

# Topical delivery of clofazimine from an avocado oil nano-emulsion

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A strong woman accepts both compliments and criticism graciously, knowing that it takes both sunshine and rain for a flower to grow - **Mandy Hale**

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# ABSTRACT

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The skin is the largest organ of the human body (Finch, 2003:29). The outermost layer of the skin, the stratum corneum, offers a protective barrier that prevents foreign substances from penetrating into the body through the skin (Madison, 2003:231). Although the skin acts as a penetration barrier towards substances, it also offers an attractive alternative for the topical administration of drugs and their transdermal delivery. The skin is considered as the preferred route for drug administration, since it avoids the first-pass liver metabolism of drugs and offers an alternative to systemic drug treatments (Gratieri *et al.*, 2013:610; Ruby *et al.*, 2014:1421; Zhang *et al.*, 2015:2713). The skin barrier can be bridged if (1) the topical/transdermal preparation, (2) the active pharmaceutical ingredients (APIs) in the preparation and (3) the skin's physicochemical properties correlate. Factors, such as drug polarity and molecular weight have proven to affect the permeation of a drug through the skin (Lipinski *et al.*, 2001:9; Yamashita & Hashida, 2003:1188).

Cutaneous tuberculosis (CTB) is a form of extra-pulmonary tuberculosis, which is sometimes caused by resistant forms of bacteria, called the *Mycobacterium tuberculosis* complex, for which there are currently no topical treatments available (Barbagallo *et al.*, 2002:319; Wirth *et al.*, 2008:2). CTB often leads to unsightly skin lesions, especially on the face, hands and feet, which are often difficult to hide. Clofazimine is classified as a riminophenazine antibiotic and it is a last-line drug that is reserved for the treatment of drug-resistant tuberculosis (Chopra & Brennan, 1996:93; Xu *et al.*, 2012:1104). CTB is currently only systemically treated with treatment regimens similar to those that are used for the treatment of pulmonary tuberculosis (Semaan *et al.*, 2008:476-477). It may consequently be advantageous to develop a topical treatment for CTB, for using concurrently with systemic treatment, in order to try and improve CTB lesions faster.

During this study, clofazimine was incorporated into dispersions, called nano-emulsions, aimed at enhancing the topical diffusion of the API. A nano-emulsion is a dispersion consisting of immiscible liquids, typically when one liquid is dispersed within the other in nano-scale droplets, by adding a surfactant or emulsifying agent to the dispersion. A nano-emulsion has droplet sizes ranging from 20 nm to 300 nm or even from 50 nm to 1000 nm (Anton & Vandamme, 2009:142; Sharma *et al.*, 2010:2; Tyagi *et al.*, 2011:1382). The nano-emulsion being prepared in this study was an oil-in-water nano-emulsion, in which natural avocado oil was used as the oil phase. Avocado oil contains linoleic acid that aids as a penetration enhancer, since it is also found naturally in the skin (Bouwstra *et al.*, 2003:7; Hussain *et al.*, 2014:22; Morena *et al.*, 2003:2220). The lipophilic clofazimine was incorporated into the oil phase of the dispersion to aid with its possible topical delivery and skin permeation (Peters *et al.*, 2000:82). The nano-

emulsion was incorporated into an emulgel to improve the viscosity of the topical application. A conventional emulsion (emulgel) was prepared for comparison purposes to determine whether the emulgel, containing the nano-emulsion, would show any advantages over the emulgel containing the coarse emulsion, with regards to topical drug delivery and skin permeation.

The aim of this study therefore was to determine whether it would be possible to deliver clofazimine topically through a natural oil (avocado oil) nano-emulsion. Three pharmaceutical preparations were formulated for the investigation of their abilities to aid in the topical delivery of the API, i.e. a nano-emulsion and two emulgel preparations (one containing a conventional (coarse) emulsion and the other containing a nano-emulsion). The targeted delivery areas of the API were the stratum corneum-epidermis and the epidermis-dermis, since CTB presents topically.

Characterisation of the three pharmaceutical preparations was performed in order to assure that the preparations were optimised to ease the possible topical delivery of clofazimine. A suitable, accurate and reliable high performance liquid chromatography (HPLC) method was developed for evaluating and quantifying the presence and concentration of clofazimine after possible topical application and skin delivery. The membrane release studies confirmed drug release from all three preparations. The nano-emulsion yielded the best drug release, compared to the emulgel formulations, while the emulgel formulation, containing the coarse emulsion, yielded a slightly better API release than the emulgel containing the nano-emulsion.

The nano-emulsion was the only pharmaceutical preparation that has yielded a limited degree of topical drug release at low values, as well as minimal, non-quantifiable traces of permeation during the diffusion studies. This could have been as a result of the low solubility of the clofazimine in avocado oil and due to the lipophilic characteristic of the API (Drugbank, 2015; O'Driscoll & Griffin, 2008:618). Traces of the API were found in the stratum corneum-epidermis, the epidermis-dermis and the receptor phase in very small and non-quantifiable amounts. The topical delivery of clofazimine during this study therefore only occurred to a very small extent and only from the nano-emulsion.

**Keywords:** clofazimine, nano-emulsion, emulgel, cutaneous tuberculosis (CTB), topical delivery, active pharmaceutical ingredient (API), conventional emulsion, coarse emulsion, avocado oil.

# UITTREKSEL

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Die vel is die grootste orgaan van die menslike liggaam (Finch, 2003:29). Die heel buitenste laag van die vel, die stratum korneum, dien as die deurlaatbaarheidsgrens wat verhoed dat vreemde stowwe die liggaam deur die vel binnedring (Madison, 2003:231). Alhoewel die vel verhinder dat vreemde stowwe die liggaam binnedring, bied dit 'n aantreklike alternatief vir die toediening vir verskeie topikale geneesmiddels en hulle transdermale aflewering. Die vel word ook beskou as die gewenste toedieningsroete vir geneesmiddelaflawering, aangesien dit die eerstedeurgangs-lewermetabolisme van geneesmiddels vermy en 'n alternatief tot sistemiese geneesmiddelbehandelings bied (Gratieri *et al.*, 2013:610; Ruby *et al.*, 2014:1421; Zhang *et al.*, 2015: 2713). Die vel se deurlaatbaarheidsgrens kan oorkom word indien (1) die topikale/transdermale preparaat, (2) die aktiewe farmaseutiese bestanddele (AFB) in die preparaat en (3) die vel se fisies-chemiese eienskappe met mekaar korreleer. Faktore, soos geneesmiddelpolariteit en molekulêre gewig het byvoorbeeld bewys dat dit die deurlaatbaarheid van 'n geneesmiddel deur die vel kan beïnvloed (Lipinski *et al.*, 2001:9; Yamashita & Hashida, 2003:1188).

Kutaneuse tuberkulose (KTB) is 'n vorm van ekstra-pulmonêre tuberkulose, wat soms veroorsaak word deur weerstandige vorms van bakterieë, genaamd die *Mycobacterium tuberculosis* kompleks, waarvoor daar tans geen topikale behandeling beskikbaar is nie (Barbagallo *et al.*, 2002:319; Wirth *et al.*, 2008:2). KTB gee aanleiding tot onooglike letsels op die vel, veral op die gesig, hande en voete, wat dit dikwels moeilik maak om weg te steek. Klofasimien word as 'n riminofenasien antibiotikum geklassifiseer en dit is 'n laaste linie behandeling wat vir geneesmiddelweerstandige tuberkulose gereserveer word (Chopra & Brennan, 1996:93; Xu *et al.*, 2012:1104). KTB word tans slegs sistemies behandel, met behulp van behandelings wat vergelykbaar met die behandeling vir pulmonêre tuberkulose is (Semaan *et al.*, 2008:476-477). Dit kan gevolglik voordelig wees om 'n topikale behandeling te ontwikkel wat tesame met die sistemiese behandeling gebruik kan word, ten einde te poog om die KTB letsels vinniger op te klaar.

Tydens hierdie studie is klofasimien in dispersies, genaamd nano-emulsies, geïnkorporeer, wat ten doel gehad het om die topikale diffusie van die AFB te bevorder. 'n Nano-emulsie is 'n dispersie wat uit onmengbare vloeistowwe bestaan, tipies wanneer een vloeistof in nano-grootte druppeltjies binne-in 'n ander gedispergeer word, deur 'n oppervlak-aktiewe stof, of 'n emulsifiseermiddel by die dispersie te voeg. Nano-emulsies het deeltjiegroottes wat tussen 20 nm tot 300 nm, of selfs tussen 50 nm tot 1000 nm strek (Anton & Vandamme, 2009:142; Sharma *et al.*, 2010:2; Tyagi *et al.*, 2011:1382). Die nano-emulsie wat tydens hierdie studie berei is, was 'n olie-in-water nano-emulsie, waarin natuurlike avokado-olie as die oliefase

gebruik is. Avokado-olie bevat linoleïensuur, wat as 'n penetrasiebevorderaar optree, omdat dit ook natuurlik in die vel voorkom (Bouwstra *et al.*, 2003:7; Hussain *et al.*, 2014:22; Morena *et al.*, 2003:2220). Die vetoplosbare AFB is in die oliefase van die dispersie geïnkorporeer om met die moontlike topikale aflewering en veldeurlaatbaarheid van die AFB behulpsaam te wees (Peters *et al.*, 2000:82). Die nano-emulsie is in 'n emuljel geïnkorporeer om die viskositeit van die topikale doseringsvorm te verbeter. 'n Konvensionele emulsie (emuljel) is voorberei ten einde te vergelyk of die nano-emulsie emuljel enige voordele bo die growwe-emulsie emuljel sou inhou, ten opsigte van topikale geneesmiddelaflawering en veldeurlaatbaarheid.

Die doel van hierdie studie was dus om te bepaal of dit moontlik sou wees om klofasimien topikaal af te lewer deur middel van 'n natuurlike olie (avokado-olie) nano-emulsie. Drie farmaseutiese preparate is geformuleer ten einde hulle vermoëns om die moontlike topikale aflewering van die AFB te bemiddel, te ondersoek, naamlik 'n nano-emulsie en twee emuljelpreparate (waarvan die een 'n konvensionele (growwe) emulsie bevat het en die ander 'n nano-emulsie). Die teikenarea van die AFB was die stratum korneum-epidermis en die epidermis-dermis, aangesien KTB topikaal voorkom.

Al drie die farmaseutiese preparate is gekarakteriseer om te verseker dat hulle oor die beste moontlike eienskappe beskik om die moontlike topikale aflewering van die AFB te bevorder. 'n Gepaste, akkurate en betroubare hoëdrukvlloeistofchromatografiese (HDVC) metode is ontwikkel om die teenwoordigheid en konsentrasie van klofasimien te evalueer en te kwantifiseer na moontlike topikale aanwending en velaflewering. Die membraanvrystellingstudies het geneesmiddelvrystelling vanaf al drie die farmaseutiese preparate bevestig. Die nano-emulsie het die beste geneesmiddelvrystelling getoon, in vergelyking met die emuljelformules, terwyl die growwe-emulsie emuljelformule tot 'n klein mate beter vrystelling as die nano-emulsie emuljelformule getoon het.

Die nano-emulsie is die enigste farmaseutiese preparaat wat 'n beperkte mate van topikale vrystelling teen lae waardes getoon het, sowel as minimale, nie-kwantifiseerbare aanduidings van deursypeling tydens die diffusie-studies. Hierdie uitkomstes kon as gevolg van die lae oplosbaarheid van klofasimien in die avokado-olie en weens die vetoplosbare eienskappe van die AFB gewees het (Drugbank, 2015 & O'Driscoll & Griffin, 2008:618). Aanduidings van die AFB is in baie klein en nie-kwantifiseerbare waardes in die stratum korneum-epidermis, die epidermis-dermis en in die reseptorfase gevind. Die topikale aflewering van klofasimien tydens hierdie studie het dus slegs tot 'n baie klein mate en slegs vanaf die nano-emulsie plaasgevind.

**Sleutelwoorde:** klofasimien, nano-emulsie, emuljel, kutaneuse tuberkulose (KTB), topikale aflewering, aktiewe farmaseutiese bestanddeel (AFB), konvensionele emulsie, growwe emulsie, avokado-olie.

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$$J = K D (C_{\text{app}} - C_{\text{rec}}) / h$$

Equation 2.1

## APPENDIX A

$$y = mx + c$$

Equation A 1

## APPENDIX C

$$\%EE = \left[ \frac{C_t - C_f}{C_t} \right] \times 100$$

Equation C.1

# CHAPTER 1

## INTRODUCTION, PROBLEM STATEMENT AND AIMS AND OBJECTIVES

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### 1.1 Introduction

Tuberculosis (TB), a disease found in humankind for many years, is caused by a bacterium known as *Mycobacterium tuberculosis* (Zink *et al.*, 2007:381), however recently it has shown resistance to treatment. Since the appearance of resistant strains of TB, last line therapy drugs have been investigated for the treatment of this disease (Caminero *et al.*, 2010:622, 626; Espinal *et al.*, 2000:2537, 2545). In some cases, CTB is a condition that is caused by MDR-TB (Vadwai *et al.*, 2011:2540). Since there is only systemic treatment available for this disease, there is a need for a topical treatment for CTB (Barbagallo *et al.*, 2002:327). However, should such a topical treatment become available, it will probably only be used for the treatment of secondary TB infections. Such a treatment method will then be a combination of systemic TB chemotherapy and the nano-emulsion containing clofazimine.

Clofazimine, a reddish dye, is a narrow spectrum chemotherapeutic for the treatment of various mycobacterial infections (Chopra & Brennan, 1996; Kieu *et al.*, 2012:141-144). This active pharmaceutical ingredient (API) is classified as a rimonphenazine and has redox properties (Bastian & Colebunders, 1999:653-654; Lu *et al.*, 2011:5185). Since the appearance of MDR-TB, this API has been re-evaluated as a potential drug for the treatment of this particular strain of disease, as well as for the treatment of CTB (Bastian & Colebunders, 1999:653-654; Ho *et al.*, 2006:272-273; Wood, 1993:788). For the purpose of this study, this API will be used for topical delivery through a nano-emulsion, an emulgel containing a nano-emulsion and an emulgel containing a coarse emulsion, each containing a natural oil, namely avocado oil.

To enable the topical delivery of an API through the nano-emulsions and emulgels, the layers of the skin and their properties must be considered. The human skin comprises three main layers, each of which is different in structure and function: the epidermis, the dermis and the hypodermis (Bartoli, 2010:22, Menon, 2002:S3-S17; Waller & Maibach, 2005:221-235). The stratum corneum is the outermost layer of the epidermis and acts as the main barrier of the skin (Bartoli, 2010:22, Menon, 2002:S3-S17; Waller & Maibach, 2005:221-235). The lipid structure and the corneocytes of the stratum corneum largely contribute towards this barrier function (Jepps *et al.*, 2013:154). The skin layer below the stratum corneum is the viable epidermis, which consists of keratinocytes comprising 40% water, 15 – 20% lipids and 40% proteins. The water in the structure of the viable epidermis allows for the diffusion of water-soluble and water-

like substances through the viable epidermis, therefore it is less permeable to lipophilic substances. Lipophilic substances show better permeation in non-polar regions of the skin, such as the stratum corneum (Jepps *et al.*, 2013:155). The layer of skin beneath the viable epidermis is the dermis (Bartoli, 2010:22, Menon, 2002:S3-S17; Waller & Maibach, 2005:221-235). The transport of molecules through the dermis occurs through diffusion in an aqueous medium (Jepps *et al.*, 2013:156).

These three layers of the skin act as a penetration barrier to keep xenobiotics, such as drugs, from entering the skin and hence the systemic circulation (Wiedersberg & Guy, 2014:84; Yamashita & Hashida, 2003:1185). In order to enable skin penetration of a drug, the physicochemical properties of the API and the skin need to correlate with each other. Factors, such as drug polarity and molecular weight, have proven to affect the permeation of a drug through the skin (Uchida *et al.*, 2015:113; Yamashita & Hashida, 2003:1188). Clofazimine has a molecular weight of 473 g/mol, hence it is smaller than 500 Da (Dalton), which is regarded as suitable for skin permeation (Kumar & Philip, 2007:634, Reddy *et al.*, 1999; TB alliance, 2008:96; Wiedersberg & Guy, 2014:84). The level of permeation a substance shows in the stratum corneum is determined by the particular polarity of the substance. Clofazimine has a log P (octanol-water partition coefficient) value of 7.132, which is too high for ideal skin penetration of a drug, as a log P between 1 and 3 is preferable (Hadgraft, 2004:292; Swart *et al.*, 2005:72; TB alliance, 2008:96). Clofazimine is lipid soluble and therefore has a low aqueous solubility of 0.3 mg/ml (Peters *et al.*, 2000:82); the ideal aqueous solubility for skin permeation is > 1 mg/ml (Naik *et al.*, 2000:319). Since clofazimine has such unfavourable physicochemical properties concerning skin permeation, it will be formulated into a suitable dosage form to promote possible topical delivery of the API.

To attempt the optimisation of the topical delivery of clofazimine, fatty acids that occur naturally in the human skin was used in the dispersions in this study, i.e. linoleic acid (18-carbon fatty acid). Linoleic acid is found in avocado oil (natural oil) (Morena *et al.*, 2003:2220) and acts as a penetration enhancer of drugs through the lipid layer of the stratum corneum (Argenta *et al.*, 2014:4738; Williams & Barry, 2012:129, 132). Penetration enhancers act via altering the lipids of the stratum corneum and cause higher levels of cohesion amongst corneocytes (Argenta *et al.*, 2014:4738). Thus avocado oil was included in the formulations made in this study for two reasons, namely as a penetration enhancer and as the oil phase for the nano-emulsion, the emulgel containing the nano-emulsion and the emulgel containing the coarse emulsion.

The topical delivery system investigated during this study was a nano-emulsion. A definition of a nano-emulsion is an oil-in-water (o/w) or a water-in-oil (w/o) system that is thermodynamically unstable, but kinetically stable, and having a droplet size ranging between 10 – 500 nm (Solans *et al.*, 2005:102; Sosnik *et al.*, 2010:550; Tadros *et al.*, 2004:303; Usón *et al.*, 2004:415). Nano-

emulsions have demonstrated to have several advantages over other pharmaceutical delivery vehicles. Nano-emulsions allow for a smaller dosage with increased bioavailability compared to other dosage forms due to the increase in retention time at a target site (Lovelyn & Attama, 2011:626; Sharma *et al.*, 2010:3; Solans *et al.*, 2005:108). Nano-emulsions offer more stability than conventional emulsions due to a smaller droplet size, especially against the common stability problems found amongst emulsions such as Oswald ripening, creaming and sedimentation (Lovelyn & Attama, 2011:626; Sharma *et al.*, 2010:2; Solans *et al.*, 2005:105). Furthermore, nano-emulsions allow for quick penetration of the API due to the large surface area offered by the nano-scale droplets. Nano-emulsions can easily penetrate the skin, thus a lower concentration of the penetration enhancer is required. These types of emulsions improve the bioavailability of hydrophobic drugs in o/w dispersions (Sharma *et al.*, 2010:3). Due to clofazimine being lipophilic in nature, it was incorporated into an o/w emulsion (Solans *et al.*, 2005:102). Avocado oil was used as the oil phase. Energy is required to form nano-emulsions and these emulsions were therefore prepared by using an ultrasonicator (Solans *et al.*, 2005:103; Usón *et al.*, 2004:415).

An emulgel formula was prepared with the nano-emulsion incorporated into an emulgel formula since nano-emulsions are very difficult to apply to the skin due to a very low viscosity. For the purpose of comparison, another emulgel formula was prepared containing a conventional emulsion. The outcome of the comparison led to the conclusion as to which preparation is preferable for the topical delivery of clofazimine.

## **1.2 Research problem**

As mentioned, there is no topical treatment available for CTB and the stratum corneum is an impermeable and excellent protective lipid barrier of the skin and thus hinders the topical delivery of drugs. The physicochemical properties of clofazimine are not ideal for topical delivery and will therefore be formulated in a nano-emulsion, together with the two emulgel formulas, in order to attempt to cross the stratum corneum and deliver clofazimine topically.

## **1.3 Aims and objectives**

The aim of this study was to deliver clofazimine topically via a nano-emulsion formulated with a natural oil containing 18-carbon fatty acids (linoleic acid), i.e. avocado oil. The effectiveness of the formulated clofazimine in a nano-emulsion was evaluated by investigating whether such dispersions would be successful in enabling clofazimine to cross the stratum corneum and to penetrate into the deeper skin layers. The nano-emulsion dispersion was compared to an emulgel containing a nano-emulsion and to a conventional emulsion, i.e. an emulgel containing a coarse emulsion in order to determine if the nano-emulsion delivers the API more effectively topically than a conventional emulsion.

