **(NE1)** through the membrane as a function of time to illustrate the average flux from 2 – 5 h ( $n = 10$ )



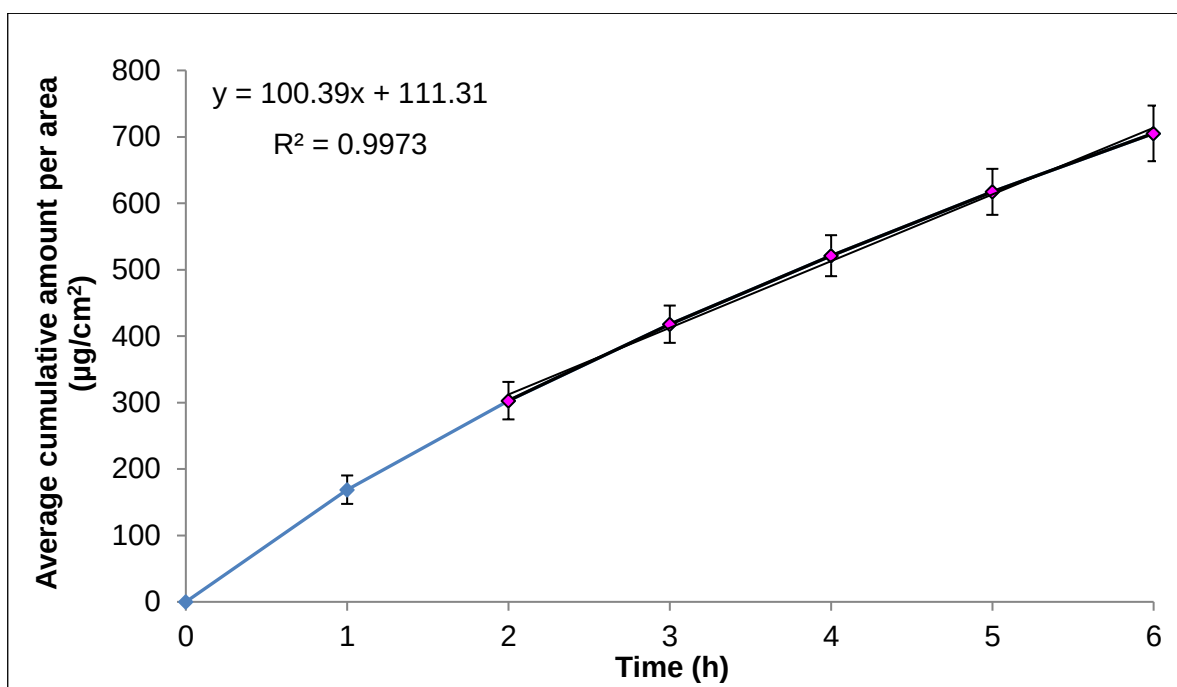
**Figure F.7:** Cumulative amount artemether per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NE1)** ( $n = 10$ )



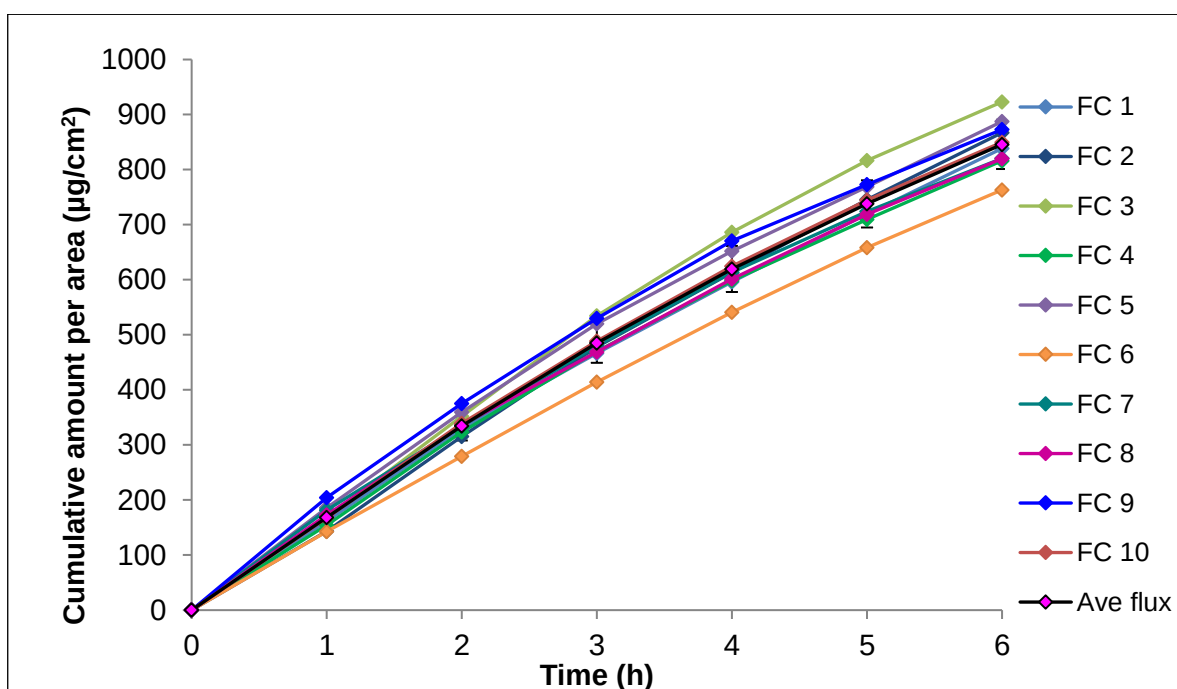
**Figure F.8:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of artemether that permeated from the **(NEG)** through the membrane as a function of time to illustrate the average flux from 2 – 6 h (n = 10)