The objectives of this study were:

- To validate a suitable high performance liquid chromatography (HPLC) method to determine the clofazimine concentrations of three pharmaceutical preparations, i.e. the test samples generated from the dispersion (nano-emulsion) and from two formulas (emulgel containing a nano-emulsion and an emulgel containing a coarse emulsion).
- To determine the aqueous solubility and octanol-buffer distribution coefficient (log D) of clofazimine.
- To prepare a nano-emulsion, an emulgel containing a nano-emulsion and an emulgel containing a coarse emulsion, all containing natural avocado oil and clofazimine.
- To characterise the nano-emulsion and the two different emulgel formulas.
- To determine whether clofazimine was released from the nano-emulsion, emulgel containing a nano-emulsion and the emulgel containing a coarse emulsion by using membrane diffusion studies.
- To investigate the possible transdermal and topical delivery of clofazimine from the dispersion and the two emulgel formulas, by utilising skin diffusion studies, followed by tape stripping, respectively.

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## CHAPTER 2

# THE TOPICAL DELIVERY OF CLOFAZIMINE FROM AN AVOCADO OIL NANO-EMULSION

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### 2.1 Introduction

Tuberculosis is a disease that is caused by a group of bacteria, called the *Mycobacterium tuberculosis* complex (Wirth *et al.*, 2008:2). Over recent years, TB strains have become resistant towards the first-line treatment regime of the disease. The appearance of resistant strains of TB has necessitated the search for and development of new drugs and treatments to curb the TB epidemic that threatens humans (Caminero *et al.*, 2010:622, 626). Currently, clofazimine is being investigated as one of the potentially new drugs for the treatment of MDR-TB. To date very little clinical data is available about its usage in the treatment of resistant TB (Caminero *et al.*, 2010:622, 626).

Clofazimine is classified as a riminophenazine for the narrow spectrum treatment of various mycobacterial infections, including MDR-TB. This active pharmaceutical ingredient (API) has a redox property that enables it to kill the MDR-TB bacterium (Chopra & Brennan, 1996:93; Yano *et al.*, 2011:10276).

In some cases of MDR-TB, a form of extra-pulmonary TB, known as CTB, is occasionally observed (Barbagallo *et al.*, 2002:319). CTB is a disease that often leads to unsightly skin lesions that can be difficult to hide. Unfortunately, up to date, no topical treatment for CTB is available on the market and it would therefore be advantageous to develop such a dosage form for this condition. As it takes a while for a systemic treatment to have a topical effect, a topical treatment will have to be used in combination with the systemic dosage form, in order to maximise the efficacy and the synergistic effect of such a combination of treatments. Therefore, because CTB is a disease that is sometimes associated with drug resistant TB (Barbagallo *et al.*, 2002:319) and having no topical treatment available, a need exists for preparing a topical formulation including clofazimine, for the possible treatment of CTB.

The human skin offers a crucial protective barrier to the body. The uppermost layer of the skin, i.e. the stratum corneum, consists of lipids and proteins that are impermeable to external and xenobiotic substances, due to its brick-and-mortar, bi-lipid structure (Jain *et al.*, 2010:63). Consequently, for a pharmaceutical formulation and a drug to penetrate the skin, it should possess certain physicochemical properties that correlate with those of the human skin. Most APIs do not possess the ideal physicochemical properties to penetrate the skin, which often

hinder skin penetration. Therefore, encapsulation of the API into a vehicle system, such as a nano-emulsion, may possibly aid in its ability to permeate through the skin.

A nano-emulsion is a pharmaceutical delivery system that is classified as either an oil-in-water (o/w), or a water-in-oil (w/o) emulsion and it is kinetically stable, but thermodynamically unstable, with a droplet size ranging between 10 nm to 500 nm (Sharma *et al.*, 2013:39; Sosnik *et al.*, 2010:550; Tadros *et al.*, 2004:303). This dispersion allows for quick and easy skin permeation, because the small droplet sizes of nano-emulsions offer a large surface area, making it possible to use less penetration enhancers (Sharma *et al.*, 2010:3). Nano-emulsions, when compared to other dosage forms, are capable of delivering smaller dosages, while offering an increased bioavailability of the API, because such dispersions have a longer retention time at the targeted delivery site (Sharma *et al.*, 2010:3; Solans *et al.*, 2005:108). Due to the lipophilic nature of clofazimine, it was decided to formulate it into an o/w nano-emulsion for the purpose of this study (Peters *et al.*, 2000:82).

A natural oil, avocado oil, was used as the oil phase in the clofazimine nano-emulsion formulation, to enhance the permeation of the drug through the skin. The nano-emulsion was also added to an emulgel formulation, to enhance the spreadability of the dispersion, as the viscosity of the nano-emulsion is close to that of water (Valenta & Schultz, 2004:257). Another emulgel formulation, containing a coarse emulsion, was formulated for comparison purposes to identify the formulation that would show the best membrane and skin permeation results.

## **2.2 Tuberculosis**

Tuberculosis is a disease that has affected the human species since time immemorial. This disease had been described in ancient Egyptian art and the bacterium, *Mycobacterium (M.) tuberculosis*, was even found in some ancient mummies (Daniel, 2006:1863; Zink *et al.*, 2007:380-381). TB is considered as having been the leading cause of microbial pathogen related deaths in history (Daniel, 2006:1862).

It was only as recent as in 1993 that the World Health Organization (WHO) declared TB a global emergency (WHO, 2012:3). This disease is most commonly found amongst immunocompromised hosts that have been diagnosed with diseases, such as cancer, diabetes, acquired immune deficiency syndrome (AIDS) and amongst patients who are on immunosuppressive drug treatments (Dannenberg, 1989:374; Prasad, 2005:127). Common symptoms and signs of this disease include a cough lasting for more than 3 weeks, chest pain, dyspnoea, weight loss and anorexia, haemoptysis, night sweats, anaemia, tachycardia and fever (Wejse *et al.*, 2008:112-113). TB may occur in a host after initial infection with the bacteria, causing a primary disease, or it may only occur years later to cause a post-primary disease (Hunter, 2011:498).

### **2.2.1 Infection with tuberculosis**

Tuberculosis presents as a pulmonary, or an extra-pulmonary disease (Hasan *et al.*, 2005:1). Firstly, TB bacilli are inhaled through infected particles. Thereafter, in the absence of an immune response, as would be the case in immunocompromised persons and if the pathogen is relatively virulent, it would lead to infection of the lungs by *M. tuberculosis* (Dannenberg, 1989:369-370). A lesion forms in the lungs and if the growth of the tubercle bacilli is not inhibited by macrophages, the bacilli and the lesion will continue to grow and the disease will soon be blood borne. However, in patients who are non-immunocompromised, the bacilli grow in the bronchi and infection will only commence if the caseous (cheese-like) centre of the lesion turns into a liquefied state thereby enabling the bacilli to spread throughout the lungs (Dannenberg, 1989:369). Often the liquefied sections of the lungs become necrotic in nature and burst and are spread throughout the lungs, as well as to the outside environment (Dannenberg, 1989:373). Liquefaction is regarded as a possible mechanism through which drug resistance by *M. tuberculosis* develops, since it enables the bacilli to spread throughout the lungs in large numbers, at this point possibly also enabling the bacilli to mutate, which in turn can lead to drug resistant strains of the disease (Dannenberg, 1989:369).

### **2.2.2 Multi-drug resistant tuberculosis**

Drug resistant TB is defined as a condition where the bacterium, which causes TB, is resistant to one or more of the drugs that are used in the treatment of TB. MDR-TB is defined as a TB strain, where the bacterium is found to be resistant to two of the first-line drugs being used in the treatment of this disease, i.e. isoniazid and rifampicin (Chon *et al.*, 1997:S122; Caminero *et al.*, 2010:621; Prasad, 2005:121). MDR-TB is suspected if the sputum of a patient still tests positive for TB, after the patient had been on TB treatment with first-line chemotherapeutic drugs for 2 months. Such patients must be re-evaluated and tested for possible drug resistance. If the sputum test outcomes are still positive after 5 to 6 months of treatment, the patient has indeed developed a resistant strain of TB and must be treated accordingly (Bastian & Colebunders, 1999:639). Types of drug resistance to treatment include acquired or primary drug resistance. Acquired drug resistance is found amongst patients, who had previously been treated for the disease with the necessary chemotherapeutic regimen, whereas primary drug resistance is found amongst patients who have never been treated for TB before (Prasad, 2005:121). This resistant TB strain especially occurs when the prescribed TB chemotherapy had been inadequate (Riley, 1993:442). Drug resistance to anti-TB chemotherapy often results from the treatment of the patient with insufficient chemotherapeutic agents, failure by the patient to drink one or more of the prescribed chemotherapeutic drugs, inconsistent taking of the chemotherapy, the prescription of a too low dosage of the drugs and failure by the practitioner to diagnose drug resistance in its early stages. Previously, this particular TB strain was mostly

caused by poor drug compliance, but recently, it has become a disease that is transmittable by a human vector (Faustini *et al.*, 2006:162; GBC Health, 2011:1-2; Johnston *et al.*, 2009:5). MDR-TB often occurs in developing countries, due to poor public health infrastructures, homelessness and poverty, patient non-compliance, the increasing prevalence of human immunodeficiency virus-acquired immune deficiency syndrome (HIV-AIDS), the lack of funds to purchase basic anti-TB chemotherapies, as well as the high cost of drug resistant TB treatments (Bastian & Colebunders, 1999:634; Mukherjee *et al.*, 2004:474). MDR-TB especially occurs as a co-infection amongst the majority of the younger HIV-AIDS positive population. This may be due to their compromised immune systems and possible mal-absorption of the TB chemotherapy, which represents as either a pulmonary, or an extra-pulmonary disease (Bastian & Colebunders, 1999:638, 647).

### **2.2.3 Extra-pulmonary tuberculosis**

Extra-pulmonary TB commonly occurs amongst HIV-TB co-infected patients. This type of TB often also presents together with pulmonary TB (Raviglione *et al.*, 1992:517, 519). Extra-pulmonary TB is found in patients with severely compromised immune systems, e.g. patients with HIV-AIDS, patients who abuse drugs intravenously and who use immunosuppressive drugs, patients with diabetes mellitus, end-stage renal disease, infancy and those with malignancies (Frankel *et al.*, 2009:19; Sharma *et al.*, 2005:553). This disease is found in about 50% of HIV-TB co-infected patients of which half of these patients also have pulmonary TB infections (Sharma *et al.*, 2005:553). Extra-pulmonary TB comprises of  $\pm 10\%$  of all TB infections, whilst CTB comprises of  $\pm 1\%$  of all extra-pulmonary cases, especially amongst immunocompromised patients (Barbagallo *et al.*, 2002:327-328; Bravo & Gotuzzo, 2007:173; Kandola & Meena, 2014:16).

#### **2.2.3.1 Cutaneous tuberculosis as a manifestation of extra-pulmonary tuberculosis**

CTB is defined as the manifestation of extra-pulmonary TB on the skin. This disease is often acquired from *M. tuberculosis*, from the Bacillus Calmette-Guérin (BCG) vaccination and from *M. bovis* (Frankel *et al.*, 2009:20). TB is often spread in the air, which at times presents on the skin, due to a direct delay being acquired from active or latent foci, or a blood borne disease can spread it. This disease can also spread if the bacteria are directly introduced to the mucosa of the skin, especially if the skin is broken (Frankel *et al.*, 2009:19).

#### **2.2.3.2 Classification of cutaneous tuberculosis**

CTB is classified into one of two classification systems, i.e. either as endogenous or exogenous forms, or as multibacillary or paucibacillary forms (Bravo & Gotuzzo, 2007:174; Frankel *et al.*, 2009:21). Although CTB comprises only a small number of the population having TB, it has

become relevant to consider a proper treatment of this disease, since the number of TB cases worldwide is continually growing (Bravo & Gotuzzo, 2007:173).

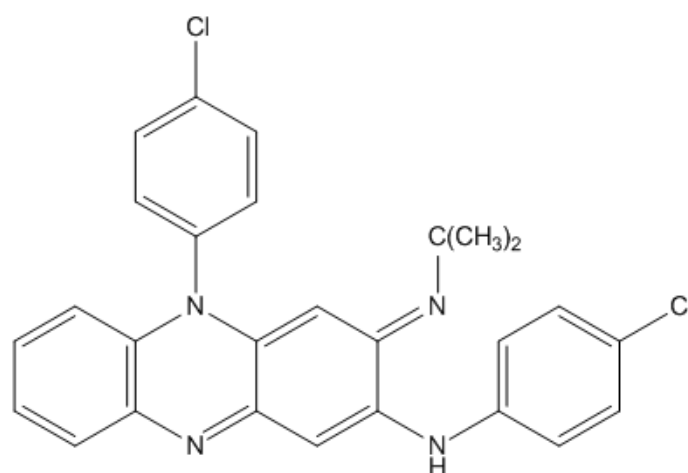
**Table 2.1:** The classification of cutaneous tuberculosis (CTB) (adapted from Bravo & Gotuzzo, 2007:174; Frankel *et al.*, 2009:21)

| Classification system               | Types                    | Sub-types   |
|-------------------------------------|--------------------------|---|
| Endogenous and Exogenous            | <b>Endogenous</b>        |   |
|                                     | (a) <i>Contiguous</i>    | Scrofuloderma and orificial TB  |
|                                     | (b) <i>Haematogenous</i> | Acute miliary TB, metastatic TB abscess (gummatous TB), lupus vulgaris and papulonecrotic tuberculids |
|                                     | (c) <i>Lymphatic</i>     | Lupus vulgaris  |
|                                     | <b>Exogenous</b>         | Tuberculous chancre, TB verrucosa cutis and lupus vulgaris  |
| Multi-bacillary and Pauci-bacillary | Multi-bacillary          | Tuberculous chancre, scrofuloderma, orificial TB, acute miliary TB and gummatous TB                   |
|                                     | Pauci-bacillary          | TB verrucosa cutis, lupus vulgaris and tuberculids  |

### 2.3 Clofazimine

Clofazimine is a red dye that was first described in 1957. It is classified as a rhimophenazine and is it used for the treatment of MDR-TB (Arbiser & Moschella, 1995:241; Bor, 1973:1451; Cholo *et al.*, 2012:290). This drug was developed for the treatment of drug resistant TB strains, because of the global crisis experienced with MDR-TB (Arbiser & Moschella, 1995:241). However, clofazimine is a second-line drug that is more toxic, but less effective in the treatment of TB and therefore seldom used to treat the disease (Xu *et al.*, 2012:1104).

Clofazimine is classified by the WHO as a Class 5 drug for the possible treatment of drug resistant strains of TB (Caminero *et al.*, 2010:622; Gopal *et al.*, 2013:1106). It was originally developed and later tested by Barry and co-workers in 1954 in Dublin for its clinical activity against MDR-TB (as cited in Bor, 1973:1451; Gopal *et al.*, 2013:1001). Studies have shown that clofazimine has good activity against MDR-TB in animal models (Gopal *et al.*, 2013:1004). Furthermore, clofazimine practically has the same chemotherapeutic potency as isoniazid and rifampicin, as found in macrophages (Reddy *et al.*, 1996:636). Although studies have shown that the inclusion of clofazimine in the treatment regimen of MDR-TB has aided with the healing of this disease, there is still doubt about its effectiveness in the treatment of MDR strains of TB (Gopal *et al.*, 2013:1002).



**Figure 2.1:** The chemical structure of clofazimine (adapted from Arbiser & Moschella, 1995:241)

### 2.3.1 Dosage and mechanism of action of clofazimine

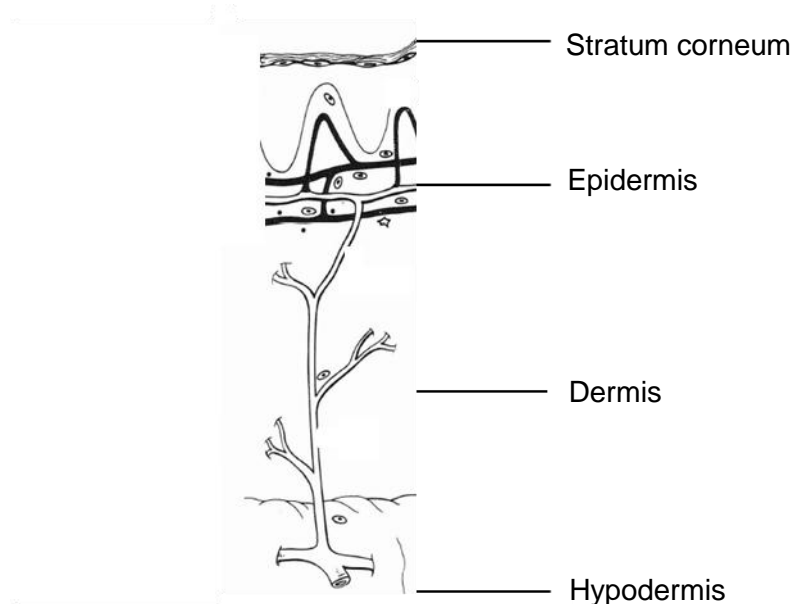
The recommended dosage for the treatment of MDR-TB with clofazimine is 100 mg daily in combination with other drugs, with the intention of keeping the bacteria from further cultivating drug resistance and to improve the treatment outcomes of clofazimine (Gopal *et al.*, 2013:1106-1107; TB alliance, 2008:97). The therapeutic blood concentration for the treatment of MDR-TB with clofazimine should range between 1 - 2 µg/ml, depending upon the particular type of MDR-TB strain that is treated (Reddy *et al.*, 1996:634).

The mechanism of action of clofazimine was thought to include binding of the drug to guanine bases in the deoxyribonucleic acid (DNA) of *M. tuberculosis*. However, its mechanism is also described as working through the redox cycling in cells and the destabilisation of the bacterial cell membranes. Clofazimine therefore increases the reactive oxidative species (ROS) (hydrogen peroxide) and their release from the neutrophils and macrophages, thereby killing MDR species (Arbiser & Moschella, 1995:241; Cholo *et al.*, 2012:291-292; Gopal *et al.*, 2013:1103; Reddy *et al.*, 1999:617, 620). Phospholipase A<sub>2</sub> is stimulated in the phagocytes, resulting in an increase in arachidonic acid and lysophosphatidylcholine in these cells, which cause the phagocytes to release superoxides, which in turn oxidase bacterial cells. Clofazimine works together with interferon gamma to kill resistant strains of TB (Gopal *et al.*, 2013:1103). Due to its lipophilic nature and longer half-life, the drug could possibly shorten the duration of the treatment of MDR-TB. Treatment of MDR-TB with clofazimine enables the drug to reverse the inhibitory effect of the MDR bacteria on the phagocytes, thereby also enhancing the immunopharmacological related effect of the macrophages (Gopal *et al.*, 2013:1106; TB alliance, 2008:97). Furthermore, the drug displays anti-inflammatory and immune properties (Gopal *et al.*, 2013:1103; Reddy *et al.*, 1999:620; TB alliance, 2008:97).

### 2.3.2 Adverse effects of clofazimine

Adverse effects that are associated with clofazimine treatments are dosage related and include red-black skin discolouration, reddish discolouration of the bodily fluids, rashes, ichthyosis, pruritus, dry skin, gastro-intestinal side effects and central nervous system effects, including sleeplessness, taste disorders, depression, fatigue, drowsiness and vertigo (Bastian & Colebunders, 1999:654; Bor, 1973:1451; Cholo *et al.*, 2012:293; Dey *et al.*, 2013:290; Gopal *et al.*, 2013:1105; TB alliance, 2008:98). The side effects of clofazimine intake, e.g. gastro-intestinal and photosensitivity can be reduced by controlling the dosage (Caminero *et al.*, 2010:626; TB alliance, 2008:98). Clofazimine can lead to higher blood sugar levels than usual and a reddish discolouration and irritation in the eyes, due to crystal deposits of the drug within the eyes (Cholo *et al.*, 2012:293; TB alliance, 2008:98). The discolouration of the skin and the bodily fluids may be present in a patient for several months to years after the cessation of clofazimine treatment. This is caused by the accumulation of the drug in all fatty tissues in the body, such as the skin and the nerves, as well as the long half-life of the drug (Arbiser & Moschella, 1995:242; Cholo *et al.*, 2012:290; Gopal *et al.*, 2013:1105; TB alliance, 2008:98).

### 2.4 Skin



**Figure 2.2:** The skin and its three main layers (adapted from Mihm *et al.*, 1976:306)

The skin is an intricate organ, consisting of numerous layers and is the largest organ in the human body (Menon; 2002:S4). It provides a unique protective barrier, as it protects the body from the external environment by preventing the penetration of xenobiotic substances and the excessive loss of bodily fluids (Yamashita & Hashida, 2003:1185). The human skin is comprised of three main layers, each different in structure and function, i.e. the epidermis, the dermis and the hypodermis (Tyagi *et al.*, 2011:1381).

### **2.4.1 Epidermis**

The epidermis is a layer of skin that comprises of lipids, packed into lamellar sheets that control the permeability barrier of the skin (Vitorino *et al.*, 2015:2699). The epidermis is composed of multiple layers of skin, consisting of avascular keratinocytes. During the maturing process, keratinocytes produce and express various lipids and proteins (Bouwstra & Ponec, 2006:2081). These layers of heterogeneous keratinocytes in the epidermis stretch from the basal layer of the skin to the top and these layers form as a result of the cells differentiating into sub-layers, through a process known as mitoses (Proksch *et al.*, 2006:160). The sub-layers of the epidermis, from the basal layer to the outermost layer of the skin include the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and the stratum corneum (Khan *et al.*, 2015:2849). The stratum corneum is known as the non-viable epidermis and the rest of the layers of the epidermis as the viable epidermis (Pathan & Setty; 2009:174).

### **2.4.2 Stratum corneum**

The stratum corneum, also referred to as the horny layer, or stratified squamous epithelium, is 10 - 15  $\mu\text{m}$  in thickness and forms the outermost layer of the epidermis (Goldstein & Williams, 2003:256; Guterres *et al.*, 2007:149; Jain *et al.*, 2010:63). It is also called the non-viable epidermis, as it consists of tightly packed flattened cells that are without nuclei in the final stages of keratinocyte differentiation, called corneocytes (Jain *et al.*, 2010:63). Epidermal differentiation is balanced through a process of loosening and shedding the top layer of dead epidermal cells, i.e. the corneocytes, through the process of desquamation (Brattsand *et al.*, 2005:198). The corneocytes are geometrically arranged and heterogeneous (Nemanic & Elias, 1980:573). Corneocytes are linked together with corneodesmosomes, which are surrounded by an extracellular lipid matrix to form the structure of the horny layer of the skin (Brattsand *et al.*, 2005:198; Haftek, 2015:483). The structure of the stratum corneum is often described in the literature as a “brick-and-mortar” structure, in which the corneocytes are represented by the “bricks” and the extracellular lipids by the “mortar” (Alexander *et al.*, 2012:27). Corneocytes are comprised of keratin filaments that are enclosed within cross-linked protein capsules (Jain *et al.*, 2010:63). The extracellular lipid matrix is filled with lipids and proteins through lamellar bodies (Elias, 2005:183). The lipids in the stratum corneum are arranged into continuous, multi-cellular sheets by the cornified envelope, known as the lamellar sheets (Eckhart *et al.*, 2013:3471).

The lipids in the stratum corneum’s structure are responsible for the barrier function of the skin. Cellular differentiation in the epidermis cornifies the stratum corneum, which in turn makes it an impermeable barrier (Jain *et al.*, 2010:63).

### **2.4.3 Dermis**

The dermis comprises of asymmetrical connective tissues and forms the layer of the skin beneath the epidermis. It is separated from the epidermis by a thin basal membrane (Monteiro-Riviere, 2006:12). The dermis is composed of two strata, the stratum papillae and the stratum reticulare (Zoschke *et al.*, 2015:2785). This layer of skin is 2 - 5 mm in thickness and is composed of collagen fibrils and connective tissues, surrounded by an amorphous substance of muco polysaccharides. The function of the connective tissue is to offer flexibility to the dermis. The collagen fibrils are responsible for the support of the dermis and help it to maintain its shape (Benson 2012:10-11).

The dermis is a well hydrated and highly vascularised layer of tissues and in accordance with its structure, it offers low resistance to the penetration of drugs. However, the transdermal delivery of lipophilic drugs may be problematic, due to the polarity of the tissues of the dermis. The vascularisation of the tissues ensures the diffusion of the molecules from the dermal-epidermal junction into the bloodstream. This enables the applied drug to penetrate transdermally from the surface of the skin into the dermis tissues at a concentration gradient. Larger molecules penetrate the skin through lymph vessels in the dermis. Sensory nerves and the appendages are located in the dermis (Benson, 2012:10).

### **2.4.4 Appendages**

The appendages that originate in the dermis include the sebaceous glands, the hair follicles, nails, apocrine sweat glands and eccrine glands. Hair follicles are found all across the skin, except on the lips, hand palms and the soles of the feet. Sebaceous glands are associated with hair follicles and are responsible for the secretion of sebum, which consists of free fatty acids, triglycerides and waxes. The sebum's functions are to offer lubrication and protection to the skin, while maintaining the skin's pH at 5. Eccrine glands comprise of about 1 in each 10 000<sup>th</sup> of the body's surface and secrete sweat during exercise, emotional stress and in response to high temperatures. Apocrine glands are appendages that include small glands found in the nipples, and in the axillary and anogenital areas (Benson, 2012:10-11).

The appendages in the dermis offer a breach in the stratum corneum barrier and enable the transport of particles through the skin (Kadadia & Conway, 2015:2823).

### **2.4.5 Hypodermis**

The hypodermis, also known as the subcutaneous tissues, is the layer of skin tissue underneath the dermis. This layer consists of loose connective tissues that are filled with lipocytes. Lipocytes are packed into fat lobules that are connected through collagen and elastin fibres (Lai-

Cheong & McGrath, 2009:224). The function of the hypodermis is to provide the skin with nerves and blood via the blood vessels. However, the main function of this layer of skin is the storage of excess energy, to ensure that the body's heat is maintained and to aid with the absorption of physical shocks to the body (Benson, 2012:11).

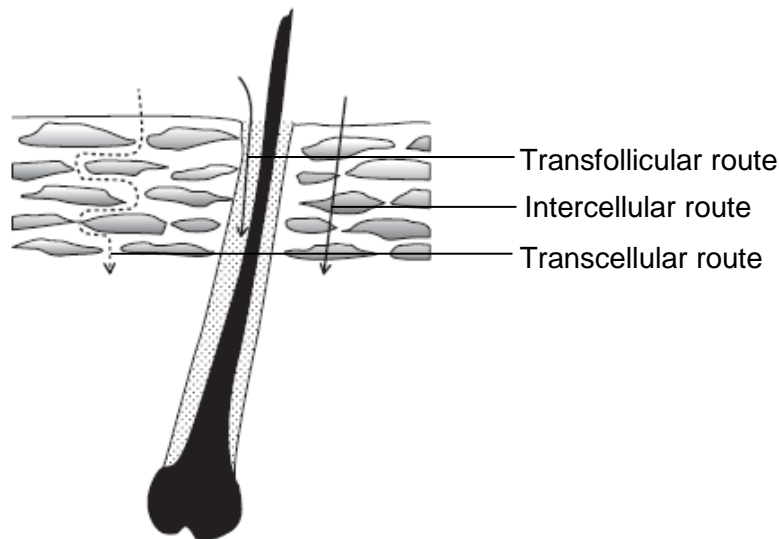
## **2.5 The three main skin penetration pathways**

The human skin offers several routes for the penetration of drugs, the main route being through the intercellular spaces (Hadgraft, 2004:292). There are three main routes for the penetration of drugs into the human skin through passive diffusion, i.e. the transappendageal, the transfollicular and the transepidermal routes (Geusens *et al.*, 2011:200; Ghosh *et al.*, 2015:2771).

The transappendageal route is only partly involved in drug penetration, as it only comprises of about 0.1% of the total skin surface area of the body. This route offers a drug penetration path through the sweat glands and hair follicles in the skin (Geusens *et al.*, 2011:200; Ghosh *et al.*, 2015:2771). Hair follicles form part of the transappendageal pathway, or shunt route for drug penetration through passive diffusion into the skin. The transappendageal route is a very important route for nano-particles, such as those found in nano-emulsion formulations, as it offers a pathway through the stratum corneum and contains nearby blood vessels, located in the dermis and in the hypodermis layers of the skin (Kadadia & Conway, 2015:2823; Lademann *et al.*, 2005:232).

The transepidermal drug permeation route can be divided into two sub-routes for skin penetration, i.e. the intercellular and the transcellular routes. An API firstly travels via the transcellular route, through the cytoplasm of the corneocytes and then through the extracellular lipids (Geusens *et al.*, 2011:200; Khan *et al.*, 2015:2848). For a drug to hence successfully permeate through the skin along the transcellular route, it needs to partition through the intercellular lipid layers, as well as through the keratinocytes (Geusens *et al.*, 2011:200). The intercellular route of transport occurs by way of passive diffusion through the lipid lamellae. Hydrophilic drugs penetrate the stratum corneum across the polar routes, such as the transcellular way, whereas lipophilic drugs penetrate the stratum corneum through non-polar routes along the lipid lamellae (Ghosh *et al.*, 2015:2771; Kadadia & Conway, 2015:2823).

The third route of skin permeation is the transfollicular, or shunt route, despite hair follicles only making up about 0.1% of the total skin surface area (Barry, 2001:101). Hair follicles, together with the sebaceous glands, provide quite a large surface area. The hair follicles extend from the dermis, through the skin and into the stratum corneum. This enables transdermal absorption below the skin surface (Verma *et al.*, 2016:1).



**Figure 2.3:** The three main skin penetration pathways for the passive diffusion of drugs into the skin (adapted from Khan *et al.*, 2015:2850)

The diffusion of drugs occurs through passive diffusion, mainly according to Fick's law, through the transepidermal, or transappendageal skin penetration routes (Cilurzo *et al.*, 2015:2733; Khan *et al.*, 2015:2850).

### 2.5.1 Mathematical models of skin permeation

Permeation through the skin along the described polar and non-polar routes happens in accordance with Fick's law of diffusion through passive diffusion (Cilurzo *et al.*, 2015:2733; Hadgraft, 2004:292). Fick's first law of diffusion is described by the following equation (Cilurzo *et al.*, 2015:2733; Hadgraft, 2004:292):

$$J = K D (C_{app} - C_{rec}) / h \quad \text{Equation 2.1}$$

Where:

J = the steady-state flux or transfer per unit area of skin;

D = the diffusion coefficient;

K = the partition of a substance between the skin and the formulation applied to the skin;

h = the intercellular pathways of the diffusion route;

$C_{app}$  = the applied concentration of the substance in the vehicle;

$C_{rec}$  = the concentration of the solution in the receptor phase.

## 2.6 Skin delivery

The topical delivery of drugs appears to have become the preferred route of drug administration in comparison with other routes (Gratieri *et al.*, 2013:610). Drug delivery in the skin occurs at the target site, i.e. topically, or through the skin into the systemic circulation, i.e. transdermally. Although the topical delivery of drugs is an ancient practice, the transdermal delivery of drugs through the skin is a novel practice in the pharmaceutical industry that is still being investigated (Zhang *et al.*, 2015: 2713).

### 2.6.1 Advantages of the delivery of drugs through the skin

Some advantages of delivering drugs through the skin include:

- The oral route causes a significant loss in the bio-availability of drugs, due to their first-pass liver metabolism when taken orally, which is not the case when administering drugs transdermally (Ruby *et al.*, 2014:1421).
- There is high patient non-compliance with the oral administration of drugs (Ruby *et al.*, 2014:1421).
- The topical and transdermal delivery routes offer non-invasive and painless drug administration methods, which improve patient compliance (Khan *et al.*, 2015:2850; Ruby *et al.*, 2014:1421).
- The avoidance of side effects, which could result in patient non-compliance and thereby a lowered drug bio-availability (Tanwar & Sachdeva, 2016:2274).
- Topical and transdermal delivery can achieve zero-order kinetics and can thus help to deliver stable blood levels over prolonged periods of time (Gratieri *et al.*, 2013:610).
- The avoidance of drugs passing through the gastro-intestinal route, which prevents drug degradation that is associated with pH and enzymes (Tanwar & Sachdeva, 2016:2279).
- Topical drugs are easily applied and are thus patient friendly (Khan *et al.*, 2015:2849).
- In the event of any adverse effects, a topical or transdermal formulation can easily be removed from the area of the skin to which it was applied (Khan *et al.*, 2015:2850).
- The delivery of drugs through the skin can be controlled, which allows for a lower drug dosage, less medication costs, less side effects and a lower dosage frequency, which could ultimately improve patient compliance (Khan *et al.*, 2015:2849).
- Another advantage of topical and transdermal delivery is that the human skin structure and biology are almost a constant, which reduce variability in drug absorption (Khan *et al.*, 2015:2849).

## 2.6.2 Disadvantages of the delivery of drugs through the skin

Topical and transdermal drug delivery also have their disadvantages and challenges, which may include the following:

- Skin and drug binding may occur that would lower the absorption and bio-availability of a drug (Tyagi *et al.*, 2011:1380).
- Possible skin allergy or irritation, due to the pharmaceutical preparation's excipients, or the API (Tyagi *et al.*, 2011:1380).
- Possible dosage dumping of the drug in topical or transdermal formulations could occur (Tyagi *et al.*, 2011:1380).
- It is difficult to create a topical or transdermal delivery system that would deliver more than 5 mg of the drug per day (Zhang *et al.*, 2015:2713).
- Dermal metabolism of the drug in the formulation could possibly occur (Tyagi *et al.*, 2011:1380).
- The release of the drug from the formulation into the skin may not occur in a controlled manner (Tyagi *et al.*, 2011:1380).
- The stratum corneum barrier could limit the transdermal delivery of the drug that is released from the formulation, resulting in a slow onset in the working of the drug and possibly low blood levels (Tanwar & Sachdeva, 2016:2279; Tyagi *et al.*, 2011:1380).

## 2.7 Physicochemical factors influencing skin permeation

The physicochemical properties of a drug that influence its percutaneous absorption include its partition coefficient, its diffusion coefficient, the drug's solubility, its pH,  $pK_a$  and ionisation, its molecular size and weight, the drug's concentration and its melting point.

### 2.7.1 Partition coefficient

The partition coefficient of a drug is defined as the re-arrangement of molecules between the oil and water phases. It is an indication of how lipophilic an API is, as well as how likely it is to show signs of skin permeation through the various hydrophilic and lipophilic layers of the skin (Aulton, 2000:243; Farahmand & Maibach, 2009:2). For a drug to permeate through the skin, an optimal partition coefficient (K) is needed (Sharma *et al.*, 2011:75). The partition coefficient measures the flux of a drug through the skin and especially through the stratum corneum. The partition coefficient plays a very crucial role when a drug has to diffuse through a membrane. When a drug diffuses from a delivery vehicle towards the membrane and through it, the partition coefficient of the stratum corneum to the vehicle is vital for ensuring that a high concentration of the diffusant is present, initially in the principal layer of the membrane (Barry, 2007:578). If the

partition coefficient is very high, a drug will easily pass through the horny layer of the skin, but will struggle to pass through the underlying aqueous layers of the skin. If the partition coefficient is very low, a drug will not be able to pass through the lipophilic layers of the stratum corneum (Barry, 2007:578; Sharma *et al.*, 2011:75).

The log D value of clofazimine was determined during this study, using octanol, which is an organic solvent that mimics the properties of biological membranes. The log D value of an API is an indication of the drug distribution that would take place during percutaneous diffusion through the lipid and hydrophilic layers of the skin (Aulton, 2002:243; Barry, 2002:512). Clofazimine has a log P (octanol-water partition coefficient) value of 7.132, which is deemed too high for ideal skin penetration of a drug, as a log P between 1 and 3 is preferable (Hadgraft, 2004:292; TB alliance, 2008:96). Clofazimine is therefore not regarded as an ideal candidate for skin permeation, because of its unsuitably high log P value.

### **2.7.2 Diffusion coefficient**

The diffusion coefficient (D) of a drug is used to measure the permeation rate of a drug through the skin under specific conditions. The diffusion of drug molecules is the lowest in the human skin, especially in the stratum corneum. The diffusion coefficient of a drug taken at a constant temperature is directly dependent upon the diffusional media and the physicochemical properties of the API and sometimes upon interactions between the diffusional media and the drug's physicochemical properties (Barry, 2007:577). The diffusion coefficient is directly proportional to the temperature of a delivery vehicle and inversely proportional to the viscosity of a vehicle (Williams, 2003:18).

Topical diffusion occurs through passive diffusion, according to Fick's law (Cilurzo *et al.*, 2015:2733; Hadgraft, 2004:292). Fick's first law of diffusion states that the amount of a substance applied to a skin surface area is directly dependent upon its concentration gradient (Benson, 2005:24; Williams, 2003:18). Fick's first law of diffusion is represented by the equation in Section 2.5.1. As stated, two very important factors influence skin permeation according to this equation, i.e. the concentration of the API that is incorporated into the particular pharmaceutical preparation and the API's partition coefficient (Cilurzo *et al.*, 2015:2733; Hadgraft, 2004:292).

### **2.7.3 Solubility**

Because the human skin consists of both lipophilic and hydrophilic layers, for an API to be able to permeate through the skin, it should also possess both lipophilic and hydrophilic properties (Williams, 2007:37). The extent to which a substance would permeate the stratum corneum is determined by its particular polarity. As mentioned, clofazimine has an unfavourably high log P value of 7.132 for skin penetration, as a log P between 1 and 3 is preferable (Hadgraft,

2004:292; TB alliance, 2008:96). Clofazimine is lipid soluble and therefore has a low aqueous solubility of 0.3 g/l at a pH of 7.8 (Peters *et al.*, 2000:82). The ideal aqueous solubility of a drug for skin permeation is above 1 mg/ml (Naik *et al.*, 2000:319). Due to the lipophilic nature of clofazimine, it is not deemed ideal for skin delivery. Since clofazimine has such unfavourable physicochemical properties for skin permeation, it was formulated into a suitable dosage form, aimed at promoting its topical delivery.

#### **2.7.4 pH, pK<sub>a</sub> and ionisation**

The pH of a formulation has a significant impact on skin permeation, by influencing the diffusivity of a drug or formulation (Cázares-Delgado *et al.*, 2005:205; Shin *et al.*, 2005:69). The ideal pH for skin permeation ranges between 5 and 9 (Naik *et al.*, 2000:319).

Skin permeation is improved when a formulation's molecules are in their most unionised form, in accordance with the pH partition hypothesis. The part of the unionised drug (lipophilic) that is applied to the skin will determine the effectiveness of the membrane gradient through the skin. The ionised species could also penetrate the stratum corneum to a lesser extent, because the ionised species show a higher aqueous solubility than the neutral species, as are found in saturated, or almost saturated solutions. These ionised species contribute significantly enough towards enhancing the flux of the formulation during the skin permeation process (Barry, 2007:576; Shargel *et al.*, 2005:382).

Drugs are usually classified as weak acids or weak bases, if their levels of dissociation and ionisation are reliant upon the pH of the body, as well as upon the pK<sub>a</sub> or pK<sub>b</sub> values of the drug (Barry, 2007:576; Hillery, 2001:23-24). An API that needs a certain amount of ionised or unionised species can be adjusted by adding a buffer solution that is two to three units less or more than the pK<sub>a</sub> and pK<sub>b</sub> of the API, respectively (Williams, 2003:70).

The pK<sub>a</sub> value of clofazimine is 8.51 (Drugbank, 2015). The highest percentage of unionised species of this API was calculated as 97% at pH 7. The pharmaceutical preparations made during this study were therefore kept at a pH as close as possible to 7, where most of the species would be in their unionised form. During this study, the pharmaceutical preparations therefore had a pH ranging between 5 and 9, to achieve a maximum level of unionised species, suitable for skin permeation.

#### **2.7.5 Molecular size and shape**

The molecular size and shape of molecules play an important role in the permeation of a solute through the skin. Lower weight molecules show more effective and faster skin permeation than molecules with a higher molecular weight (Barry, 2007:578; Sharma *et al.*, 2011:75). However, it is difficult to determine the real effect of molecular size on the speed and effectiveness with

which a drug would permeate the skin and its layers, as it is almost impossible to separate the effect that the drug size has on the flux across the skin, from the changes in solubility, due to the partition coefficient. The partition coefficient of a drug is dependent upon its molecular size. Since the molecular shape of a drug is impossible to separate from the partition coefficient, it is impossible to determine the effect of the molecular shape on the permeation of a drug through the skin (Barry, 2007:578).

Drugs that have a molecular mass below 500 Da (Dalton) are more likely to penetrate the stratum corneum. Because clofazimine has a lower molecular weight of 473 g/mol, it is regarded as suitable for skin permeation (Kumar & Philip, 2007:634; Lipinski *et al.*, 2001:9; Reddy *et al.*, 1999; TB alliance, 2008:96).

### **2.7.6 Drug concentration**

Since the permeation of a drug through the skin occurs in accordance with Fick's law of diffusion, the flux of a solute is directly dependent upon the concentration gradient across the stratum corneum barrier. Generally, the flux of the drug will be higher if the concentration gradient is higher (Barry, 2007:577; Sharma *et al.*, 2011:75). To ensure a maximal drug flux through the skin, the donor solution should be saturated. The concentration gradient and the chemical potential gradient play important roles in skin diffusion (Barry, 2007:577).

During this study, the concentration of clofazimine that was present in the dispersion was 0.0142% m/v. The concentration of clofazimine in both the emulgel formulations was 0.0071% m/v. It was therefore expected that the topical permeation of clofazimine from the emulgel formulations would be lower than that from the nano-emulsion.