**Figure F.9:** Cumulative amount artemether per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEG)** (n = 10)



**Figure F.10:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of artemether that permeated from the **(CEG)** through the membrane as a function of time to illustrate the average flux from 2 – 6 h (n = 10)



**Figure F.11:** Cumulative amount artemether per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(CEG)** (n = 10)

The **(NE1)** had the highest average flux ( $1\,011.4 \pm 66.968 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), followed by the **(NEG)** ( $127.57 \pm 12.178 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ) and lastly, the **(CEG)** ( $100.39 \pm 9.294 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ). When evaluating the average %released, the **(NE1)** presented with the highest release of the API from the formulation ( $\pm 3$  times greater than the semi-solid dosage forms). When comparing the two semi-solids, the **(NEG)** presented with a 1.887% higher release than the **(CEG)**.

The large difference between the average flux values could be ascribed to the viscosity and droplet size of the formulations. Naturally, nano-emulsions present with very low viscosity compared to a nano-emulgel or conventional emulgel, which are greatly viscous. It has been proposed that lower viscosity can lead to faster and better release of an API from the dispersion (Chime *et al.*, 2014:97). When the two semi-solids, i.e. the **(NEG)** and the **(CEG)**, were compared, the **(NEG)** presented with a better average flux than the **(CEG)**. This permeation difference between the **(NEG)** and the **(CEG)** can be confirmed through literature, since one of the disadvantages of emulgels is that they are constituted of large droplets, which limit permeation (Baibhav *et al.*, 2011:66).

### F.3.4 *In vitro* skin diffusion results

Following the membrane release studies, *in vitro* Franz cell skin diffusion studies and tape stripping were conducted and would determine whether the aim of the study was achieved: to deliver artemether topically resulting in localised targeting of CTB lesions. Data obtained through these experiments could indicate whether artemether was found within the receptor phase, which represents the systemic circulation, i.e. blood flow (transdermally), or whether it was retained within the SCE (topically) or ED (topically).

#### F.3.4.1 Transdermal diffusion

All samples collected through the skin diffusion studies were analysed by means of HPLC. Data gathered presented the average %artemether that had diffused through the skin after 12 h, as well as the average amount of artemether that had diffused per area ( $\mu\text{g}/\text{cm}^2$ ) after 12 h.

During the diffusion studies, any data from outlier cells (which includes cells that had depleted prior to experiment completion or leaked, etc.) was removed from the dataset. At the start of each of the diffusion studies, ten Franz cells were used. For the skin diffusion study evaluating the **(NE1)**, one Franz cell had depleted before completion and was therefore removed from data. The delivery of the semi-solids (**(NEG)** and **(CEG)**) was investigated through a pilot study utilising only three Franz cells.

**Table F.3:** Concentration of artemether quantified in the receptor phase

| Formulation | n | Average %diffused (%) | Average amount diffused per area ( $\mu\text{g}/\text{cm}^2$ ) |
|-------------|---|-----------------------|----------------------------------------------------------------|
| (NE1)       | 9 | $0.050 \pm 0.010$     | $7.011 \pm 1.499$                                              |
| (NEG)       | 3 | 0.000                 | 0.000                                                          |
| (CEG)       | 3 | 0.000                 | 0.000                                                          |

After HPLC analysis, it was quantified that artemether was present in the receptor phase of the **(NE1)** samples. Initially, a diffusion study was conducted over a 12 h period with extractions every 2 h, which resulted in very low concentrations; consequently, it was decided that the skin diffusion studies should be repeated with a single extraction after 12 h. From Table F.3, the average %artemether diffused from the **(NE1)** was very low as only  $0.050 \pm 0.010\%$  of the initial amount of artemether (in the formulation) diffused through the skin into the receptor phase; consequently, only a small amount of artemether also reached the systemic circulation. This concentration of artemether, found in the receptor phase, was still lower than the LOD ( $10.16 \mu\text{g}/\text{ml}$ ) and LLOQ ( $20.32 \mu\text{g}/\text{ml}$ ) values (as determined in Section A.3.7), yet it was still quantified and reported. In essence, this could suggest that artemether did not permeate into the systemic circulation. No concentration of artemether was quantified when the receptor phase of the **(NEG)** and the **(CEG)** was analysed, hence, it did not diffuse through the skin from the semi-solid dosage forms into systemic circulation.

The lack of artemether found transdermally could possibly be ascribed to the API itself or to the delivery system used. The systemic circulation, mostly hydrophilic, presents unfavourable for the lipophilic and weak aqueous soluble artemether, therefore limiting the quantification thereof. Lipophilic APIs could also be retained within the lipophilic layers, such as the SCE, therefore decreasing permeation into the circulation (Yourick *et al.*, 2008:120). Looking at the delivery system, nano-emulsions could aid in targeted delivery of an API when the lipophilicity is high. Formulating a lipophilic API within the oil droplets of the oil phase could aid in targeted delivery through slow release (Hörmann & Zimmer, 2016:87). It has been proposed that unionised molecules facilitate better permeation across lipid layers, whereas ionised molecules show little to no degree of penetration into the stratum corneum (Barry, 2002:511; Williams, 2003:38). Since the **(NE1)**, the **(NEG)** and the **(CEG)** measured with a pH of 6.85, 5.14 and 5.86, respectively, low unionised species of 0.11%, 5.44% and 1.09% were calculated for each of the three formulations. As a result, the lack of unionised species of artemether within each of the formulations (the **(NE1)**, the **(NEG)** and the **(CEG)**) could be used as an explanation for no quantification thereof in the systemic circulation. It can also be proposed that the initial concentration within the three formulations were too low to result in systemic delivery.