### **2.7.7 Melting point**

A direct correlation exists between a compound's melting point and its solubility. A lower melting point is associated with better solubility and skin permeation of a drug (Williams, 2003:37). The preferable melting point of an API for crossing the stratum corneum is at a temperature of 200°C (Naik *et al.*, 2000:319). Since clofazimine has a melting point of 210°C to 212°C (Drugbank, 2015), it was not deemed ideal for topical delivery.

## **2.8 Chemical penetration enhancers**

Chemical penetration enhancers are chemicals that hydrate the skin, and they are pharmacologically inert and non-damaging to the skin (Barry, 2001:106). Chemical penetration enhancers typically include poly-alcohols, water, alcohols, amines, amides, azones and their derivatives, terpenes, fatty acids, pyrrolidones, sulphoxides, alkanes, surfactants (anionic, non-ionic and cationic), esters and phospholipids, among others (Barry, 2001:106; Naik *et al.*, 2000:321). They are used to assist with skin permeation, and to enhance the flux of a drug, by

making it possible for molecules that normally show poor skin permeation to penetrate the skin and its layers (Barry, 2001:106; Naik *et al.*, 2000:321). Chemical penetration enhancers act in various ways to enhance skin permeation through the stratum corneum. These methods include one of three basic methods, namely (1) interaction with proteins in cells, (2) improved drug partition into the horny layer and (3) disruption of the stratum corneum's lipid structures (Pathan & Setty, 2009:175).

Although chemical penetration enhancers should exert certain ideal characteristics, only a few possess some of these qualities, including a rapid working action, being non-toxic, non-allergenic, non-irritating and pharmacologically inert. Furthermore, these chemicals should be easily removed from the skin once transdermal delivery of the drug is achieved to allow for the skin's barrier properties to return back to normal (Pathan & Setty, 2009:175).

## **2.9 Nano-emulsions**

### **2.9.1 Nano-carriers**

A nano-carrier is defined as a drug carrier that has a size smaller than 1  $\mu\text{m}$ , ranging between 50 - 500 nm (Simone *et al.*, 2008:2). Nano-carriers have a large surface area that enhances drug delivery to the skin. Typically, these systems act by enhancing the drug penetration process (Bolzinger *et al.*, 2012:163; Borm & Müller-Schutte, 2006:235). The drug dosage that is required in a formulation can thus be reduced to lower the toxicity of the drug in the body, while the system enhances its bioavailability (Khan *et al.*, 2015:2850). Nano-particles carry colloidal particles with ease, which give them a binding ability and the ability to absorb compounds (Kakadia & Conway, 2015:2825). Additionally, nano-carriers can improve the bioavailability of hydrophobic drugs and they are effortlessly manufactured, sterilised and controlled. Additional advantages being offered by lipid-based nano-carriers include that they are non-toxic and biodegradable (Rupenagunta *et al.*, 2011:2069-2070).

### **2.9.2 Nano-particles that are present in nano-emulsions**

Nano-particles in nano-based carriers are defined as polymeric or lipid particles, with a size range below 300 nm. Nano-particles are typically formulated in the form of nano-emulsions (Anton & Vandamme, 2009:142). Nano-emulsions are defined as a mixture of immiscible liquids, typically when one liquid is dispersed within the other in nano-scale droplets, with a surfactant or emulsifying agent being added to the dispersion, ranging from 20 - 300 nm, or even from 50 - 1000 nm in size (Anton & Vandamme, 2009:142; Sharma *et al.*, 2010:2; Tyagi *et al.*, 2011:1382).

### 2.9.3 Different kinds of nano-emulsion systems

Nano-emulsions are classified into three types, i.e. oil-in-water (o/w), water-in-oil (w/o) and bi-continuous nano-emulsions. Oil-in-water nano-emulsions are emulsions in which oil droplets are dispersed into a continuous aqueous phase. Lipophilic drugs can be incorporated into an o/w nano-emulsion and the drug is dissolved in the oil phase (Weiss *et al.*, 2006:R3). In w/o nano-emulsions, the water phase is dispersed as droplets through a continuous oil phase. Bi-continuous nano-emulsions are classified as nano-emulsions in which micro-domains of water and oil are co-dispersed. A typical example of these types of nano-emulsions is an emulsion that is formulated into another emulsion. Typically, these types of emulsions are composed of droplets of a liquid that are dispersed into a droplet of another liquid that is dispersed into a continuous phase (Kumar & Singh, 2012:40).

For the purpose of this study, an o/w dispersion was prepared and the lipophilic clofazimine was incorporated into the oil phase of the dispersion.

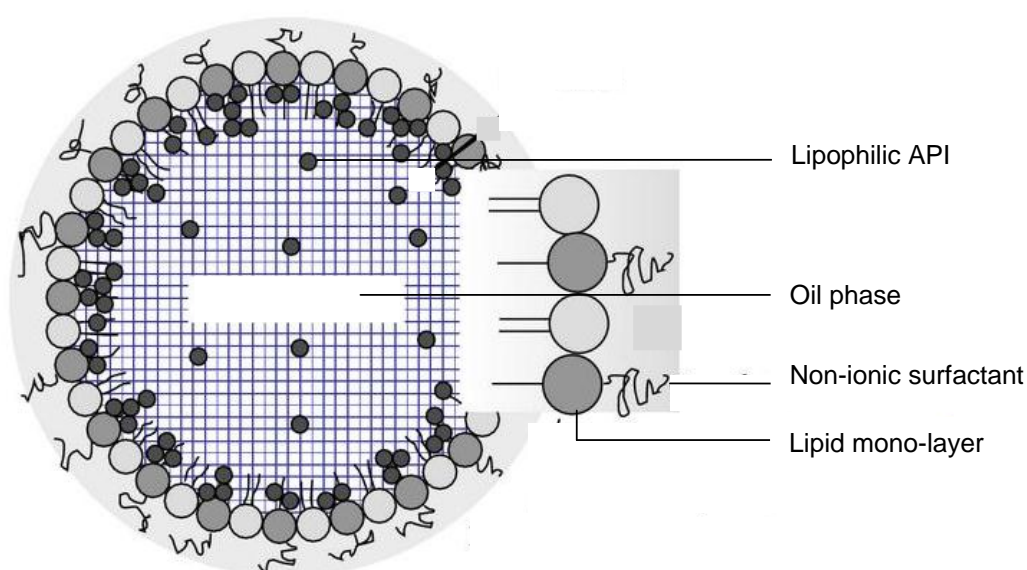
### 2.9.4 Nano-emulsions

A nano-emulsion is typically formulated by using an aqueous phase, an oily phase, surfactants and/or co-surfactants and emulsifying agents (Jaiswal *et al.*, 2015:124; Mason *et al.*, R637). These kinds of emulsions are used to deliver both lipophilic and hydrophilic drugs. By adding surfactants and/or co-surfactants to a nano-emulsion formulation, the interfacial film in between the droplets is reduced in order to decrease the free energy in the system to form thermodynamically stable emulsion droplets. Emulsifying agents are added to the formulation to reduce the interface tension area between the two different phases, to form a fresh interfacial area in an emulsion, which assists with the nano-emulsion formation (Jaiswal *et al.*, 2015:124).

Furthermore, the surfactants and oil within the formulation can possibly act as penetration enhancers through the stratum corneum, by interacting with the skin's lipids, by fluidising the stratum corneum barrier and thus enhancing skin permeation of the topically applied formulation. Fluidisation occurs through the hydration and swelling of the skin's corneocytes and proteins in the lipid bi-layers of the skin. This leads to the disruption of the lipid bilayers of the skin and the opening of aqueous pores to enable skin permeation of the released API from the applied nano-emulsion formulation (Thong *et al.*, 2007:275, 277, 278).

Nano-emulsions comprise of very large surface to mass ratios and are extremely target specific, which make them incredibly attractive as pharmaceutical delivery vehicles (Ravi & Padma, 2011:2, 4). As mentioned, because nano-emulsions are thermodynamically stable and regarded as being kinetically stable, due to the low levels of kinetic energy in the system, instabilities within these formulations are limited (Anton & Vandamme, 2011:979). Instabilities

that may occur in nano-emulsions include Oswald ripening, flocculation, creaming and coalescence. Such instabilities, however, rarely occur, since nano-emulsions possess such small droplet sizes (Anton & Vandamme, 2011:979). These nano-scaled droplets are able to overcome gravitational forces with Brownian motion, which leads to a higher diffusion rate than the rate of sedimentation, or creaming that result from gravitational forces (Sharma *et al.*, 2010:2-3; Tadros *et al.*, 2004:303). Smaller droplets of nano-emulsions offer a much higher dispersity as is found amongst conventional emulsions, which in turn keeps the system from flocculating and from forming common emulsion instabilities. Additionally, the usage of non-ionic surfactants also helps to lessen nano-emulsion instabilities (Sharma *et al.*, 2010:3-4). Below is an illustrated image of one of the droplets in an o/w nano-emulsion.



**Figure 2.4:** Illustration of a nano-emulsion droplet with the oil phase and the API incorporated into the droplet (adapted from Anuchapreeda *et al.*, 2012:9)

### 2.9.3.1 Nano-emulsion instabilities

When the interfacial area of nano-emulsion droplets is reduced, it typically leads to flocculation, which results in the coalescence of droplets, as well as Oswald ripening (Anton *et al.*, 2008:186). Oswald ripening is the most common instability that occurs in nano-emulsions, and it is caused by differences in the solubility of the smaller and the larger droplets, as well as by the nano-emulsions' polydispersity (Anton & Vandamme, 2011:979; Sharma *et al.*, 2010:2). Oswald ripening causes smaller droplets with a large radius of curvature to change into larger droplets with a small radius of curvature. This inevitably leads to the coagulation of the droplets into larger droplets, thereby shifting the droplet size distribution to much larger sizes. The nano-emulsion becomes turbid from Oswald ripening, especially after being stored for prolonged

periods of time (Sharma *et al.*, 2010:5). Such instability can be prevented by adding a polymeric surfactant, or a combination of surfactants in order to increase the elasticity of the formulation and to keep the charge of the electrical double layer constant (Sharma *et al.*, 2010:5; Tadros *et al.*, 2004:303-304).

Other instabilities found amongst nano-emulsions include sedimentation and creaming (Sharma *et al.*, 2010:5). When the free energy in a nano-emulsion system is reduced, especially at the interfacial area, it leads to the growth of the emulsion droplets, causing instabilities, such as creaming and sedimentation (Anton *et al.*, 2008:187). Sedimentation is caused by Van der Waal's forces between the droplets, which cause the droplets to attract each other and to subsequently result in flocculation, droplet aggregation and growth and hence the instability of nano-emulsions. However, the small droplet sizes and the use of non-ionic surfactants in nano-emulsion formulations prevent the occurrence of flocculation. Droplet coalescence is prevented by adding a multi-cellular surfactant film in the formulation. This surfactant efficiently spreads across the droplets in the nano-emulsion, which prevents droplet aggregation and growth (Sharma *et al.*, 2010:5). If weak interfacial forces exist between droplets, they aggregate, which results in changes in the size distribution and thus in creaming of the nano-emulsion (Dickinson *et al.*, 1997:517). Creaming is an instability found amongst nano-emulsions, during which the droplets of the emulsion rise to the top of the emulsion (Robins, 2000:265). This instability is prevented by the Brownian motion forces being stronger than the effect of gravitational forces on the nano-emulsion, which keeps the droplets in dispersion (Tadros *et al.*, 2004:303).

### **2.9.3.2 Energy required in formulating nano-emulsions**

A certain amount of energy is required to successfully manufacture nano-emulsions, by employing one of two main methods, i.e. high energy methods, or low energy methods (Sharma *et al.*, 2010:5). Low and high pressure energy is utilised to make nano-emulsions by using machinery, such as high pressure homogenisers, ultrasonicators (low pressure) and micro-fluidisers (high pressure) (Jafari *et al.*, 2006:484; Jafari *et al.*, 2008:1195; Sharma *et al.*, 2010:3).

#### **2.9.3.2.1 High energy methods**

High energy methods employ mechanical energy to form nano-emulsions. Such methods use mechanical forces to break up larger droplets into smaller nano-sized droplets (Yukuyama *et al.*, 2015:3). The advantages of using high energy methods include control over particle sizes, the ability to acquire the desired properties of the nano-emulsion, the ability to manufacture at industrial level and also to decide between different stabilities, rheologies and emulsification colours (Lovelyn & Attama, 2011:627). However, high energy methods are costly, are often not

reproducible on large scale and also do not take thermolabile drugs into consideration during the formulation process (Lovelyn & Attama, 2011:627; Sharma *et al.*, 2010:3).

Despite the disadvantages, nano-emulsions are often prepared by employing high energy methods. The ultrasonication method is considered the best way to reduce the droplet sizes of emulsions to produce nano-emulsions (Sharma *et al.*, 2010:4). This method utilises a sonicator to create smaller nano-scaled droplets through interfacial waves and cavitations (Anton *et al.*, 2008:188). Ultrasonic energy is hence used to reduce the larger droplets into smaller nano-scale droplets (Chen *et al.*, 2011:357). The limitations of this high energy method are:

- It is unsuitable for large scale manufacturing and is it used for the production of small nano-emulsions batches only.
- The high levels of heat that are generated through the energy being applied to the system can lead to the decomposition of the API and other emulsion components.
- The droplet size distribution is inadequate (Chen *et al.*, 2011:357; Sharma *et al.*, 2010:4; Yukuyama *et al.*, 2015:3).

An alternative high energy method being used to prepare nano-emulsions uses a high pressure homogeniser, or a piston homogeniser (Lovelyn & Attama, 2011:627). High pressure homogenisation can be employed for lipophilic or hydrophilic drugs in the formulation. Typically, the drug is formulated in a medium that has the same hydrophilic-lipophilic balance (HLB) value than the API (Chen *et al.*, 2011:357). High pressure is applied to a pre-formulation, consisting of an aqueous and an oil phase, a surfactant and/or a co-surfactant to form a nano-emulsion with droplet sizes of about 1 nm (Lovelyn & Attama, 2011:627; Sharma *et al.*, 2010:4). To enable the formation of nano-emulsions, this machinery uses forces, such as turbulence, hydraulic shear and cavitation that are continually applied to the system in order to obtain the desired droplet size and droplet size distribution (Lovelyn & Attama, 2011:627). This method is used to firstly form coarse emulsions through the forces being obtained from the high pressure, and then to generate API nano-crystals. Through the pressure that is applied to the system during the formulation process and through a certain number of cycles by the high pressure homogeniser, nano-emulsions with a certain droplet size and droplet size distribution eventually form (Chen *et al.*, 2011:357). Typically, surfactants, or a combination of surfactants are added to the system to reduce droplet coalescence (Lovelyn & Attama, 2011:627). Limitations of this method include heat production during preparation and thus the decomposition of components in the nano-emulsion, while this method only allows for o/w fluid based emulsion formulations when the oil phase is less than 20% of the emulsion, and it is unsuitable for large-scale manufacturing (Sharma *et al.*, 2010:4).

The micro-fluidisation method is another high pressure method that is discussed for the purpose of this study. Pre-emulsions are prepared by using an inline homogeniser. The oil and aqueous phases are added together and forced by means of a positive displacement pump through the central chamber of the micro-fluidiser at high speed, through micro-channels that are situated in the interaction chamber. Inside the interaction chamber, forces, such as cavitation, shear force and shear impact are used to form nano-sized droplets (Anton *et al.*, 2008:188; Chen *et al.*, 2011:357; Jafari *et al.*, 2006:475; Lovelyn & Attama, 2011:278). The pre-emulsion hence flows through the micro-channels and are exposed to an impingement area, which leads to the formation of nano-sized droplets and hence a nano-emulsion. The nano-emulsion is then filtered under a nitrogen purge to remove the larger sized droplets from the emulsion, to ensure a uniform droplet size distribution (Lovelyn & Attama, 2011:278). The pressure that is applied to the pre-emulsion leads to the larger droplets splitting into smaller ones, as long as the shear being applied to the system is more than the Laplace pressure of the emulsion (Tadros *et al.*, 2004:305). Micro-fluidisation is a high pressure nano-emulsification formation method that is deemed suitable for large scale industrial manufacturing purposes (Lovelyn & Attama, 2011:278).

### 2.9.3.2.2 Low energy methods

Low energy, low pressure methods are more widely employed in the manufacturing of nano-emulsions these days, since the extent of degradation that is associated with high energy methods is largely reduced, because no such high levels of heat are generated in the process, and also because large scale manufacturing is much easier through low energy methods (Yukuyama *et al.*, 2015:5). Low energy methods do not require expensive machinery, but, instead, they utilise the chemical potential energy being stored in the system of the prepared pre-emulsion (Sharma *et al.*, 2010:3). The chemical potential energy in the system is used to promote phase transition in the emulsion in order to form nano-scaled droplets, by changing the parameters that impact on the HLB of the system. Such parameters include temperature and the composition of the pre-emulsion. One advantage over high energy methods is an improved stability of nano-emulsions that have been prepared by employing low energy methods (Sharma *et al.*, 2010:5). Typical low energy methods that are used include the phase inversion temperature method (PIT) and the spontaneous emulsification method (Sharma *et al.*, 2010:3).

Shinoda *et al.* (1968 & 1969) were the founders of the PIT method (Sharma *et al.*, 2010:3), which is an organic, low pressure method that does not utilise any solvents (Anton *et al.*, 2008:190). The PIT method uses the temperature dependent solubility of non-ionic surfactants (e.g. polyoxyethylene) and changes the water and oil affinities as a function of temperature (Lovelyn & Attama, 2011:278). The pre-emulsion is formed by adding together the oil and aqueous phases, an emulsifying agent and a non-ionic surfactant (Lovelyn & Attama, 2011:278). This mixture is slowly heated, which causes the surfactant's HLB value to change from lipophilic to more hydrophilic (Anton & Vandamme, 2009:142). Chemical energy is used to create phase inversions to allow for the formation of nano-emulsions (Sharma *et al.*, 2010:3). This low energy method of nano-emulsion formation can be used on an industrial scale, since it allows for temperature sensitive drugs to be formulated into nano-emulsions, without losses as a result of decomposition (Anton *et al.*, 2008:190). The phase transitions are controlled by either varying the temperature at a consistent composition of the pre-emulsion, or by varying the composition of the pre-emulsion at a consistent temperature (Sharma *et al.*, 2010:3). The PIT method is based upon the differences in the solubility of the polyoxyethylene surfactant, in relation to temperature differences. An increased temperature causes the surfactant to become more lipophilic, due to the fact the polymer chain dehydrates during the process. If the temperature is drastically lowered and the formulation allowed to cool down, a positive charge is formed in the surfactant's monolayer, which thus leads to the formation of a high curvature, swollen micellar solution, that results in the surfactant becoming more hydrophilic in nature (Lovelyn & Attama, 2011:278; Sharma *et al.*, 2010:3). The PIT method occurs through one of two possible processes, either through a transitional or catastrophic inversion process. The catastrophic inversion method uses a mixture of surfactants at a constant temperature to

change the HLB value of these surfactants. Transitional inversion occurs by changing the electrolyte concentration and/or by changing the temperature to change the HLB value of the system (Lovelyn & Attama, 2011:278). Nano-emulsions are formed by a low energy PIT method through nano-metric templates. When the coarse dispersion is diluted in cold water, or cooled rapidly to a temperature above or equal to that of the PIT, nano-emulsions will form (Anton & Vandamme, 2009:142). This low energy nano-emulsification method is typically used to formulate o/w nano-emulsions (Anton *et al.*, 2008:188).

The spontaneous emulsification method is another low energy method that is used to prepare nano-emulsions, without applying any heat and/or any organic solvents (Anton & Vandamme, 2009:143; Lovelyn & Attama, 2011:628). Spontaneous emulsification is acquired by slowly mixing an aqueous phase into a mixture of oils, a surfactant and a water-miscible solvent at room temperature (Anton *et al.*, 2008:188; Anton & Vandamme, 2009:143; Lovelyn & Attama, 2011:628-629). This method works through solvent diffusion and by decreasing the Gibb's free energy of the system and increasing the entropy of the system (Anton *et al.*, 2008:188; Anton & Vandamme, 2009:143). The solvent displaces itself from the hydrophobic phase into the hydrophilic phase. Turbulence forms at the water/oil interface, causing a non-equilibrium state where these two phases are variegated. An increase in the interfacial area occurs from the quick transfer of the hydrophilic phase from the oil into the aqueous phase, to result in a metastable emulsion. To acquire nano-scaled droplets, a very high solvent-oil ratio is required. Sometimes macro-molecules, monomers and polymers are added to the pre-emulsion to enhance the formation of smaller droplets (Anton & Vandamme, 2009:143). A disadvantage of this particular low energy method is that it does not offer thermodynamic stability to the formulated nano-emulsion, due to high levels of kinetic energy and long term colloidal stability (Lovelyn & Attama, 2011:629).

Another low energy method for preparing nano-emulsions that is discussed for the purpose of this study, is the solvent displacement method. An oily solvent is dispersed in a water soluble organic solvent, such as acetone, ethanol, or ethyl methyl ketone. The formation of nano-emulsions is aided by the rapid diffusion of the organic solvent, by removing it from the newly formed nano-emulsion by means of vacuum evaporation. It is possible for a nano-emulsion to form spontaneously by adding a combination of organic solvents, together with small amounts of oil to the water phase, without the aid of a surfactant. This method of nano-emulsion formation can therefore generate a nano-emulsion at room temperature, by stirring all the components together. Disadvantages of this method include the usage of toxic organic solvents, but they can be removed from the formulated nano-emulsion through additional inputs. Although these organic solvents can be easily removed in a laboratory, it cannot be done during large-scale production in a factory. Lastly, another disadvantage of this method is that a very

high solvent-oil ratio is required to successfully produce the small droplet sizes (Lovelyn & Attama, 2011:278).

## **2.10 Conclusion**

The topical delivery of drugs has proven to offer many opportunities for the potential development of delivery systems for the non-invasive administering of drugs through the skin. The properties of the API, clofazimine, which was investigated during this study, are unfortunately very unfavourable for the purpose of topical delivery, as it is very lipophilic (high log P value) and has a very low aqueous solubility. However, during this study, the topical delivery of clofazimine through nano-emulsion dispersions was attempted, due to the advantages of such dispersions and the lipophilic nature of the drug. Emulgel formulations were prepared for the purpose of comparison, one containing a nano-emulsion and the other a coarse emulsion. The purpose was to investigate whether the nano-emulsion would demonstrate any possible advantages with regards to membrane and skin permeation, over a conventional emulsion. In this study, the aim was to deliver clofazimine topically, using nano-emulsions and emulgels, despite this API having such unfavourable skin permeation properties.

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## **CHAPTER 3**

# **ARTICLE FOR PUBLISHING IN THE EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES**

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Chapter 3 is written in research article format, especially with the goal of publishing in the European Journal of Pharmaceutical Sciences. The article was written in US English according to the author's guide, which is included in Appendix J. The article was justified in the dissertation to ease reading of the article. The Masters student's contribution to the article included research on the preparation of nano-emulsions and emulgels and the characterisation thereof, as well as membrane- and skin diffusion studies performed on the pharmaceutical preparations. The Masters student was also included in the writing of the article.

**Pharmaceutical preparations containing clofazimine for the possible treatment of drug-resistant cutaneous tuberculosis**

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## **Abstract**

Cutaneous tuberculosis (CTB) is a disease that is often associated with multidrug-resistant tuberculosis (MDR-TB). The disease causes unsightly lesions on the skin of an infected person which is difficult to hide. Currently, there is no topical treatment available for the treatment of CTB, since only systemic drug treatment is available on the market.

Nano-emulsions and emulgel formulas (an emulgel containing a nano-emulsion and an emulgel containing a coarse emulsion), all containing clofazimine and avocado oil were formulated for the purpose of this study. These preparations were each characterized to determine their physical properties that could affect topical delivery. Membrane release and skin diffusion studies were performed on the various preparations to test whether the pharmaceutical preparations would yield any clofazimine release and topical delivery.

All three pharmaceutical preparations yielded the optimal physical properties for the purpose of membrane release and topical delivery studies. The membrane release studies demonstrated that all the preparations had clofazimine release. The skin diffusion and tape stripping studies did not show any signs of transdermal or topical delivery, which could be due to the small concentration of clofazimine that dissolves in the oil phase of the nano- and coarse emulsions.

**Keywords:** Nano-emulsion, Characterization, Emulgel, Cutaneous tuberculosis, Coarse emulsion, Franz cell diffusion

## 1 **1 Introduction**

2 Cutaneous tuberculosis (CTB) is a form of extra-pulmonary tuberculosis (TB), which could  
3 be caused by multidrug-resistant tuberculosis (MDR-TB) (Ramesh *et al.*, 2015; Ramirez-  
4 Lapausa, 2015). It leads to lesions on the skin of the infected person (Bravo & Gotuzzo,  
5 2007). Currently, there is no topical treatment available for this disease, only systemic drug  
6 treatment similar to that of the treatment for pulmonary TB (Semaan *et al.*, 2008).

7 Clofazimine is pharmacologically classified as a narrow spectrum riminophenazine anti-biotic  
8 for the treatment of mycobacterial infections such as MDR-TB. Clofazimine kills via  
9 disrupting the DNA (deoxyribonucleic acid) of mycobacteria via the release of reactive  
10 oxygen species (Drugbank, 2015; Hoagland, 2016). Therefore, an investigation was done  
11 regarding the topical delivery of clofazimine. A suitable topical delivery vehicle was chosen  
12 for the attempt to deliver clofazimine topically, which was formulated and characterized in  
13 order to assess the physical properties of the pharmaceutical preparations.

14 Three pharmaceutical preparations, which comprised of a nano-emulsion and two emulgel  
15 formulas (one of which contained a coarse emulsion and the other a nano-emulsion), were  
16 prepared during this study. The nano-emulsion was compared to an emulgel formulation, as  
17 it has a very fluidly consistency and is not easy to apply onto the skin (Thakur *et al.*, 2012).  
18 Avocado oil (natural oil) and clofazimine were included in all of the pharmaceutical  
19 preparations. The preparations were characterized and compared to each other to  
20 determine which of them presented the ideal physiochemical properties. The  
21 pharmaceutical preparations were also tested using membrane and skin diffusion studies in  
22 order to determine which of them yielded release (if any) and which yielded the best skin  
23 diffusion data (if any).

24 The stratum corneum, the outermost layer of the human skin, is responsible for the barrier  
25 function of the skin. The lipid and proteins embedded in the brick-and-mortar bi-lipid  
26 structure of the stratum corneum makes the skin impermeable to external substances such  
27 as drugs (Fore-Phliger, 2004).

28 Clofazimine was incorporated into a nano-emulsion in order to aid with possible skin

29 permeation, since the stratum corneum hinders the permeation of drugs. A nano-emulsion  
30 is defined as a pharmaceutical dispersion prepared as either an oil-in-water (o/w) dispersion,  
31 or a water-in-oil (w/o) dispersion. These dispersions exhibit kinetic stability and  
32 thermodynamic instability and have a nano-metric droplet size ranging between 10-1000 nm  
33 (Kanwale *et al.*, 2015; Mangale, 2015). This particular dispersion allows easy and swift skin  
34 penetration as it offers a large surface area due to such a small droplet size, making skin  
35 permeation easier without the use of penetration enhancers (Sharma *et al.*, 2010).  
36 Clofazimine was also incorporated into an o/w nano-emulsion, since it is very lipophilic of  
37 nature (Peters *et al.*, 2000; Mishra *et al.*, 2014). Avocado oil was chosen as the oil phase of  
38 the nano-emulsion as it is a natural oil high in linoleic acid, which is also found in the skin  
39 (Argenta *et al.*, 2014:4738; Morena *et al.*, 2003:2220; Williams & Barry, 2012:129, 132).  
40 This could possibly enhance the penetration of the nano-emulsion through the skin. The  
41 dispersion was prepared using a probe-sonicator and a sonication bath.  
42 The objectives of the study were to prepare pharmaceutical preparations that included  
43 clofazimine, such as the nano-emulsion preparation and the two-emulgel formulas. These  
44 pharmaceutical preparations were also characterized to determine their ideal physical  
45 properties. Thereafter, membrane and skin studies were performed to see if there was a  
46 possibility that some of the formulas would yield drug release and/or deliver clofazimine  
47 topically.

## 48 **2 Materials and methods**

### 49 **2.1 Materials**

50 Clofazimine (active pharmaceutical ingredient (API)) and avocado oil (oil phase) was  
51 donated as part of the MRC Flagship Program. The Span<sup>®</sup> 60 (lipophilic non-ionic  
52 surfactant) was purchased from Fluka (Midrand, RSA), and the Tween<sup>®</sup> 80 (hydrophilic non-  
53 ionic surfactant) and liquid paraffin (emollient) from Merck (Midrand, RSA). The xanthan  
54 gum (gelling agent) was purchased from Warren Chem Specialties (Cape Town, RSA). The  
55 other solvents and reagents used were all analytical grade chemicals.

56

## 57 **2.2 Methods**

### 58 **2.2.1 Compatibility studies between clofazimine and avocado oil**

59 Compatibility studies were performed to determine whether clofazimine and avocado oil  
60 were compatible with each other before starting with the preparation of a nano-emulsion.  
61 Avocado oil and clofazimine were separately weighed off in a weight ratio of 0.06:1.00 (w/w);  
62 a mixture of these two components was also weighed off in the same weight ratio for the  
63 compatibility study. A 2277 Thermal Activity Monitor (TAM) (TA Instruments, USA),  
64 equipped with an oil bath, with a stability of 100  $\mu$ K over 24 hours, was used to perform the  
65 compatibility analysis. The calorimeters were kept a temperature of 32°C and 60°C,  
66 respectively.

### 67 **2.2.2 Determination of the solubility of clofazimine in avocado oil**

68 The solubility of clofazimine in avocado oil was determined by heating an excess amount of  
69 the API in the oil phase with the Span<sup>®</sup> 60, at a temperature of 60°C on a magnetic stirring  
70 plate until it dissolved. The mixture was stirred overnight on a low heat and on the following  
71 day, 200  $\mu$ l of the mixture was drawn off and dissolved in tetrahydrofuran. The mixture was  
72 centrifuged at a speed of 20000 rpm for 20 min at a temperature of 25°C. The supernatant  
73 (oil phase) was drawn off to determine the amount of the clofazimine dissolved in the oil  
74 phase. High performance liquid chromatography (HPLC) analysis was done to determine  
75 the concentration of the clofazimine that dissolved in the oil phase of the nano-emulsion.

### 76 **2.2.3 High performance liquid chromatography analysis**

77 An HPLC method was developed and validated for the purpose of analysis of samples  
78 containing clofazimine at the Analytical Technology Laboratory (ATL), North-West  
79 University (NWU), Potchefstroom Campus, RSA. An Agilent 1200 series, equipped with an  
80 Agilent 1200 pump, a diode array detector, an auto sampler injection mechanism and  
81 ChemStation Rev. A.10.01 software (Agilent Technologies, Palo Alto, CA) was used to  
82 perform all the analysis in this study. The UV-detector was set at a wavelength of 284 nm  
83 to detect clofazimine peaks. The mobile phase that was used comprised of an acetonitrile  
84 and 0.005 M octane sulphonic acid (pH 3.5) mixture in a ratio of 65:35. The mobile phase

85 flow rate was set at 1.0 ml/min; the runtime was 10.0 min and the retention time was  $\pm 3.6$   
86 min. All analyses performed were done in a laboratory with a temperature-controlled  
87 environment (25°C). The lower limit of quantification (LLOQ) for this method was 0.10330  
88  $\mu\text{g/ml}$  and the percentage relative standard deviation (%RSD) was 3.338%. The limit of  
89 detection (LOD) for this method was calculated as 0.01290  $\mu\text{g/ml}$  and the %RSD was  
90 0.024%.

#### 91 **2.2.4 Preparation of nano-emulsions**

92 O/w nano-emulsions with clofazimine were prepared by combining the low and high-energy  
93 methods via a self-emulsification method and probe and bath sonication. The method is  
94 described point wise as follows:

- 95 • All the required excipients were measured and weighed off for the required amounts  
96 to prepare a hydrophilic:lipophilic surfactant ratio of 3:2.
- 97 • The distilled water and the Tween<sup>®</sup> 80 were heated to 60°C on a magnetic stirring  
98 plate until completely homogenous.
- 99 • The Span<sup>®</sup> 60 and the avocado oil were heated to 60°C on a magnetic stirring plate  
100 until completely dissolved and homogenous.
- 101 • The clofazimine was dissolved in the oil phase and the Span<sup>®</sup> 60 at 60°C.
- 102 • The heated oil phase was drawn up with a syringe, slowly added to the reheated  
103 water phase and mixed at a temperature of 60°C for 5 min.
- 104 • Thereafter, the macro-emulsion that formed was probe-sonicated for 3 min and bath-  
105 sonicated for another 15 min.

#### 106 **2.2.5 Method for the formulation of emulgels**

107 Two emulgel formulas containing clofazimine were prepared for the purpose of comparison  
108 to the nano-emulsion. One emulgel formula contained a nano-emulsion and the other a  
109 coarse emulsion. Firstly, a nano-emulsion was prepared as described in Section 2.2.4. The  
110 coarse emulsion was prepared in the same way as the nano-emulsion, but without any  
111 sonication. Each emulgel formula was prepared separately. The method for preparing the

112 emulgels was as follows:

- 113 • Distilled water was measured to the desired amount and gelling agent was weighed  
114 off.
- 115 • The water was heated to  $\pm 40^{\circ}\text{C}$  on a magnetic stirring plate.
- 116 • The gelling agent was added to the heated water, whilst being homogenized using a  
117 Heidolph RZR 2041 homogenizer (Germany), at a power setting of 340 W with a  
118 frequency of 50 Hz and a speed of 777 rpm.
- 119 • The oil phase of the emulgel including liquid paraffin, Tween<sup>®</sup> 80 and Span<sup>®</sup> 60 was  
120 heated to  $\pm 80^{\circ}\text{C}$  on a magnetic stirring plate until completely dissolved and  
121 homogenous.
- 122 • The coarse emulsion was added to the water phase and homogenized at a speed of  
123 777 rpm until homogenous.
- 124 • Lastly, the oil phase was added to the water phase and homogenized at a speed of  
125 13500 rpm until the emulgel cooled to  $\pm 25^{\circ}\text{C}$ .

## 126 **2.2.6 Characterization of the emulgel and nano-emulsion formulas**

### 127 **2.2.6.1 Light microscopy**

128 Light microscopy was performed on the nano-emulsion and the emulgel formulas to  
129 investigate whether any of the oil droplets were present in the formulas. The light  
130 microscope used was a Nikon Eclipse E4000 microscope (Nikon, Japan Linkam THMS600)  
131 equipped with a Nikon DS-Fi1 camera. A small sample of each emulgel was placed  
132 separately on a 20 mm microscope slide. Each formula was viewed individually on the  
133 microscope with a 40x magnification.

### 134 **2.2.6.2 Zeta-potential determination**

135 The zeta-potential of the dispersion and the emulgel formulas and the nano-emulsion were  
136 determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The  
137 pharmaceutical preparations were each diluted separately; 2 ml of the preparation in 10 ml  
138 distilled water. The sample was then injected into a clean plastic cuvette. The zeta-potential

139 was measured in millivolt (mV); triplicate zeta-potential readings were taken with freshly  
140 prepared samples on the same day to ensure reliable average values.

#### 141 **2.2.6.3 Droplet size and size distribution determination**

142 The droplet size and size distribution of the pharmaceutical preparations was determined  
143 using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The pharmaceutical  
144 preparations were freshly prepared and 2 ml of each sample was diluted separately with 10  
145 ml of distilled water. Each sample was then injected into a clean plastic cuvette and  
146 triplicate readings were taken of each sample.

#### 147 **2.2.6.4 pH determination**

148 The pH of the pharmaceutical preparations was determined using a Mettler Toledo Seven  
149 Compact pH meter (Mettler Toledo, Switzerland). Readings were taken in triplicate each  
150 time with a freshly prepared sample to ensure reliable average readings.

#### 151 **2.2.6.5 Viscosity determination**

152 The viscosity of the pharmaceutical preparations was determined using a Brookfield  
153 Viscometer (model DV-II+, Stoughton, Massachusetts, USA), equipped with rotating  
154 spindles and Helipath attachments. Triplicate viscosity readings were taken separately for  
155 each pharmaceutical preparation from freshly prepared samples to ensure reliable average  
156 values. The viscosity was determined by inserting the rotating spindle into each individual  
157 preparation at a predetermined speed. The data collected by the viscometer was gathered  
158 by Wingather™ software, which was pre-programmed to gather and record the data of the  
159 32 readings taken at 10 sec intervals. The average viscosity value of each separate sample  
160 was calculated from the data.

#### 161 **2.2.6.6 Entrapment efficacy of the nano-emulsion**

162 The entrapment efficacy (EE%) of the nano-emulsion was determined using a Beckman  
163 Coulter Optima L-100 XP ultra-centrifuge (Beckman Coulter, South Africa) with a 50.2Ti  
164 fixed rotor. The nano-emulsion samples were centrifuged at a speed of 25000 rpm for a  
165 period of 30 min at 25°C, in order to separate the water phase and the oil phase of the nano-  
166 emulsion. One ml of the supernatant containing the oil phase was removed and diluted with

167 9 ml of ethanol (99%). The oil phase was filtered with 0.45 µm polyvinylidene difluoride  
168 (PVDF) filters and analyzed with HPLC. The EE% was calculated using the following  
169 equation of Kurakula *et al.* (2012):

170  $EE\% = \frac{C_t - C_f}{C_t} \times 100$  **Equation 1**

171 where  $C_t$  is the total API in the oil phase of the dispersion and  $C_f$  is the concentration of free  
172 un-encapsulated drug (x value).

### 173 **2.2.7 Membrane diffusion studies**

174 Membrane diffusion studies were performed on the nano-emulsion and the two emulgel  
175 formulas to determine if clofazimine was released from these pharmaceutical preparations,  
176 each preparation having been used, respectively, as the donor phase during the membrane  
177 diffusion studies. Three membrane diffusion studies were performed, one study for each of  
178 the different pharmaceutical preparations, with a placebo formulation as a control. Twelve  
179 vertical Franz cells were used, of which two contained the placebo formulation (preparation  
180 without the API), during each of the membrane diffusion studies. The method used during  
181 the membrane diffusion studies is discussed point wise:

- 182 • The donor and receptor phases were prepared.
- 183 • The donor phase was placed in a water bath at a temperature of 32°C (the same as  
184 the temperature on the skin's surface).
- 185 • The receptor phase was placed in a water bath at a temperature of 37°C (the same  
186 as the temperature of the blood in the body).
- 187 • Vacuum grease (Dow Corning®; Sigma-Aldrich, Germany) was rubbed onto both the  
188 donor and receptor compartments of the vertical Franz cells to enable them to seal  
189 properly.
- 190 • A magnetic stirring rod was positioned in the receptor compartment of each of the  
191 vertical Franz cells.

- 192 • Porafil cellulose nitrate filters, with a pore size of 20 µm (Separations, Johannesburg,  
193 South Africa), were carefully positioned on the receptor compartment of each vertical  
194 Franz cell.
- 195 • The two Franz cell compartments (the donor and receptor compartments) were  
196 mounted on each other and sealed with vacuum grease.
- 197 • Each vertical Franz cell was fastened with a horseshoe clamp, thereby preventing the  
198 two compartments from shifting and in turn, preventing leakage.
- 199 • Analytical grade ethanol (pH 7.0; 2 ml) was carefully injected into each receptor  
200 phase to prevent any air bubbles.
- 201 • Each pharmaceutical preparation containing clofazimine (1 ml) was placed into the  
202 donor compartment of the first 10 vertical Franz cells.
- 203 • Each placebo preparation (1 ml) was placed into the donor compartment of the two  
204 outstanding vertical Franz cells.
- 205 • The donor compartment of each vertical Franz cell was covered with a double layer  
206 of Parafilm® to prevent leakage.
- 207 • The 12 vertical Franz cells were placed into a Grant® water bath (Grant Instruments,  
208 UK) on a Variomag® magnetic stirrer (Variomag, USA) set at a temperature of 37°C.
- 209 • The receptor compartments of the 12 vertical Franz cells were placed in the water  
210 bath to keep them temperature controlled at 37°C; this led to the reaching and  
211 maintaining of the skin's surface temperature of 32°C within the donor compartment.
- 212 • The magnetic stirrer was set to stir at a speed of 750 rpm throughout each  
213 membrane diffusion study.
- 214 • The membrane diffusion studies spanned over a full 6 h. After every one hour, each  
215 receptor phase was extracted and refilled with fresh analytical grade ethanol (pH 7.0;  
216 37°C).

- 217       • HPLC was performed on each of the samples that were obtained from the membrane  
218       diffusion study to enable the determination of the concentration of clofazimine  
219       released during each individual study.

## 220   **2.2.8 Skin diffusion studies**

### 221   **2.2.8.1 Skin preparation for the skin diffusion studies**

222   Female Caucasian skin was ethically obtained following abdominoplasty surgery (ethical  
223   approval reference number: NWU-00114-11-A5). The skin was subsequently dermatomed  
224   using a Zimmer™ electronic dermatome (Germany), model 8821 to obtain a thickness of  
225   400 µm.