#### F.3.4.2 Tape stripping results

The tape stripping technique produced data about whether topical delivery (within the SCE or within the ED) of artemether was achieved (OECD, 2004:24). In this study, the topical delivery of a novel CTB treatment was aimed at retaining the lipophilic artemether within the epidermis. The epidermis served as target site, since CTB, with its various superficial presentations, can be found within the epidermal layers (Frankel *et al.*, 2009:20, 23). Hence, artemether's physicochemical properties propose that it will remain within the lipophilic stratum corneum and not permeate further to the circulation after application.

Table F.4 represent the results obtained through the tape stripping experiment, providing the average concentration ( $\mu\text{g/ml}$ ) of artemether present in the SCE against the average concentration in the ED.

**Table F.4:** The average concentration of artemether present in the SCE and the ED gathered through tape stripping after the 12 h skin diffusion studies

| Formulation | N | Average concentration in SCE ( $\mu\text{g/ml}$ ) | Average concentration in ED ( $\mu\text{g/ml}$ ) |
|-------------|---|---------------------------------------------------|--------------------------------------------------|
| (NE1)       | 9 | $21.173 \pm 9.842$                                | 0.000                                            |
| (NEG)       | 3 | 0.000                                             | 0.000                                            |
| (CEG)       | 3 | 0.000                                             | 0.000                                            |

##### F.3.4.2.1 Concentration in stratum corneum-epidermis

In the SCE, concentrations of artemether were only detected for the **(NE1)** and not for the two semi-solids. Yourick *et al.* (2008:120) suggests that a lipophilic API, such as artemether, would deliver a higher concentration of the API in the SCE as opposed to the hydrophilic layers, such as the ED. Table F.4 illustrates the average concentration of artemether in the SCE was  $21.173 \pm 9.842 \mu\text{g/ml}$ , consequently the quantified concentration of artemether within the SCE could be ascribed to the fact that both the API and the stratum corneum presents as lipophilic. This could aid in the retention of artemether within the stratum corneum lipids, since it can partition into the stratum corneum with ease. The **(NE1)** being a two-phase dispersion, i.e. containing lipophilic and hydrophilic properties, could also result in targeted delivery.

When the samples of the semi-solids, i.e. the **(NEG)** and **(CEG)**, were analysed, no artemether was quantified within the SCE. A possible explanation could be that the concentrations of artemether within the semi-solids were lower than that within the **(NE1)**; therefore less artemether to target or permeate through the skin. Being more viscous formulations with larger droplets than the **(NE1)**, can also propose weaker permeation of the **(NEG)** and the **(CEG)** (Chime *et al.*, 2014:97).

#### F.3.4.2.2 Concentration in epidermis-dermis

As seen from Table F.4, no concentration of artemether was quantified in the ED for all three formulations (the **(NE1)**, the **(NEG)** and the **(CEG)**). This could be ascribed to the fact that this layer provided a hydrophilic environment and not a favourable lipophilic environment. In being lipophilic, artemether, was retained within the SCE and therefore was not possible to diffuse to further layers. This could be the reason why artemether was only found in the SCE and not in the ED for **(NE1)**. As no other concentration of artemether was detected in the SCE for the semi-solids, it was not expected that any artemether would be found within the ED – results confirmed this. As mentioned in Section F.3.4.1, unionised molecules results in better permeability compared to ionised molecules, showing weaker permeability (Barry, 2002:511; Williams, 2003:38). Through calculations, very low unionised species were measured, respectively, for the three formulations, the **(NE1)**, the **(NEG)** and the **(CEG)**. Consequently, the lack of unionised species of artemether within each of the formulations could be used as an explanation for no quantification in the ED.

#### F.4 Conclusion

During the *in vitro* diffusion studies, membrane release studies were an indication of the API release properties from the optimised **(NE1)** and the two semi-solids, i.e. the **(NEG)** and the **(CEG)**. It should be noted that membrane release studies are merely a method to determine API release and that no biological processes were present since a synthetic membrane was used. These release results can therefore be either much higher or lower than the values found when skin diffusion experiments are performed.

All three of the formulations, the optimised **(NE1)**, the **(NEG)** and the **(CEG)**, presented with optimal characteristics (as determined in Appendix C and Appendix E, respectively) for *in vitro* skin diffusion. Membrane release studies presented promising, as API release was found for each of the formulations. Consequently, the optimised **(NE1)** presented with a much higher flux ( $1\,011.4 \pm 66.968 \mu\text{g}/\text{cm}^2.\text{h}$ ) than that of the two semi-solids, i.e. the **(NEG)** ( $127.57 \pm 12.178 \mu\text{g}/\text{cm}^2.\text{h}$ ) and **(CEG)** ( $100.39 \pm 9.294 \mu\text{g}/\text{cm}^2.\text{h}$ ). When the flux of the **(NEG)** and the **(CEG)** were compared the results were as expected, the **(NEG)** presented with better flux than the **(CEG)**.

As mentioned, the aim of this study was to deliver artemether topically (epidermis as the target site) for the treatment of CTB. It was hoped that by the application of a **(NE1)**, or a semi-solid (the **(NEG)** and the **(CEG)**), that direct delivery of artemether could be achieved. Artemether concentrations were only quantified in the SCE ( $21.173 \pm 9.842 \mu\text{g}/\text{ml}$ ) when delivered through the optimised o/w **(NE1)**. No concentrations of artemether were present in the ED of the **(NE1)** samples. Quantified concentrations of artemether within the SCE and ED were also not found

for the **(NEG)** and the **(CEG)**. Therefore, these results could indicate that no topical delivery of artemether was achieved through the semi-solids, whilst the optimised **(NE1)** could possibly result in topical delivery.