### 226   **2.2.8.2 Franz cell skin diffusion studies**

227   Vertical Franz cells were used for the performance of skin diffusion studies. The Franz cells  
228   were placed in a pre-heated water bath at a temperature of 37°C. The dermatomed skin  
229   was placed on the receptor phase of the Franz cells with the stratum corneum facing  
230   upwards. After assembly of the donor and receptor phases, they were sealed with vacuum  
231   grease and clamped together with horseshoe clamps. The pharmaceutical formulations  
232   were each used to perform three skin diffusion studies, respectively. The receptor phase of  
233   each Franz cell was filled with phosphate buffer solution (PBS; pH 7.4) and a mixture of 99%  
234   analytical grade ethanol (pH 7) in a ratio of 1:9 (ethanol:PBS). Each Franz cell's donor  
235   phase was covered with a double layer of Parafilm®, thereby preventing evaporation. Each  
236   volume removed from the Franz cells after sampling was immediately replaced with the fresh  
237   and preheated ethanol:PBS (9:1) mixture. Each receptor phase sample was extracted and  
238   transferred into vials and analyzed using HPLC to determine if any diffusion of clofazimine  
239   took place during each individual skin diffusion study.

### 240   **2.2.8.3 Tape stripping**

241   Each Franz cell was taken apart after carefully extracting the fluid (buffer solution mixture) in  
242   the receptor phase. Each piece of skin was removed from the receptor phase of each Franz  
243   cell and dabbed dry to remove any excess pharmaceutical formulation. The 12 skin samples  
244   were fastened with pins onto Parafilm® on a solid wooden surface. Sixteen small 3M

245 Scotch<sup>®</sup> Magic<sup>™</sup> tape strips were cut per skin sample. The first tape strip attached to each  
246 skin area was discarded as it was contaminated with the donor phase and each of the  
247 remaining 15 tape strips were placed on the skin, one at a time, and pulled off. The tape  
248 strips with the stratum corneum-epidermis attached to them were transferred into individual  
249 polytops for each Franz cell used and filled with 5 ml of the extraction solution consisting of  
250 ethanol (99%). The pieces of skin that remained (consisting of the epidermis-dermis) were  
251 cut into small pieces and each individual piece was placed in a polytop containing 5 ml of  
252 extraction solution. The polytops were covered with Parafilm<sup>®</sup> and placed in the refrigerator  
253 overnight at a temperature of 4°C, to ensure the possible clofazimine present would dissolve  
254 in the extraction solution. All the polytops' contents were analyzed using HPLC.

### 255 **3 Results**

#### 256 **3.1 Nano-emulsion and emulgel visual appearance**

#### 257 **3.2 Solubility of clofazimine in avocado oil**

258 Clofazimine was shown to have a solubility of 1.557 mg/ml in avocado oil and Span<sup>®</sup> 60.

#### 259 **3.3 Compatibility study**

260 The compatibility study revealed clofazimine and avocado oil were compatible with each  
261 other at a temperature range between 32°C and 60°C during a 24 hr period. At a  
262 temperature of 32°C, the heat flow had a value of  $1.3815 \pm 1.4545 \mu\text{W/g}$ , which is plotted as a  
263 curve in magenta in Figure 1. The curve demonstrates no sudden increase or decrease,  
264 and therefore there were no incompatibilities present between clofazimine and avocado oil at  
265 a temperature of 32°C. The average heat flow measured for the compatibility study at 60°C  
266 was found to be  $252.25 \pm 226.20 \mu\text{W/g}$ , which also yielded a straight magenta line (Figure 2),  
267 also indicating no incompatibilities were present.

268

#### 269 **Figure 1**

270

#### 271 **Figure 2**

272

273 **2.4 Characterization results**

274 **2.4.1 Light microscopy results**

275 Light microscopy results of the emulgel formulas containing clofazimine are shown below in  
276 Figure 3. As observed, the oil droplets of the dispersions are still present. The emulgel  
277 containing the coarse emulsion, Figure 3.a, had larger droplets and displayed more signs of  
278 droplet aggregation than in Figure 3.b for the nano-emulsion.

279

280 **Figure 3**

281 **2.4.2 Zeta-potential results**

282 The nano-emulsion had a zeta-potential of  $-31.27 \pm 0.81$  mV, the emulgel formula containing  
283 the nano-emulsion had a zeta-potential value of  $-49.4 \pm 0.85$  mV and the emulgel formula  
284 containing the coarse emulsion  $-41 \pm 0.70$  mV. All the pharmaceutical preparations had a  
285 highly negative zeta-potential and were therefore stable (Patel *et al.*, 2013:34).

286 **2.4.3 Droplet size and size distribution results**

287 The nano-emulsion had an average droplet size of  $65.97 \pm 0.4$  nm, which fell within the size  
288 range for nano-emulsions (Pey *et al.*, 2006:144). The polydispersity index was shown as  
289  $0.216 \pm 0.01$ , indicating a narrow droplet size distribution. The emulgel formula containing the  
290 nano-emulsion had an average droplet size of  $881 \pm 0.08$  nm and the emulgel formula  
291 containing the coarse emulsion,  $1133 \pm 0.04$  nm. The emulgel formula containing the nano-  
292 emulsion had a polydispersity index of  $0.585 \pm 0.05$  and the emulgel formula containing the  
293 coarse emulsion,  $0.463 \pm 0.08$ . The nano-emulsion had a better polydispersity index and a  
294 smaller average droplet size than the emulgel formulas and therefore had better stability  
295 than the emulgel formulas (Patel *et al.*, 2013:34). The emulgel formula containing the nano-  
296 emulsion had a smaller droplet size than the emulgel formula containing the coarse  
297 emulsion. The emulgel formula containing the coarse emulsion had a smaller droplet size  
298 distribution and a more homogenous spread of the oil droplets through the emulgel formulas  
299 than the emulgel formula containing the nano-emulsion.

300

#### 301 **2.4.4 pH results**

302 The pH of the nano-emulsion and the two emulgel formulas were determined. The nano-  
303 emulsion had an average pH of  $6.61\pm 0.02$ , the emulgel formula containing the nano-  
304 emulsion had a pH of  $5.96\pm 0.01$  and the emulgel formula containing the coarse emulsion  
305 had a pH of  $5.75\pm 0.01$ . The pH of all of the pharmaceutical preparations fell within the pH  
306 range regarded as safe for skin usage and therefore would not cause skin irritation (Paudel  
307 *et al.*, 2010:118).

#### 308 **2.4.5 Viscosity results**

309 The viscosity of the nano-emulsion was measured as  $1.64\pm 0.03$  cP, the emulgel formula  
310 containing the nano-emulsion as  $657.47\pm 5.14$  cP and the emulgel formula containing the  
311 coarse emulsion as  $964.4\pm 14.84$  cP. The emulgel formulas with the higher viscosity values  
312 would therefore be easier to apply to the skin.

#### 313 **2.4.6 Drug entrapment of clofazimine in the nano-emulsion**

314 The amount of clofazimine entrapped in the oil phase of the nano-emulsion was found to be  
315 95.62%, which was quite high (Lui *et al.*, 2007:159).

#### 316 **2.5 Membrane diffusion study results**

317 The three membrane diffusion studies were performed on the three pharmaceutical  
318 preparations, one membrane diffusion study per preparation. All of the pharmaceutical  
319 preparations showed release of clofazimine during the membrane diffusion studies. Since  
320 some of the Franz cells showed signs of leakage, they were excluded from the results as  
321 they could cause faulty data entries, and ultimately, faulty final deductions.

322 Table 1 illustrates the average flux values ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ), as well as the average %API released  
323 from each membrane diffusion study over a period of 6 h.

324

#### 325 **Table 1**

326 Figure 4 illustrates the %clofazimine that was released from each of the pharmaceutical  
327 preparations during the three membrane diffusion studies performed.

328

329 **Figure 4**

330 As illustrated, the nano-emulsion had the highest average flux, followed by the emulgel  
331 formula containing the coarse emulsion and lastly, the emulgel formula containing the nano-  
332 emulsion. The clofazimine in the emulgel formula containing the nano-emulsion probably  
333 shifted to the top of the emulgel, leading to a smaller flux value than seen in the emulgel  
334 formula containing the coarse emulsion.

335 **2.6 Transdermal diffusion studies**

336 Skin diffusion studies were performed on the three pharmaceutical preparations. Both  
337 emulgel formulas did not display any skin permeation; however the nano-emulsion displayed  
338 small non-quantifiable results during topical delivery. These results were not used, as they  
339 were unreliable and under the values of the LOD and the LLOQ. Although none of the  
340 values were quantifiable, there was a small amount of clofazimine present in the skin. If the  
341 concentration gradient was increased, or another topical delivery system was used, possible  
342 quantifiable values could have been obtained from topical delivery.

343 **3 Summary**

344 The nano-emulsion displayed the most favorable physical properties overall for the purpose  
345 of skin diffusion studies, the emulgel formula containing the nano-emulsion displayed less  
346 favorable properties and the emulgel formula containing the coarse emulsion the least  
347 favorable properties.

348 The compatibility study showed that clofazimine was indeed compatible with the avocado oil  
349 phase at temperatures ranging between 32°C and 60°C. Thus, the oil phase was found to  
350 be a suitable ingredient for synthesizing nano-emulsions containing clofazimine.

351 The solubility of clofazimine in the oil phase of the nano-emulsion and coarse emulsion was  
352 determined. It was found that clofazimine had very low solubility in avocado oil.

353 The physical properties of the nano-emulsion and the emulgel formulas were determined.

354 The light microscopy micrographs illustrated that emulsion oil droplets were present in the  
355 pharmaceutical preparations, with the nano-emulsion having the smallest droplet size,  
356 followed by the emulgel formula containing the nano-emulsion and lastly, the emulgel

357 formula containing the coarse emulsion. The zeta-potential displayed negative values,  
358 which were an indication of emulsion stability against droplet aggregation. The emulgel  
359 formula containing the nano-emulsion had the smallest zeta-potential value, followed by the  
360 emulgel formula containing the coarse emulsion and lastly, the nano-emulsion. The droplet  
361 size and distribution results showed that the nano- emulsion had a droplet size that fell within  
362 nano-scale droplet size ranges with a good particle distribution. The emulgel containing the  
363 nano-emulsion displayed larger droplets that still fell within the acceptable ranges and it  
364 displayed a good particle distribution. Lastly, the emulgel containing the coarse emulsion  
365 had displayed the droplet size of a typical coarse emulsion and portrayed a good particle  
366 distribution. The pH values of all of the pharmaceutical preparations were suitable for safe  
367 skin application. The viscosity of the nano-emulsion was very low, as it was a very watery  
368 formulation. The emulgel formulas had a higher viscosity than the nano-emulsion. Lastly,  
369 the EE% of clofazimine in the oil phase of the emulsion proved to be high and consequently,  
370 it was deemed satisfactory.

371 The membrane diffusion studies indicated that release took place from all three of the  
372 pharmaceutical preparations. The nano-emulsion had the highest release, followed by the  
373 emulgel formula containing the coarse emulsion and the least release was from the emulgel  
374 formula containing the nano-emulsion. The skin diffusion studies done on the three  
375 preparations did not yield any significant results, probably due to the low concentration of  
376 clofazimine that dissolves in the oil phase of the emulsion. Although none of the skin  
377 diffusion study values was quantifiable, some of the clofazimine was visible in small  
378 concentrations. If another oil phase was incorporated into the nano-emulsion, in which  
379 clofazimine was more soluble, or if the concentration gradient was increased or another  
380 topical pharmaceutical formula was used, quantifiable API delivery could take place during  
381 future topical diffusion studies.

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391 diffusion studies.

392 **Conflict of Interest**

393 The authors declare no conflict of interest.

394

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472 **Tables**

473 **Table 1:** Average flux and average %released for clofazimine obtained during membrane  
474 release studies of the three different preparations after 6 h

| <b>Pharmaceutical preparation</b>  | <b>Average flux<br/>(<math>\mu\text{g}/\text{cm}^2\cdot\text{h}</math>)</b> | <b>Average<br/>%released (%)</b> |
|------------------------------------|---|----------------------------------|
| Nano-emulsion                      | 2.71 $\pm$ 0.66   | 6.18 $\pm$ 1.40                  |
| Emulgel containing nano-emulsion   | 0.31 $\pm$ 0.04   | 1.38 $\pm$ 0.17                  |
| Emulgel containing coarse emulsion | 0.56 $\pm$ 0.04   | 1.41 $\pm$ 0.67                  |

475

476

477 **Figure legends**

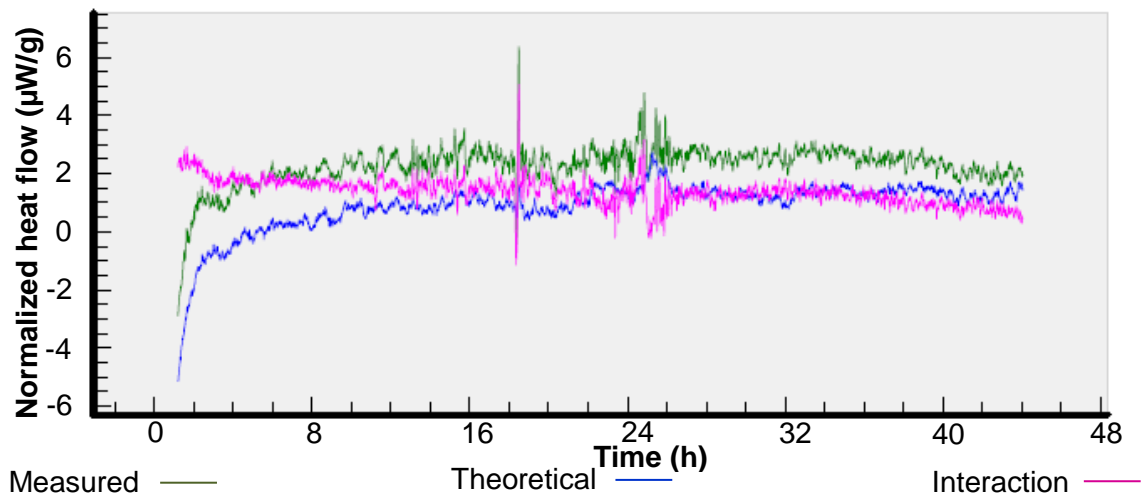
478 **Figure 1:** Combined graph depicting the measured, theoretical and interaction heat flow  
479 obtained for a mixture of avocado oil and clofazimine in a ratio of 0.06:1.00 (%w/w) at 32°C.

480 **Figure 2:** Combined graph depicting the measured, theoretical and interaction heat flow  
481 obtained for a mixture of clofazimine and avocado oil in a ratio of 0.06:1.00 (%w/w) at 60°C.

482 **Figure 3:** Micrographs of a) the nano-emulsion, b) the emulgel containing the coarse  
483 emulsion and c) the emulgel containing the nano-emulsion, with a 100 µm scale indicated.

484 **Figure 4:** Average %clofazimine released during the membrane diffusion studies for the  
485 three pharmaceutical preparations.

486

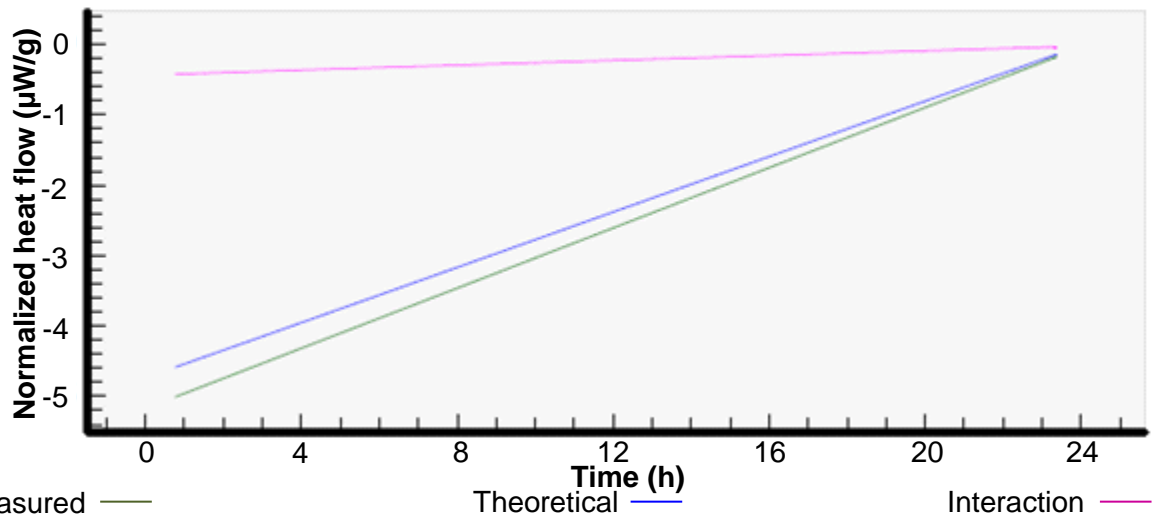


487

488 **Figure 1:** Combined graph depicting the measured, theoretical and interaction heat flow

489 obtained for a mixture of avocado oil and clofazimine in a ratio of 0.06:1.00 (%w/w) at 32°C

490

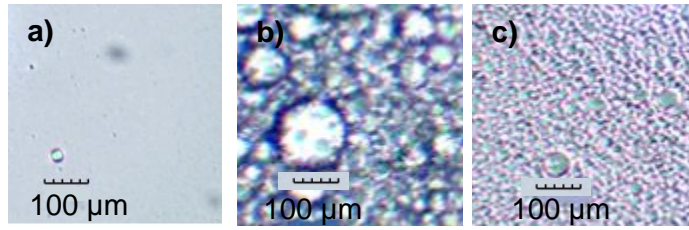


491

492 **Figure 2:** Combined graph depicting the measured, theoretical and interaction heat flow

493 obtained for a mixture of clofazimine and avocado oil in a ratio of 0.06:1.00 (%w/w) at 60°C

494

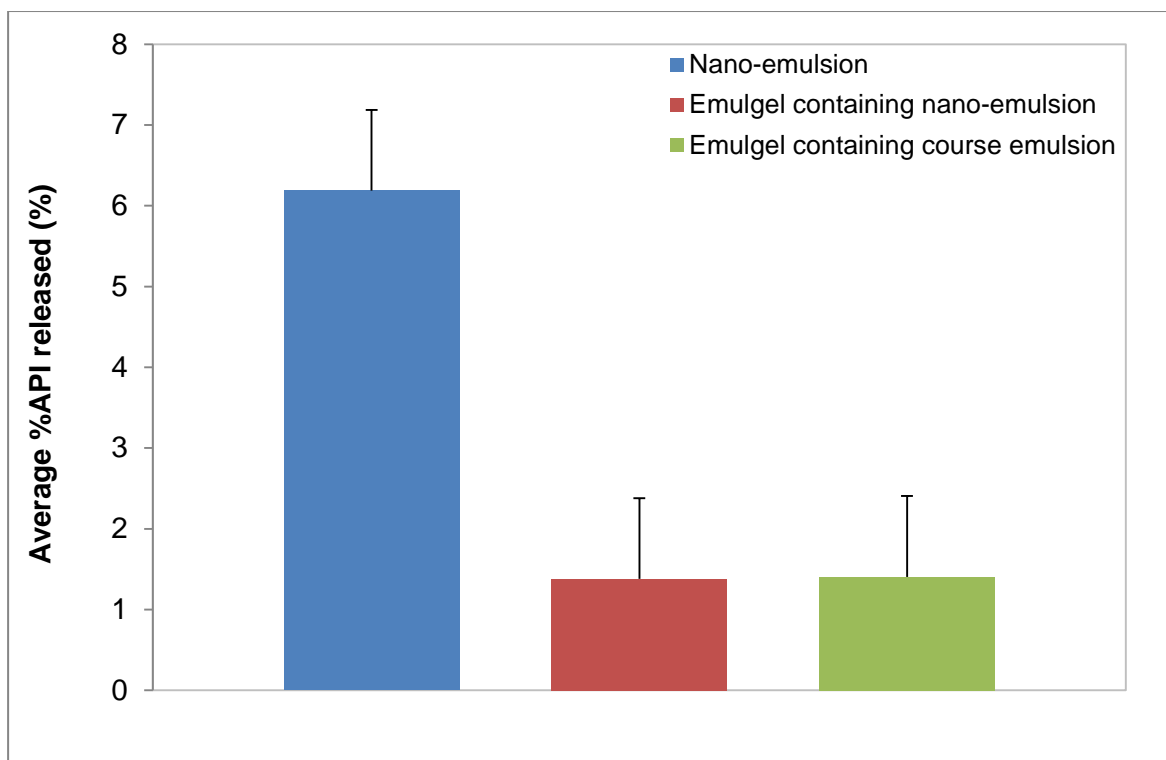


495

496 **Figure 3:** Micrographs of a) the nano-emulsion, b) the emulgel containing the coarse

497 emulsion and c) the emulgel containing the nano-emulsion, with a 100 μm scale indicated

498



499

500 **Figure 4:** Average %clofazimine released during the membrane diffusion studies for the  
501 three pharmaceutical preparations

502

## CHAPTER 4

### CONCLUSION AND FUTURE RECOMMENDATIONS

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The stratum corneum is the outermost layer and the main protective barrier of the skin (Heath & Carbone, 2013:978) and therefore the main limiting factor in the topical delivery of drugs (Prausnitz *et al.*, 2012:2065).