The experimentally determined aqueous solubility of artemether ( $0.1053 \pm 0.0022$  mg/ml in water and  $0.090 \pm 0.0030$  mg/ml in PBS (pH 7.4)), as determined in this study, suggested that artemether would not permeate ideally through the skin in comparison to the ideal log D of  $2.35 \pm 0.1170$ , which could indicate effective skin permeation. Due to the lipophilic nature of artemether, it is proposed it should accumulate within the stratum corneum. This could explain the amount of artemether found in the SCE of the **(NE1)** samples. The very small amounts of artemether detected within the receptor phase of the **(NE1)** samples could be ascribed to artemether's weak aqueous solubility (as described in Section F.3.1), since artemether was unable to dissolve within the PBS. Therefore, the fact that PBS instead of ethanol (as with membrane studies) was used as receptor phase medium could also influence the results or the lack thereof. The very low concentrations used within the semi-solid dosage forms, could also explain why no results for these formulations were found during the skin diffusion. The semi-solid dosage forms also contained large amounts of water, which is more hydrophilic, therefore not ideal for the lipophilic artemether and could result in weaker permeability.

Although mainly ionised, a very small concentration of artemether was quantified within the receptor phase of the **(NE1)** samples, which could possibly be attributed to the fact that the lipophilic component of the **(NE1)** successfully retained artemether within the SCE, consequently saturating the SCE. The hydrophilic phase of the **(NE1)** could as a result facilitate the permeation through the ED into the receptor phase. It can be proposed that a formulation with a higher artemether concentration could possibly be detected within the ED.

It can be said that artemether was released from all three formulations (the **(NE1)**, the **(NEG)** and the **(CEG)**), as found during membrane release studies. Yet, when the three formulations were investigated during *in vitro* skin diffusion studies, artemether was only quantified within the SCE for the optimised **(NE1)**, but not for the semi-solid dosage forms. The optimised **(NE1)** was therefore the most successful test formulation, as it attained the aim of the study.

Hence, the following conclusions can be made:

- \* A possibility to deliver artemether topically does exist.
- \* The most ideal formulation for topical delivery is through an optimised o/w nano-emulsion.
- \* The nano-emulgel and conventional emulgel presented as least favourable for the topical delivery of artemether.

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# APPENDIX G:

## CYTOTOXICITY STUDIES OF AN OPTIMISED O/W NANO-EMULSION CONTAINING ARTEMETHER

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### G.1 Introduction

An important step in pre-clinical development of novel drug delivery systems is to determine whether the API, and even more so the formulation containing the API, presents with any toxic effects to humans or human tissue (Astashkina *et al.*, 2012:83). According to Riss *et al.* (2013:1), assays based on cell cultures are aimed at establishing whether the test formulation poses with any cytotoxic effects and whether it would be safe to use. Consequently, cytotoxicity studies are based on the measurement of the integrity (cell viability) of cell membranes and whether it remains intact or whether cell death occurs, after a predetermined incubation and treatment period (Niles *et al.*, 2009:33,35; Riss *et al.*, 2011:103). These toxicity studies measure the growth, viability and/or proliferation of a cell (ATCC, 2011:1; Li *et al.*, 2015:617). *In vitro* cytotoxicity studies can be expressed as the response of a cell to external factors (ATCC, 2011:1).

Cytotoxicity studies are conducted *in vitro* and are merely a prediction of what occurs *in vivo* within a system (Fotakis & Timbrell, 2005:171). It must be noted that a direct correlation between the *in vitro* cytotoxicity results and the possible *in vivo* result cannot be drawn (Yoon *et al.*, 2012:634). This can be ascribed to a great difference between *in vitro* and *in vivo* circumstances, of which the pharmacokinetic processes are the most important. Absorption, distribution, metabolism and excretion are pharmacokinetic processes absent during *in vitro* studies (Yoon *et al.*, 2012:634). Therefore, cytotoxic results are merely an indication of the possible toxic effects of the API and/or the formulation on cell lines.

Formulations were prepared and investigated during this study as novel topical CTB treatment of which an o/w nano-emulsion containing 0.8% (w/v) artemether and 5% (w/v) safflower oil ((**NE1**)) was the main focus. Since the skin comprises three layers, each consisting of various cell types of which the most important is the keratinocytes, toxicity of the optimised formulation ((**NE1**)) needed to be evaluated on humans or cell cultures of human nature (López-García *et al.*, 2014:44). It has been stated that artemether presents with low toxicity and little irritation (Gao *et al.*, 2013:134). To determine whether artemether and the optimised (**NE1**) presented with any toxic effects, *in vitro* cytotoxicity assays were performed on cell cultures (Riss *et al.*, 2011:103). Although a variety of cytotoxicity assays exist, the lactate dehydrogenase (LDH) leakage and methylthiazol tetrazolium (MTT) assay are the most popular assays to utilise (Fotakis & Timbrell, 2005:171).

Consequently, in this study a MTT assay was employed on HaCaT cells. It must be noted that the cytotoxicity studies were not conducted on the semi-solid dosage forms as they were not the core focus of this study. These semi-solids were also too viscous and unsuitable for the aqueous environment of *in vitro* cell cultures.

The aim of this section of the study was to determine whether or not:

- \* artemether presented with cytotoxicity against HaCaT cells and
- \* whether the dispersions, i.e. the nano-emulsion without artemether or the nano-emulsion with artemether, presented with cytotoxicity against HaCaT cells.

## **G.2 Cell culture toxicity studies**

### **G.2.1 Selection of an appropriate cell line**

The selection of an appropriate cell line is of great importance, therefore a few selection criteria need to be taken into account. These criteria include type of cell specie, functional characteristics, finite or continuous, normal or transformed cell lines, growth conditions and characteristics, to name but a few (Gibco®, 2016:18). In order to meet the aims of this study, an appropriate cell line (for *in vitro* cytotoxicity studies) had to be selected that was primarily of human tissue and origin. Consequently, HaCaT cells were selected as an appropriate cell line. They are viewed as spontaneously, immortalised cells of a proliferative epidermal nature, hence, appropriate to use as cell line during the cytotoxic evaluation of a topical drug delivery system ((**NE1**)) and a API (artemether) proposed for topical use (López-García *et al.*, 2014:44).

### **G.2.2 Selection of an appropriate drug concentration to use**

It must be noted that concentrations used during these cytotoxic studies were greater than what are normally utilised due to the concentration present in the formulation used during *in vitro* skin diffusion studies (Appendix F). It was decided that three concentrations would be applied as treatment to the cells, namely a 0.5% (40 µg/ml), a 1.0% (80 µg/ml) and a 2.0% (160 µg/ml) concentration.

#### **G.2.2.1 Treatment**

The HaCaT cells were each treated for 12 h at 37 °C (5% CO<sub>2</sub>, 95% humidity) with three treatments in three different concentrations. The cell lines were treated with a placebo nano-emulsion (containing no artemether, further referred to as (**PNE1**)), an optimised nano-emulsion containing artemether ((**NE1**)) and an artemether stock solution (further referred to as (**ArtS**)). The concentrations of the stock solution that needed to be added were determined using the concentration of artemether within the (**NE1**) and the amount that will be present in each well of

the well plate (200 µl). Consequently, the cell lines were treated with a 0.5%, a 1.0% and a 2.0% concentration of each treatment for 12 h, respectively.

The formulation of the **(PNE1)** and the **(NE1)** are discussed in Section C.2.1. The **(ArtS)** was prepared to provide the same concentrations as within the formulations, **(PNE1)** and **(NE1)**. Therefore, an artemether dilution was prepared by dissolving 50 mg of artemether within 15 ml methanol, so that the final concentration of methanol exposed to the cell culture was never greater than 5% (v/v). Each concentration was further prepared by taking an amount of the artemether dilution and placing it in a clean test tube. To this, an amount representing 95% (v/v) of Dulbecco's Modified Eagle Medium (DMEM) was added to finally represent 0.5%, 1.0% and 2.0%, respectively. The same principal was applied for the **(PNE1)** and the **(NE1)** treatments: amounts of the **(PNE1)** and the **(NE1)** representing the same concentration as the prepared **(ArtS)** samples were transferred to a test tube, respectively and were made up to volume with DMEM. A group of cells were left untreated to serve as a control group.

### G.2.3 Non-assay experimental procedures

All experiments were carried out in the Mammalian Cell Culture Laboratory, situated in the Laboratory for Applied Molecular Biology (LAMB) of the North-West University (NWU), Potchefstroom Campus, RSA, under aseptic conditions. All procedures prior and during the cytotoxic studies included cell cultivation, feeding, seeding and the treatment of cell lines were done within LAMB by Me A Brümmer.

#### G.2.3.1 Materials

**Table G.1:** Materials used during the *in vitro* cytotoxicity study

| Product                                                     | Supplier       | Batch number  |
|-------------------------------------------------------------|----------------|---------------|
| DMEM with high glucose, 4.0 mM L-glutamine, sodium pyruvate | HyClone™       | AB216032      |
| MEM Non-Essential Amino Acid (MEM NEAA) (100x)              | HyClone™       | AAB199680     |
| L-Glutamine                                                 | HyClone™       | Not available |
| Penicillin/Streptomycin (Pen/Strep)                         | Lonza™         | 5MB068        |
| Trypan Blue solution (0.4%)                                 | Sigma-Aldrich® | RNBC9030      |
| MTT (methylthiazol tetrazolium)                             | Sigma-Aldrich® | MKBR4419V     |
| Phosphate Buffered Saline (1x)                              | HyClone™       | AAD201744     |
| Trypsin-Versene® (EDTA)                                     | Lonza™         | 5MB168        |
| Foetal Bovine Serum (FBS)                                   | HyClone™       | RZM35923      |
| Dimethyl sulfoxide (DMSO)                                   | Sigma-Aldrich® | S2BD1790V     |

The materials used during the conduction of the cytotoxicity studies are illustrated in Table G.1 and were only handled in the laminar airflow cabinet to ensure sterility and prevent contamination of the cell cultures.

### **G.3 *In vitro* toxicity testing**

The *in vitro* cytotoxicity testing was performed on the HaCaT cells to establish any possible change in the amount of viable cells. Cytotoxicity was conducted by performing an MTT assay. The HaCaT cells were seeded in a 96-well plate at a density of 20 000 per well. These plates were incubated at 37 °C (5% CO<sub>2</sub>, 95% humidity) for 24 h to ensure the cells attached to the well plate surface. The HaCaT cells were maintained in a cell culture flask (75 cm<sup>2</sup>) containing DMEM, or high glucose adequate growth medium, supplemented with 10% FBS, 1% MEM NEAA, 4 mM L-glutamine and 1% of Pen/Strep. It was then cultured in a CO<sub>2</sub> incubator at standard culturing conditions (37 °C, 95% humidity and 5% CO<sub>2</sub>).