Cutaneous tuberculosis (CTB) is a disease that presents on the skin of an infected person and can it be caused by *Mycobacterium tuberculosis*, specifically by the drug resistant strains thereof (Bañuls *et al.*, 2015:1261; Ramesh *et al.*, 2001:393). CTB is a form of extra-pulmonary tuberculosis that could be associated with multidrug-resistant tuberculosis (MDR-TB). CTB causes unsightly lesions on the skin that are often difficult for the infected person to hide (Ho, 2003:130, 132; Ramesh *et al.*, 2001:393).

Clofazimine is a riminophenazine antibiotic that is classified by the World Health Organization (WHO) as a last-line drug for the treatment of MDR-TB. It is listed in the WHO Group 5 section of medications (Dooley *et al.*, 2013:1353). No topical treatment is currently available for CTB. During this study, three different topical preparations, each containing clofazimine and natural avocado oil, were formulated, i.e. a nano-emulsion, an emulgel containing the nano-emulsion and an emulgel containing a coarse emulsion. The nano-emulsion and coarse emulsion were each incorporated into an emulgel to improve their skin spreadabilities. The three topical preparations were applied during experiments to investigate whether clofazimine would be delivered topically, despite its unfavourable physiochemical properties with regards to topical delivery. The three preparations were optimised and characterised in an attempt to improve the topical delivery of clofazimine from these formulations. The formulations had initially been tested by performing membrane release studies and by analysing the collected samples on the HPLC, to determine whether the API had been released from each formulation. The possible release of clofazimine from the formulations was then tested on human skin, by performing skin diffusion studies, followed by tape stripping and the samples were also analysed on the HPLC. The results of the nano-emulsion were compared during both the membrane and skin diffusion studies to the emulgel containing the nano-emulsion and the emulgel containing the coarse emulsion, to identify the preparation that had demonstrated the best clofazimine delivery.

The objectives of this study were:

- To validate a suitable HPLC method to determine the clofazimine concentrations of three pharmaceutical preparations, i.e. the test samples generated from the dispersion (nano-

emulsion) and from two formulas (emulgel containing a nano-emulsion and an emulgel containing a coarse emulsion).

- To determine the aqueous solubility and octanol-buffer distribution coefficient (log D) of clofazimine.
- To prepare a nano-emulsion, an emulgel containing a nano-emulsion and an emulgel containing a coarse emulsion, all containing natural avocado oil and clofazimine.
- To characterise the nano-emulsion and the two different emulgel formulas.
- To determine whether clofazimine was released from the nano-emulsion, emulgel containing a nano-emulsion and the emulgel containing a coarse emulsion by using membrane diffusion studies.
- To investigate the possible transdermal and topical delivery of clofazimine from the dispersion and the two emulgel formulas, by utilising skin diffusion studies, followed by tape stripping, respectively.

A suitable HPLC analytical method for clofazimine had been validated with the assistance and expertise of Professor Jan du Preez. The method was proven to be accurate, precise, repeatable and reliable and could it hence be used for the analysis of the clofazimine concentrations in all of the experimental samples being generated during this study.

The aqueous solubility and *n*-octanol-buffer distribution coefficient (log D) of clofazimine were determined. It was confirmed that clofazimine was very lipophilic and virtually insoluble in water, in accordance with findings, as reported in the available literature (Cholo *et al.*, 2012:290; Drugbank, 2015; O'Driscoll & Griffin, 2008:618).

After optimisation of the nano-emulsion, by choosing the optimal surfactant ratio of 3:2 (Span<sup>®</sup> 60:Tween<sup>®</sup> 80), it was characterised by employing transmission electron microscopy (TEM), zeta-potential, light microscopy, droplet size and droplet size distribution, and entrapment efficiency (EE%) analytical techniques. The concentration of the clofazimine being entrapped in the oil phase was also determined, before its incorporation into the nano-emulsion. The emulgel formulations were also characterised by means of the following parameters, i.e. zeta-potential, light microscopy, viscosity, droplet size and droplet size distribution.

The characterised optimised nano-emulsion, containing clofazimine, with the 3:2 surfactant ratio showed similar results than those of the nano-emulsion with the 1:4 surfactant ratio (Span<sup>®</sup> 60:Tween<sup>®</sup> 80). However, the optimised nano-emulsion had more favourable characteristics, such as its average droplet size (65.97 ± 0.4 nm) and distribution results that indicated that the droplets were small and that they had a narrow size distribution, which were both indicative of a stable dispersion (Patel *et al.*, 2013:34).

The average zeta-potential ( $-31.27 \pm 0.81$  mV) of the optimised nano-emulsion was highly negative, which indicated that the dispersion would be safe from possible instabilities, such as droplet aggregation (De Morias *et al.*, 2006:110). The pH of the optimised nano-emulsion ( $6.61 \pm 0.02$ ) was favourably near the physiological pH of the skin of 4.0 - 7.4 (Hadgraft & Valenta, 2000:243) and no skin irritation would occur (Paudel *et al.*, 2010:118). The entrapment efficiency (EE%) indicated that 95.62% of the clofazimine had been entrapped in the oil phase of the nano-emulsion. As expected, the average viscosity ( $1.64 \pm 0.03$  cP) of the nano-emulsion was close to that of water. The light microscopic results showed that very small droplets had formed during the preparation of the nano-emulsion. Generally, the characterisation of the nano-emulsion showed that the formulation had optimal properties for a topical formulation.

The two emulgel formulations (one containing the nano-emulsion and the other containing the coarse emulsion) were also characterised, by using light microscopy, droplet size and droplet size distribution, zeta-potential, pH and viscosity as parameters. The light microscopic results showed that emulsion droplets had been present in both these formulations, with the emulgel containing the nano-emulsion having the smaller droplets. The average zeta-potential results of  $-49.4 \pm 0.85$  mV for the emulgel containing the nano-emulsion and  $-41 \pm 0.70$  mV for the emulgel containing the coarse emulsion, showed that the emulgel formulations were both stable, as they were both highly negatively charged (Patel *et al.*, 2013:34), with the emulgel containing the nano-emulsion having been more negatively charged than the other. The droplet sizes and droplet size distribution results indicated that the emulgel containing the nano-emulsion had smaller droplets ( $881 \pm 0.08$  nm) and a narrower size distribution (Pdl = 0.463) than the emulgel containing the coarse emulsion ( $1133 \pm 0.04$  nm and Pdl = 0.585), and the first mentioned emulgel therefore was considered the more stable formulation of the two.

The average pH values of both the emulgel formulations were measured in triplicate and the results showed that the emulgel containing the nano-emulsion ( $5.96 \pm 0.01$ ) had a slightly higher pH than the other ( $5.75 \pm 0.01$ ). Both emulgel formulations were, however, purposefully formulated with pH values near that of the safe physiological range of the skin (pH 4.0 - 7.4) (Hadgraft & Valenta, 2000:243) and no skin irritation would therefore occur (Paudel *et al.*, 2010:118). Lastly, the average viscosities of both the emulgel formulations were determined. The emulgel containing the nano-emulsion ( $657.47 \pm 5.14$  cP) had a lower viscosity than the emulgel containing the coarse emulsion ( $964.4 \pm 14.84$  cP) and was it therefore expected that the first would show better release of the API during the diffusion studies (Li *et al.*, 2011:1008; Valenta & Schultz, 2004:264).

The membrane diffusion studies that had been performed on the nano-emulsion and the two emulgel formulations had aimed at excluding the non-release of clofazimine as a possible

reason, in the event that the API was released from the formulations, whilst no topical delivery occurred. The nano-emulsion and the two emulgel formulations all demonstrated drug release during the membrane diffusion studies. The nano-emulsion showed the highest (best) release of the API ( $6.187 \pm 1.40\%$ ), followed by the emulgel containing the coarse emulsion ( $1.406 \pm 0.67\%$ ) and lastly, the emulgel containing the nano-emulsion ( $1.380 \pm 0.17\%$ ). The emulgel formulations showed a lower concentration of the released clofazimine, which may have been ascribed to the fact that the emulgel formulations had much higher viscosities than the dispersion, which would have slowed down the release of the API (Li *et al.*, 2011:1008; Valenta & Schultz, 2004:264). Another reason for the emulgel showing a lower release may also have been due to the lower concentrations of clofazimine that had been present in these formulations.

Skin diffusion and tape stripping tests were performed on the nano-emulsion and the two emulgel formulations to determine whether any transdermal and/or topical diffusion of clofazimine had occurred from the three preparations. The outcomes from the skin studies indicated that only a very small amount of topical delivery had occurred from the nano-emulsion and almost no transdermal delivery, while no topical, nor transdermal delivery had occurred from both emulgel formulations. Furthermore, for the nano-emulsion, no API was found in the stratum corneum-epidermis, whereas a very small concentration of the API was found in the epidermis-dermis, as well as in the receptor phases. All these results were mostly very low and non-quantifiable.

In conclusion, future recommendations include:

- Seeking for an alternative oil phase for use in the nano-emulsion, in which clofazimine would be more soluble.
- Incorporating a higher concentration of clofazimine into the oil phase of the nano-emulsion to improve the skin permeation results.
- Improving the emulgel formulations to enhance the skin permeation of clofazimine.
- Incorporating clofazimine into alternative pharmaceutical preparations to possibly enhance skin permeation of the drug.
- Investigating the *in vitro* efficacy of the API being entrapped in the nano-emulsion and emulgel formulation on tuberculosis cells (*M. tuberculosis* H37Rv strain), to determine whether the preparations would be able to enhance the healing of CTB in combination with the existing systemic dosage form.

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# APPENDIX A

## DEVELOPMENT AND VALIDATION OF AN HPLC ANALYTICAL METHOD FOR CLOFAZIMINE

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### A.1 Introduction

The purpose of an analytical method validation process is to ensure that the method would provide precise, accurate and reproducible data for the samples being analysed under the specified parameters and conditions. The tests that were performed during the validation process included accuracy, precision, selectivity, sensitivity, reproducibility and stability (Bansal & De Stefano, 2007:109-112). During the course of this study, HPLC analysis was used for the determination of the log D values and the aqueous solubility of clofazimine, after applying the methodologies described in Appendix F, sections F.2.4 and F.2.5. Thereafter HPLC analysis was used to determine the concentration of clofazimine in the receptor phase of the Franz cells, after applying the membrane release and skin diffusion methodology as described in Appendix F, sections F.2.6 to F.2.9.

### A.2 Chromatographic conditions

The analytical method used in this study was developed in conjunction with Prof Jan du Preez at the Analytical Technology Laboratory (ATL) of the North-West University (NWU) (Potchefstroom Campus). Since the validation was done in conjunction with another student, Ewald Janse van Rensburg, both students used the same data sets. The following parameters were employed for the development of this analytical method:

|                               |  |
|-------------------------------|--|
| <b>Analytical instrument:</b> | Agilent 1100 series HPLC system, equipped with a gradient pump, UV detector, auto-sampler and a ChemStation Rev. A.10.02 data acquisition and analysis software. |
| <b>Column:</b>                | Venusil C <sub>18</sub> column, 150 x 4.6 mm and 5 µm particle size (Agilent Technologies, Newark, DE).  |
| <b>Mobile phase:</b>          | An acetonitrile and a 0.005 M octane sulphonic acid (pH 3.5)/octane sulfonic acid mixture (65:35), were used.  |
| <b>Stop time:</b>             | 10 min   |
| <b>Flow rate:</b>             | 1.0 ml/min   |
| <b>Injection volume:</b>      | 25 µl  |
| <b>Detection:</b>             | UV at 284 nm   |

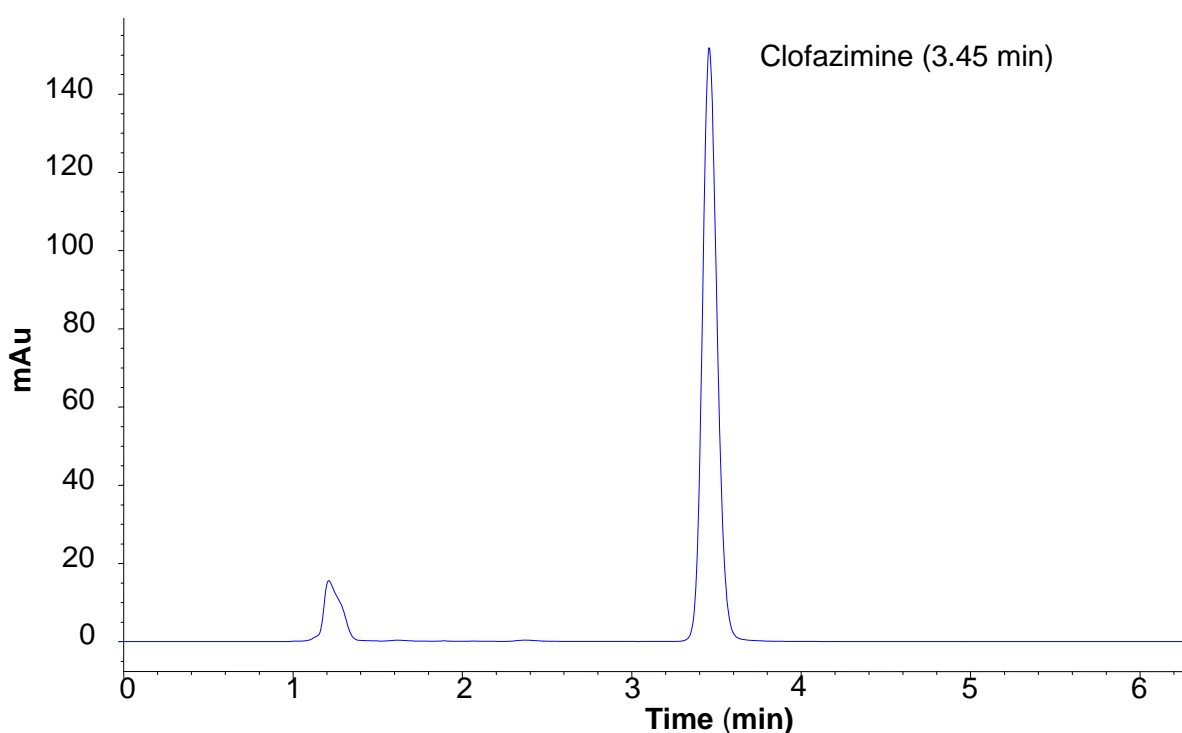
**Retention time:**  $\pm 3.6$  min

**Solvent:** Methanol (100%)

### A.3 Preparation of the standard solution

Clofazimine was dissolved in methanol for the preparation of the standard solution. 20 mg of clofazimine was weighed and diluted to 100 ml with methanol. This mother solution yielded a concentration of 200  $\mu\text{g/ml}$ . 5 ml of this solution was diluted to 50 ml with methanol to yield a concentration of 20  $\mu\text{g/ml}$ . Lastly, 5 ml of the second solution was diluted to 50 ml with methanol to yield a concentration of 2  $\mu\text{g/ml}$ .

Figure A.1 demonstrates the HPLC chromatograph of the clofazimine standard solution (2  $\mu\text{g/ml}$ ), with the peak having eluted at 3.45 min. The chromatograph for clofazimine was almost identical for all of the HPLC validation parameters tested, with minor shifts in the retention times.



**Figure A.1:** HPLC chromatograph of the clofazimine standard solution (2  $\mu\text{g/ml}$ )

### A.4 Validation parameters

During the HPLC validation process, the parameters that were investigated included accuracy, linearity, specificity, precision, ruggedness, system repeatability, limit of detection (LOD) and the lower limit of quantification (LLOQ).

#### A.4.1 Limit of detection and lower limit of quantification

The LOD is defined as the smallest possible concentration at which a particular analyte can be detected and identified from the background noise (Armbruster *et al.*, 1994:1233; FDA, 2001:20). The LLOQ is defined as the smallest amount of the analyte in a sample that can be determined quantitatively with acceptable precision and accuracy (FDA, 2001:20).

Table A.1 depicts the LOD and LLOQ results obtained during these analyses. The standard deviation (SD) and percentage relative standard deviation (% RSD) are also given.

**Table A.1:** Summary of the limit of detection and lower limit of quantification results for clofazimine

| Injection volume (µl) | 2.5        | 5.0        | 10.0       | 15.0       | 20.0       |
|-----------------------|------------|------------|------------|------------|------------|
| Concentration(mg/ml)  | 0.00001290 | 0.00002580 | 0.00005165 | 0.00007750 | 0.00010330 |
| Peak area             | 3.3        | 6.3        | 11.6       | 16.3       | 20.7       |
|                       | 3.4        | 6.5        | 11.5       | 16.2       | 20.8       |
|                       | 3.5        | 6.3        | 11.5       | 15.8       | 20.7       |
|                       | 3.3        | 6.4        | 11.6       | 16.0       | 20.6       |
|                       | 3.4        | 6.2        | 11.3       | 16.1       | 20.7       |
|                       | 3.3        | 6.3        | 11.1       | 16.2       | 20.6       |
|                       | 3.1        | 6.0        | 11.2       | 16.2       | 20.7       |
| Mean peak area        | 3.32       | 6.29       | 11.40      | 16.11      | 20.67      |
| SD                    | 0.11       | 0.13       | 0.18       | 0.17       | 0.06       |
| %RSD                  | 3.34       | 2.15       | 1.55       | 0.13       | 0.02       |

To determine the LOD and the LLOQ of clofazimine, a series of standard solutions at different concentrations were prepared. These standard samples of low concentration were each injected seven consecutive times for HPLC analysis. According to the acceptance parameters for the LLOQ and the LOD, the % RSD had to be  $\leq 15\%$  and  $\leq 20\%$ , respectively. The LLOQ for this method was measured at 0.01290 µg/ml and the % RSD was 3.338%. The LOD for this method was 0.10330 µg/ml and the % RSD 0.024%. Consequently, both the LOD and the LLOQ complied with the acceptable % RSD ranges.

#### A.4.2 Linearity

Linearity can be defined as the ability of an analytical technique to attain test results that are directly proportional to the analyte's concentration in a sample (ICH, 2005:5). Standard samples were prepared to include clofazimine concentrations ranging from 0.1 µg/ml - 150.6 µg/ml as illustrated in table A 2. The linear range of clofazimine was determined through linear regression analysis, by plotting the obtained HPLC peak areas against the relevant concentrations of clofazimine in the prepared standard solutions. A linear regression coefficient

( $R^2$ ) of more than 0.99 should be achieved during linear regression analysis, as this is an indication that the analytical system is reliable and stable (Araujo, 2009:2229). The equation used for the linear regression was adapted Araujo (2009:2229) and is illustrated below:

$$y = mx + c$$

**Equation A.1**

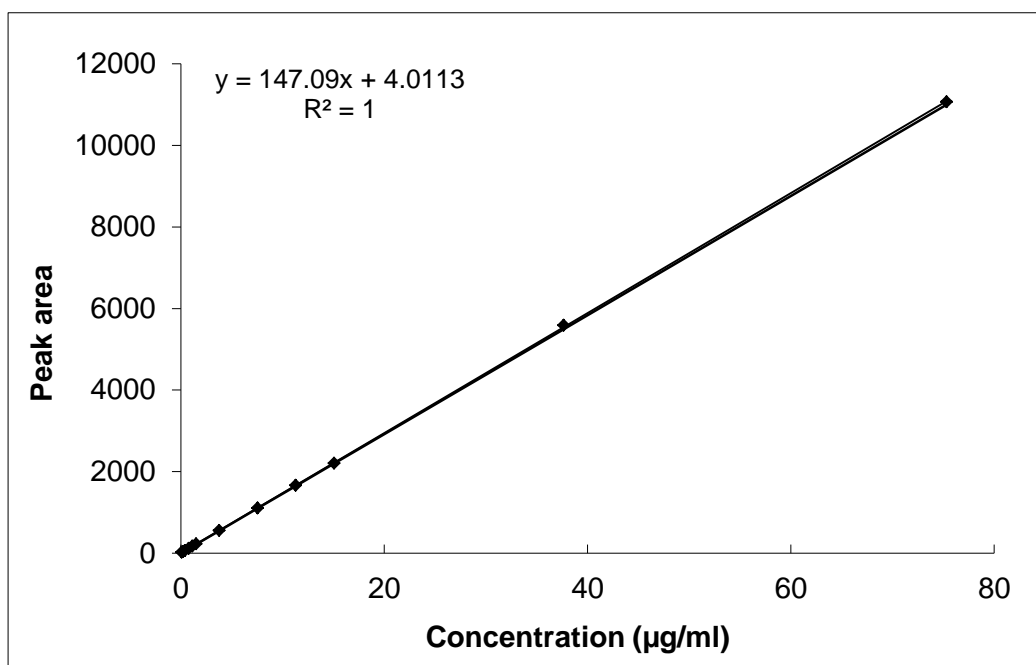
Where:

y= ratio of the peak area

m= slope of the straight line

x= analyte concentration ( $\mu\text{g/ml}$ )

c= y – intercept of the straight line



**Figure A.2:** Linear regression curve of clofazimine, constructed by plotting the generated HPLC peak areas against each relevant concentration ( $\mu\text{g/ml}$ )

For the determination of the linearity of clofazimine, three standard solutions at different concentrations (1.506  $\mu\text{g/ml}$ , 15.06  $\mu\text{g/ml}$ , 150.6  $\mu\text{g/ml}$ ) were prepared and each standard sample thereafter injected in duplicate into the HPLC. Subsequently, a linear regression analysis was performed by plotting the average concentration ( $\mu\text{g/ml}$ ) against the peak area. The calculated  $R^2$  value of 0.999975413590131  $\approx 1$  that was obtained is therefore a sure indication that the analytical method is stable and reliable (Araujo, 2009:2229).