#### **G.3.1 MTT colorimetric assay**

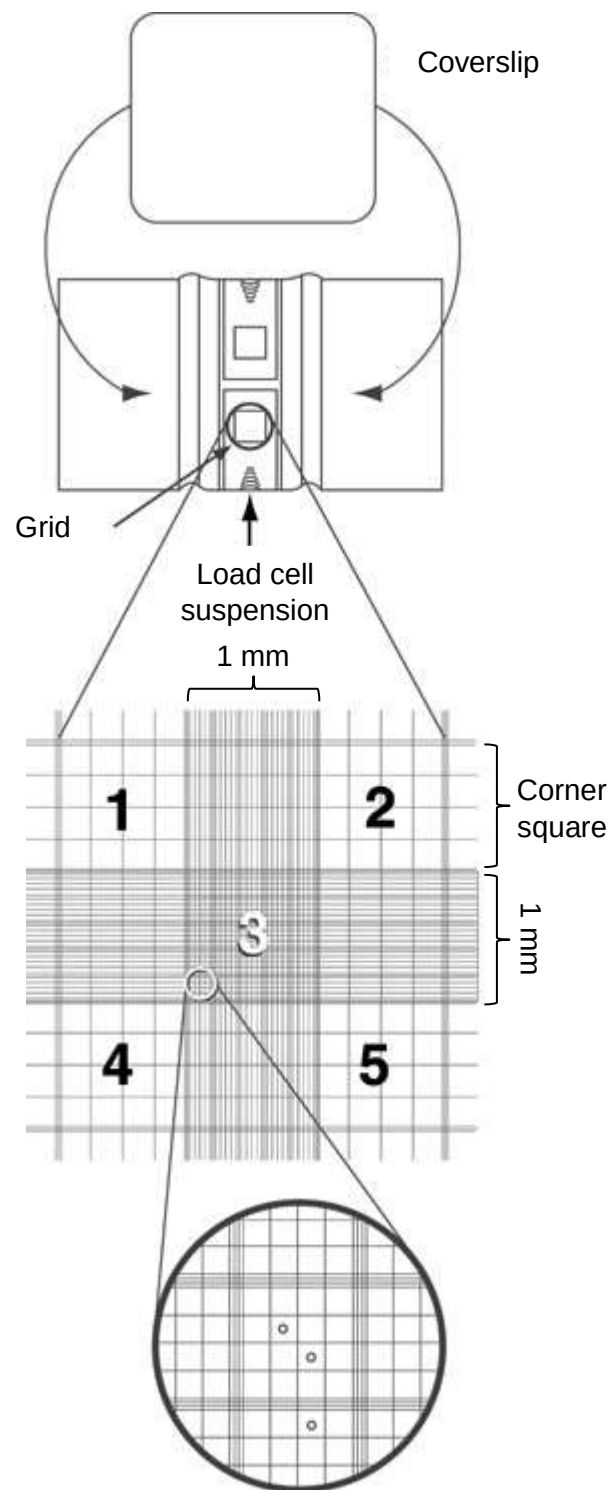
To determine *in vitro* cell viability, after the exposure to the treatments, an MTT assay was conducted. It is known that the dehydrogenase enzymes in the mitochondria of metabolically active cells will change the water soluble MTT into an insoluble, purple formazan. This purple formazan is therefore indicative of cell viability, as it is unable to cross cell membranes, therefore remaining in the healthy cells (Fotakis & Timbrell, 2006:172). This is used to determine the cell viability.

The MTT reagent (2 mg/ml) was added to the HaCaT cell lines 24 h following treatment (as described in Section G.2.2.1) and the 96-well plates were incubated for a further 3 h until a purple precipitate was visible. Once visible, the growth medium was removed, the excess MTT reagent was carefully removed and the purple formazan was dissolved by adding 200 µl DMSO to each well plate (Lee *et al.*, 2012:17) and left at room temperature for 2 h. The amount of formed purple formazan was then spectrophotometrically measured with the SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) at 570 nm (630 nm is normally used as the reference wavelength) using the SoftMax® Pro 6.2.1 Software (Riss *et al.*, 2013:2).

##### **G.3.1.1 Determination of cell viability**

Consequently, cell viability can be determined since the purple formazan is unable to cross cell membranes and therefore remains in the healthy, viable cells (Fotakis & Timbrell, 2006:172). All experiments were conducted on cells that were at least 95% viable. Determination of viable cells was conducted using the Trypan Blue exclusion test, using a haemocytometer (Figure G.1). A counting mixture consisting of 25 µl Trypan blue (0.4%), 15 µl Phosphate

Buffered Saline and 10  $\mu\text{l}$  of the cell suspension was prepared and incubated for 3 min at room temperature ( $\pm 25^\circ\text{C}$ ). The counting mixture was then pipetted sufficiently and 10  $\mu\text{l}$  was extracted. The pipette, filled with the well-suspended cell mixture, was placed at the edge of the cover slip and the contents were slowly expelled. Consequently, the fluid was drawn into the chamber.



**Figure G.1:** A haemocytometer slide (adapted from Phelan, 1996:A.3.F.9)

The cells were counted under a microscope; the counting chamber of the haemocytometer can be divided into nine large squares resulting in a total surface area of 9 mm<sup>2</sup>. All the live cells (purple formazan colour) were counted starting in each corner square and then the centre square; five squares per side of the counting chamber were provided in total. All the counted live cells were added together and the total was divided by two resulting in the averages of the two sides, this was further divided by five (average per square). The final amount was then multiplied with  $5 \times 10^4$  (dilution factor) to obtain the number of cells per ml of cell suspension ( $C_1$ ). The cell concentration was then multiplied with the total volume of cell suspension to determine the cells present in the suspension (Gouws, 2014:5-6). After the determination of the amount of cells present in the suspension, the %cell viable after treatment was calculated using Equation G.1 (Adapted from Abcam, 2016):

$$\% \text{cell viable} = (\text{number of stained cells} / \text{total number of cells}) \times 100 \quad \text{Equation G.1}$$

### G.3.1.2 MTT colorimetric assay results and discussion

#### G.3.1.2.1 MTT assay results on HaCaT cells

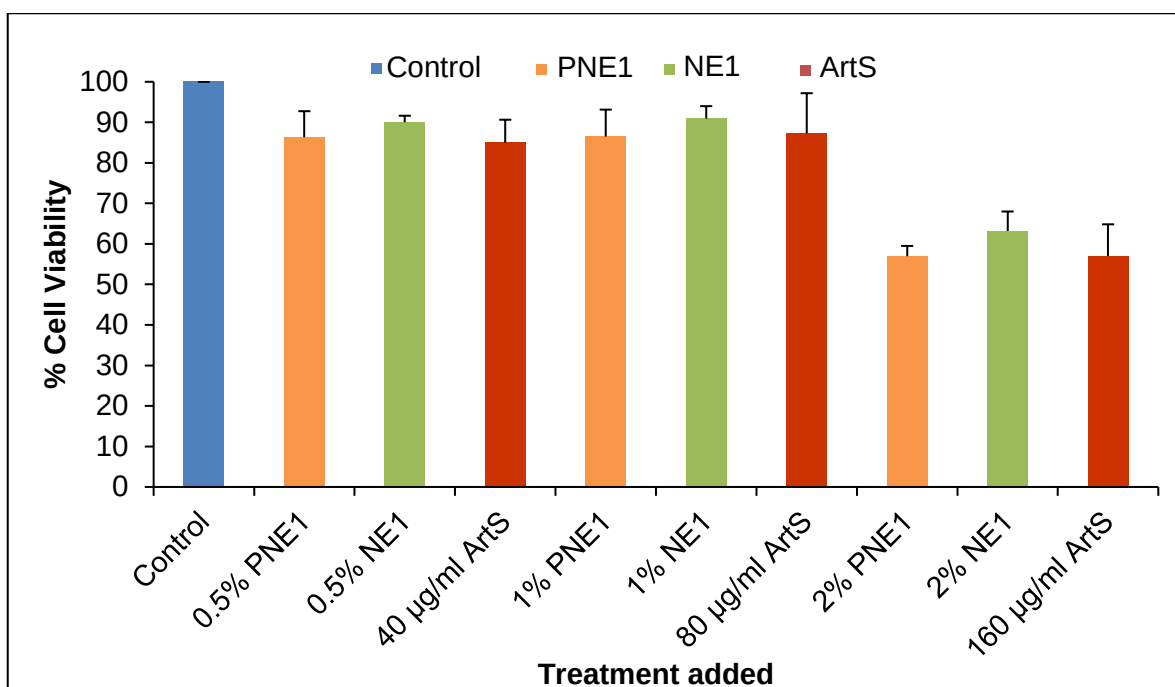
López-García *et al.* (2014:44) stated that a treatment or compound can be determined as cytotoxic according to the following percentage of cell viability (it must be noted that these guidelines are normally assay and cell line specific):

- \* **non-cytotoxic:** > 80%
- \* **weak cytotoxicity:** 80 – 60%
- \* **moderate cytotoxicity:** 60 – 40%
- \* **strong cytotoxicity:** < 40%

For the HaCaT cell line, the MTT %cell viability assessed after 12 h of treatment with the **(PNE)**, the **(NE1)** and the **(ArtS)** is represented in Table G.2. HaCaT cells were treated respectively with the **(PNE)**, the **(NE1)** and the **(ArtS)** treatment in a 0.5%, a 1.0% and a 2.0% concentration. A set of cells were left untreated and served as a control group.

**Table G.2:** %Cell viability of HaCaT cell after treatment

| %Cell viability | Concentration | (PNE1) | (NE1)  | (ArtS) | Control |
|-----------------|---------------|--------|--------|--------|---------|
|                 | 0.5%          | 86.332 | 90.032 | 84.938 | 100.000 |
|                 | 1.0%          | 86.501 | 90.951 | 87.288 |         |
|                 | 2.0%          | 56.971 | 63.178 | 56.995 |         |



**Figure G.2:** The cytotoxic effects of the various concentrations (0.5%, 1.0% and 2.0%) of the **(PNE)**, the **(NE1)** and **(ArtS)** on HaCaT cell viability

Table G.2 and Figure G.2 are a representation of the percentage of cells still viable after being treated with **(PNE)**, **(NE1)** and **(ArtS)** in three different concentrations, respectively. A concentration dependent decrease and trend can be viewed for all three of the treatments. When the %cell viability of the 0.5% concentration were examined, no noticeable difference was observed for the different treatments (**(PNE)**, **(NE1)** and **(ArtS)**); the same was true for the %cell viability of the 1.0% concentration, however for the 2% concentration, a greater cell death was noted for the three different treatments.

When taking the guidelines as proposed by López-García *et al.* (2014:44) into account, the following can be concluded from Figure G.2 – the %cell viability of the different treatments (**(PNE)**, **(NE1)** and **(ArtS)**) were relatively high for the 0.5% and the 1.0% concentrations since these concentrations yielded percentages above 80%. Therefore, both concentrations (0.5% and 1.0%) for the different treatments (**(PNE)**, **(NE1)** and **(ArtS)**) were considered to be non-cytotoxic. Conversely, at a higher concentration of 2.0%, a drastic decrease in the %viable cells was seen for all three formulations. The %cell viability of the different treatments, (**(PNE)** and **(ArtS)**), for the 2% concentration ranged between 60% and 40% and can subsequently be classified as having a moderate cytotoxicity, while the **(NE1)** of the 2.0% concentration is considered as weak cytotoxic with a %cell viability of 63.178%.

It is observed that the %cell viability for the **(PNE)** and the **(ArtS)** treatments was lower than for the **(NE1)** at all three concentrations (0.5%, 1.0% and 2.0%). Consequently, the **(NE1)** presented as the least cytotoxic treatment, as observed in the 0.5% and the

1.0% concentration, whilst the **(ArtS)** resulted in the highest %cell death. It can therefore be proposed that the **(NE1)**, when compared to the **(PNE)** and the **(ArtS)**, had the same concentration dependent effect on the HaCaT cells – all three of the treatments were therefore relatively safe to be used on the skin in low concentrations of 0.5% and 1.0%.

Through various cytotoxicity studies of artemether, as well as other artemisinin derivatives, conducted on different cell lines, it was determined that artemether presented with low cytotoxicity effects against HaCaT cells (Mikaeiloo *et al.*, 2016:183). A study conducted by Wu *et al.* (2015:7072) found that when cell death of HaCaT cells occurred, through artemether, a concentration dependent cell death effect was visible, which correlated with findings in this study. Since no known literature on cytotoxicity studies conducted on HaCaT cells treated with a nano-emulsion containing artemether was found, this study contributes to the lack of information.

It must be said that the lipophilic nature of the nano-emulsions (**(PNE)** and **(NE1)**) could possibly influence the results obtained through the MTT assay. Studies have shown that agents with a lipophilic nature can reduce the MTT a great deal (Stockert *et al.*, 2012:786). With this said, the MTT assay was a relative identification of the cytotoxicity effects of the formulation and of the API, yet further investigation, especially *in vivo* experiments would provide the most accurate results.

#### G.4 Conclusion

The MTT assay, as cytotoxicity evaluation, provided information about whether the formulation **(PNE1)** or artemether **(ArtS)** or the combination thereof (**(NE1)**) presented as cytotoxic against human epidermal cells. Although the lipophilic nature of the formulation and the API could possibly affect the MTT assay results, a MTT assay was still employed, since it is the primary cytotoxicity assay used. It can also be said that although lipophilic, the largest component of the **(NE1)** was water therefore the lipophilic effects might be relatively small, but would only be confirmed through another assay or through *in vivo* experiments.

The results obtained through these cytotoxicity studies concluded that the optimal nano-emulsions (**(PNE1)** and **(NE1)**) and artemether **(ArtS)**, respectively, presented with very little toxic effects on human epidermal cells in low concentration (equal to or less than 1.0%). At the 2.0% treatment of the **(PNE)**, the **(NE1)** or the **(ArtS)** (180 µg/ml) the %cell viability decreased drastically, yet was still viewed as weak to moderately cytotoxic. The **(PNE1)** and the **(NE1)** can be regarded as non-cytotoxic in low concentration (equal to or less than 1.0%), since cell viability was yielded above 80% for both. Due to this, and as the **(NE1)** presented with the least cell death, it can be proposed this optimised formulation remained relatively safe and may possibly be used as a novel CTB treatment in low concentrations.

It should be explained that *in vitro* determinations are not a direct indication of what would occur *in vivo*, yet these results could suggest that no or little side effects or toxicity would occur at the target site. It is recommended that another assay, such as an LDH assay, should be employed, and *in vitro* efficacy studies against *M. tuberculosis* cells should be conducted. This could provide a clearer indication of the effects of artemether and/or the nano-emulsion against this bacterium and consequently determine the effectiveness of the **(NE1)** and or artemether against CTB.

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# APPENDIX H:

## THE INTERNATIONAL JOURNAL OF PHARMACEUTICS: GUIDE FOR AUTHORS

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### Introduction

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

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

Note: For details on pharmaceutical nanotechnology, see Editorials in 279/1-2 281/1, and 288/1.

### Types of paper

#### (1) Full Length Manuscripts

#### (2) Rapid Communications

- a. These articles should not exceed 1500 words or equivalent space.
- b. Figures should not be included otherwise delay in publication will be incurred.
- c. Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

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

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Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

*Examples:* 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

**List:** References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

### **Examples:**

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Reference to a website:

Cancer Research UK, 1975. Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/> (accessed 13.03.03).

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# APPENDIX 1:

LANGUAGE EDITING CERTIFICATE - ENGLISH

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## *Work Certificate*

|         |                                                                                                                          |
|---------|--------------------------------------------------------------------------------------------------------------------------|
| To      | Esmari van Jaarsveld                                                                                                     |
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| Date    | 21/11/2016                                                                                                               |
| Subject | Formulation and topical delivery of a safflower oil nano-emulsion containing artemether                                  |
| Ref     | GS/EvJ /01                                                                                                               |

I, Gill Smithies, certify that I have proofed the following,

Chapters 1 to 4, Appendices A to G, the Abstract and Acknowledgements:  
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This certificate is to certify that the Afrikaans language editing on this thesis, written by **ESMARI VAN JAARVELD (22758569)** was done by Mrs. S L. van Niekerk.

Mrs. Van Niekerk is since 2008 a registered and accredited Language Editor for the Potchefstroom Campus of the North-West University. During this time she has done the language editing of hundreds dissertations in English as well as Afrikaans. She finishes her studies for a degree in Education in December 1964 and received her National Education Teacher's Diploma.

After a few years in Education she worked as journalist with various newspapers. She retired as a veteran journalist after more than 35 years

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15 November 2016