**Table A.2:** Linearity results for clofazimine

| <b>Standard solution<br/>(µg/ml)</b> | <b>Peak area ratio</b> |
|--------------------------------------|------------------------|
| 0.1                                  | 18.3                   |
| 0.2                                  | 32.6                   |
| 0.4                                  | 58.4                   |
| 0.7                                  | 113.7                  |
| 1.1                                  | 171.2                  |
| 1.5                                  | 228.6                  |
| 3.7                                  | 554.7                  |
| 7.5                                  | 1101.7                 |
| 11.3                                 | 1662.0                 |
| 15.1                                 | 2201.8                 |
| 37.6                                 | 5587.1                 |
| 75.3                                 | 11062.2                |
| 112.9                                | 16539.1                |
| 150.6                                | 21903.9                |
| <b>R<sup>2</sup></b>                 | <b>1</b>               |
| <b>Slope</b>                         | <b>147.09</b>          |
| <b>Intercept</b>                     | <b>0</b>               |

#### **A.4.3 Accuracy**

The term accuracy, or trueness, is defined as the nearness of agreement between the values obtained and the actual concentration of the analyte (ICH, 2005:4). A specific concentration range (200 µg/ml, 20 µg/ml and 2 µg/ml) was used to assess the accuracy of this HPLC analytical procedure. Three different concentrations of clofazimine were prepared. From these three concentrates, three samples of each were prepared and injected in duplicate into the HPLC.

**Table A3:** Accuracy results for clofazimine

| Concentration spiked ( $\mu\text{g/ml}$ ) | Peak area 1 | Peak area 2 | Mean peak area | Recovery         |              |
|---|-------------|-------------|----------------|------------------|--------------|
|   |             |             |                | $\mu\text{g/ml}$ | %            |
| 18.6                                      | 2681.5      | 2675.0      | 2678.3         | 18.7             | 100.4        |
| 15.5                                      | 2248.2      | 2241.8      | 2245.0         | 15.6             | 100.5        |
| 21.3                                      | 3023.9      | 3052.5      | 3038.2         | 21.3             | 99.9         |
| 43.4                                      | 6221.8      | 6224.4      | 6223.1         | 44.0             | 101.4        |
| 36.2                                      | 5232.3      | 5225.4      | 5228.9         | 36.9             | 102.1        |
| 49.7                                      | 7081.2      | 7065.7      | 7073.5         | 50.1             | 100.9        |
| 93.1                                      | 13282.7     | 13291.9     | 13287.3        | 94.6             | 101.6        |
| 77.5                                      | 11162.1     | 11166.1     | 11164.1        | 79.4             | 102.5        |
| 106.4                                     | 15039.1     | 15030.0     | 15034.6        | 107.1            | 100.6        |
|   |             |             |                | <b>Mean</b>      | <b>101.1</b> |
|   |             |             |                | <b>SD</b>        | <b>0.8</b>   |
|   |             |             |                | <b>%RSD</b>      | <b>0.8</b>   |

For the accuracy test to be successful, the accuracy of the performed analytical instrument should fall between the mean recovery values of 98% - 102% (Du Preez, 2010a:6). The mean recovery of this method was 101.1 %, which was found to comply with the acceptable criteria. This HPLC method is thus found to be sufficiently accurate for the analysis of clofazimine.

#### A.4.4 Precision

Precision can be defined as the nearness in the agreement between several measurements obtained from various samples of the same homogeneous sample under certain prescribed conditions (ICH, 2005:4). Precision can be categorised into two sub-sections, i.e. intra-day and inter-day precision. To determine the precision of an HPLC method, a minimum of three concentrations must be analysed yielding an analysis result falling within the range of expected concentrations (FDA, 2001:5).

##### A.4.4.1 Intra-day precision

To observe the within-day precision of the method used, intra-day precision measurements must be performed to establish whether the system is reproducible on the same day under identical conditions (Araujo, 2009:2227). For these analyses, nine clofazimine samples were prepared in the laboratory on the same day. Each sample was injected in duplicate. Because this method involves no sample preparation and that the contents of the acceptor cells can simply be withdrawn and directly transferred into HPLC vials for analysis, it was possible to execute the accuracy and intra-day precision experiments simultaneously.

**Table A4:** Intra-day precision results for clofazimine

| Concentration spiked ( $\mu\text{g/ml}$ ) | Peak area 1 | Peak area 2 | Mean peak area | Recovery         |              |
|---|-------------|-------------|----------------|------------------|--------------|
|   |             |             |                | $\mu\text{g/ml}$ | %            |
| 18.6                                      | 2681.5      | 2675.0      | 2678.3         | 18.7             | 100.4        |
| 15.5                                      | 2248.2      | 2241.8      | 2245.0         | 15.6             | 100.5        |
| 21.3                                      | 3023.9      | 3052.5      | 3038.2         | 21.3             | 99.9         |
| 43.3                                      | 6221.8      | 6224.4      | 6223.1         | 44.0             | 101.4        |
| 36.2                                      | 5232.3      | 5225.4      | 5228.9         | 36.9             | 102.1        |
| 49.7                                      | 7081.2      | 7065.7      | 7073.5         | 50.1             | 100.9        |
| 93.1                                      | 13282.7     | 13291.9     | 13287.3        | 94.6             | 101.6        |
| 77.5                                      | 11162.1     | 11166.1     | 11164.1        | 79.4             | 102.5        |
| 106.4                                     | 15039.1     | 15030.0     | 15034.6        | 107.1            | 100.6        |
|   |             |             |                | <b>Mean</b>      | <b>101.1</b> |
|   |             |             |                | <b>SD</b>        | <b>0.8</b>   |
|   |             |             |                | <b>%RSD</b>      | <b>0.8</b>   |

The acceptance criterion for intra-day precision is a % RSD of not more than 2 % (McPolin, 2009:84). Table A.4 lists the % RSD as having been 0.8 %, and therefore the HPLC method complies with the stated 2 % acceptance limit.

#### A.4.4.2 Inter-day precision

Inter-day precision, also called intermediate precision, is used to express the variations that could occur in the laboratory during the preparation of the standard samples on two or more days (ICH, 2005:5). This particular analysis was conducted over a period of three consecutive days. For these analyses, samples of 52  $\mu\text{g/ml}$  were prepared in triplicate.

For the inter-day precision outcome to comply, the SD should be less than 2 % (Johnson & Van Buskirk, 1996:97). As per Table A.5, the SD on days 1 and 3 were within the acceptance criterion. Although the SD on day 2 (2.1) was slightly higher (0.1 %) than the acceptance criterion, this was still regarded as acceptable. The overall SD between the 3 days complied with the acceptance criterion.

**Table A5:** Inter-day precision results for clofazimine

| Parameters  | Day 1        | Day 2       | Day 3       | Between days |
|-------------|--------------|-------------|-------------|--------------|
|             | 101.4        | 102.3       | 98.1        |              |
|             | 102.1        | 99.92       | 98.1        |              |
|             | 100.9        | 97.1        | 98.3        |              |
| <b>Mean</b> | <b>101.5</b> | <b>99.7</b> | <b>98.2</b> | <b>99.8</b>  |
| <b>SD</b>   | <b>0.5</b>   | <b>2.1</b>  | <b>0.1</b>  | <b>1.8</b>   |
| <b>%RSD</b> | <b>0.5</b>   | <b>2.6</b>  | <b>0.2</b>  | <b>1.84</b>  |

#### A.4.5 System repeatability

System repeatability is defined as the precision associated with an analytical method over a short period of time (Ermer, 2001:763). A 100 % clofazimine standard solution was prepared and injected six times consecutively.

**Table A.6:** System repeatability results for clofazimine

| <b>Injection number</b> | <b>Peak area</b> | <b>Retention time (min)</b> |
|-------------------------|------------------|-----------------------------|
| 1                       | 5307.0           | 3.883                       |
| 2                       | 5274.5           | 3.891                       |
| 3                       | 5268.0           | 3.889                       |
| 4                       | 5271.0           | 3.872                       |
| 5                       | 5268.8           | 3.868                       |
| 6                       | 5284.9           | 3.874                       |
| <b>Mean</b>             | <b>5279.0</b>    | <b>3.879</b>                |
| <b>SD</b>               | <b>13.69</b>     | <b>0.009</b>                |
| <b>%RSD</b>             | <b>0.26</b>      | <b>0.224</b>                |

The acceptance criterion for system repeatability states that the % RSD values obtained for the six determinations per sample may not vary by more than 2 % (Shabir, 2003:62). As shown in Table A.6, the % RSD was found to be 0.224 % for the retention times and 0.26 % for the peak areas. The % RSD outcomes were thus well below 2 %, which was indicative of a minimum SD for this clofazimine analytical method, which proved that the system's repeatability complied with the acceptance criterion.

#### A.4.6 Stability

Stability is a test performed as a measure of the chemical stability of an API under specified conditions in a particular environment over a pre-determined time period (FDA, 2001:21). Standard samples containing clofazimine were prepared as described before in section A.3. Each sample was injected at hourly intervals over a period of 24 hr. Each peak area was compared to the peak area obtained at time 0 hr. Table A.7 depicts the results obtained.

**Table A.7:** Stability results for clofazimine

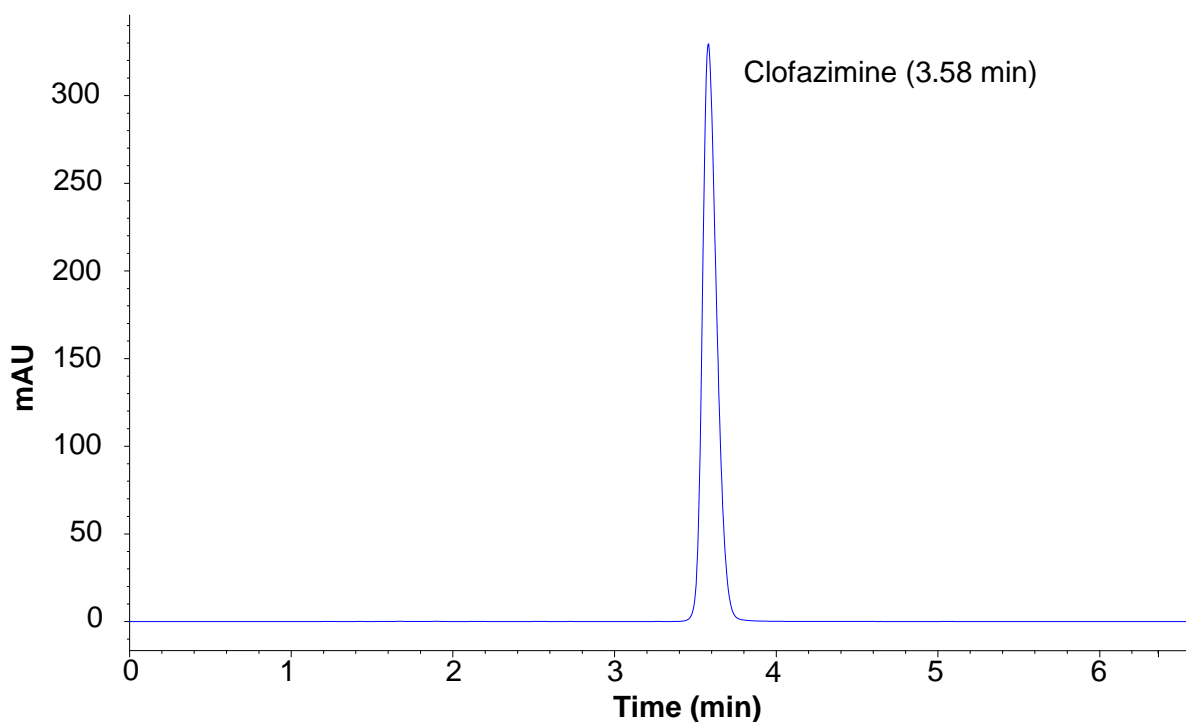
| <b>Time (h)</b> | <b>Peak area</b> | <b>% Recovery</b> |
|-----------------|------------------|-------------------|
| <b>0</b>        | 5300.8           | 100.0             |
| <b>1</b>        | 5359.3           | 101.1             |
| <b>2</b>        | 5338.3           | 100.7             |
| <b>3</b>        | 5320.3           | 100.4             |
| <b>4</b>        | 5321.2           | 100.4             |
| <b>5</b>        | 5290.7           | 99.8              |
| <b>6</b>        | 5283.9           | 99.7              |
| <b>7</b>        | 5299.4           | 100.0             |
| <b>8</b>        | 5287.8           | 99.8              |
| <b>9</b>        | 5288.7           | 99.8              |
| <b>10</b>       | 5295.9           | 99.9              |
| <b>11</b>       | 5299.4           | 100.0             |
| <b>12</b>       | 5305.8           | 100.1             |
| <b>13</b>       | 5297.4           | 99.9              |
| <b>14</b>       | 5311.7           | 100.2             |
| <b>15</b>       | 5312.6           | 100.2             |
| <b>16</b>       | 5293.2           | 99.9              |
| <b>17</b>       | 5296.8           | 99.9              |
| <b>18</b>       | 5290.3           | 99.8              |
| <b>19</b>       | 5288.8           | 99.8              |
| <b>20</b>       | 5291.1           | 99.8              |
| <b>21</b>       | 5286.8           | 99.7              |
| <b>22</b>       | 5292.4           | 99.8              |
| <b>23</b>       | 5278.4           | 99.6              |
| <b>24</b>       | 5305.0           | 100.1             |
| <b>Mean</b>     | <b>5301.40</b>   | <b>100.00</b>     |
| <b>SD</b>       | <b>17.72</b>     | <b>0.33</b>       |
| <b>%RSD</b>     | <b>0.33</b>      | <b>0.33</b>       |

For optimum sample stability, the samples should not be used, or kept for a longer period than it would take the analyte to degrade with a percentage of 2 % (Shabir, 2003:59). The results obtained showed a RSD % of 0.33 % that had proven that virtually no degradation of clofazimine had occurred over the duration of the 24 hr. Therefore clofazimine would stay stable during the duration of the 12 h skin diffusion studies performed.

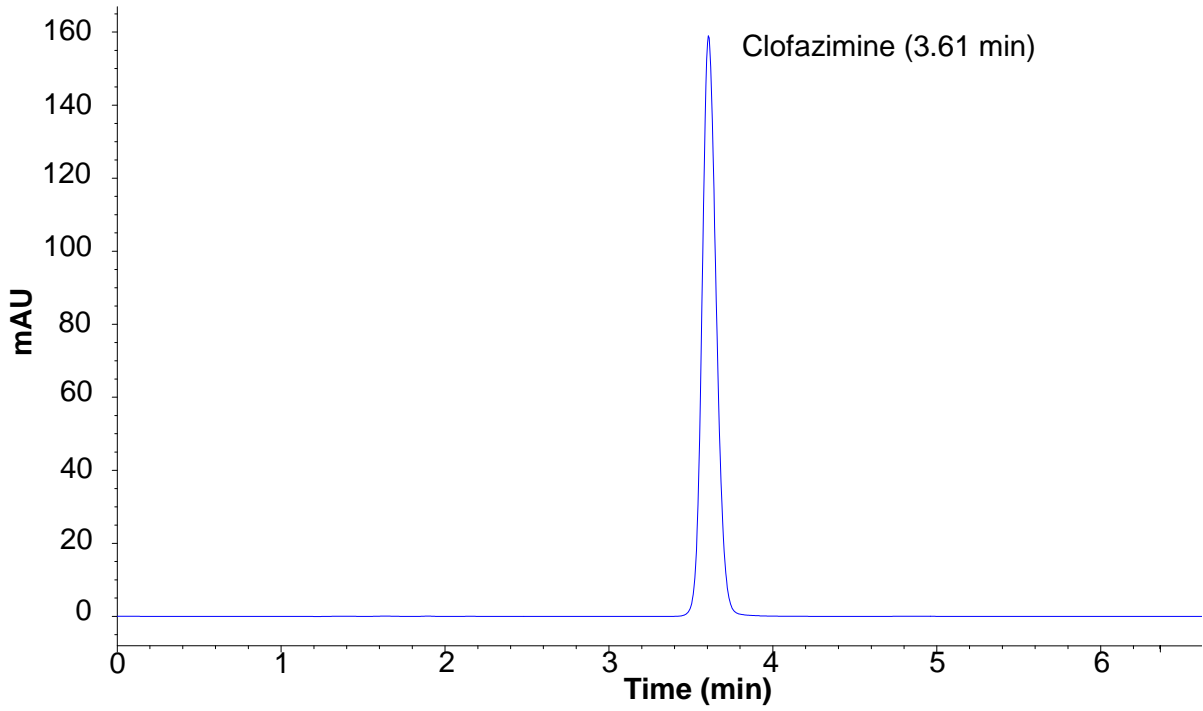
#### A.4.7 Specificity

Specificity is defined as the ability to quantify a particular analyte, despite the presence of other compounds, such as degraded analytes, impurities, or matrix compounds in the samples being analysed. For an analytical method to be deemed successful, the method should be able to differentiate between compounds that have structures very similar to that of the analyte (Shabir, 2003:62).

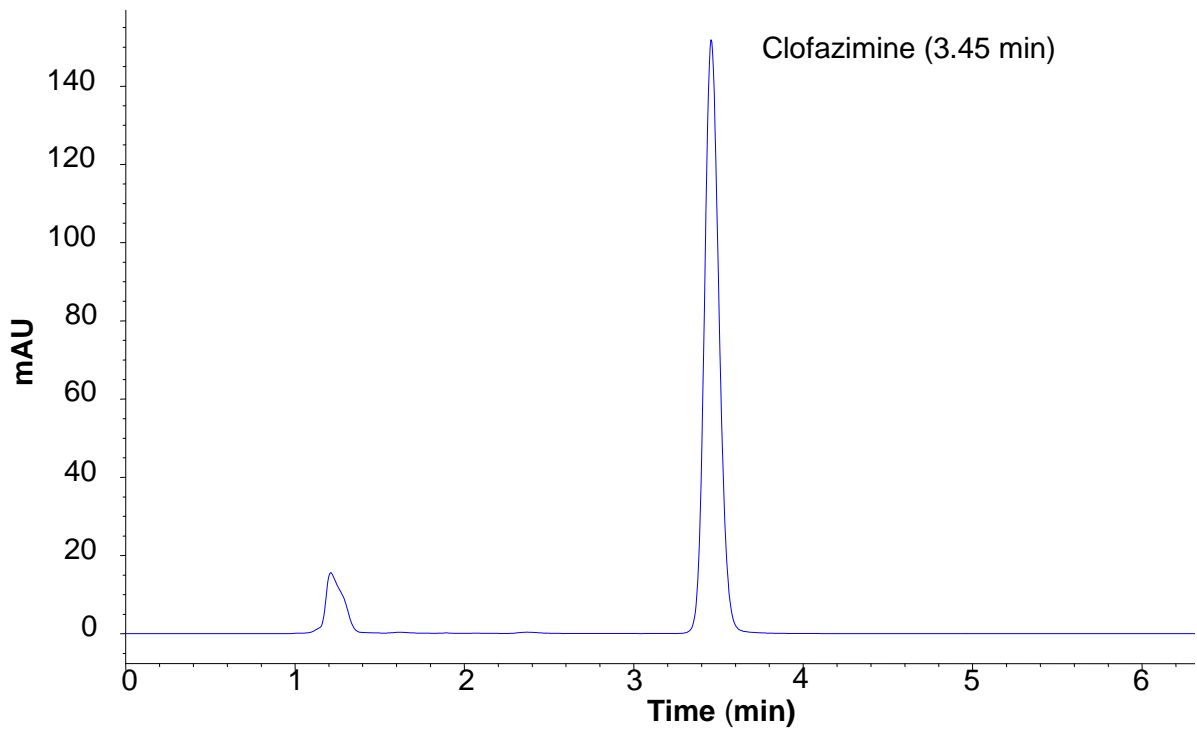
To establish specificity, a 100 % standard solution was prepared and 1 ml of this was transferred into four different test tubes. 1 ml of water, 1 ml of hydrochloric acid (1.865 g/mol), 1 ml of sodium hydroxide (1.8251 g/mol) and 1 ml of hydrogen peroxide (1.9992 g/mol) each was added to a different test tube. These samples were kept overnight and analysed the following morning.



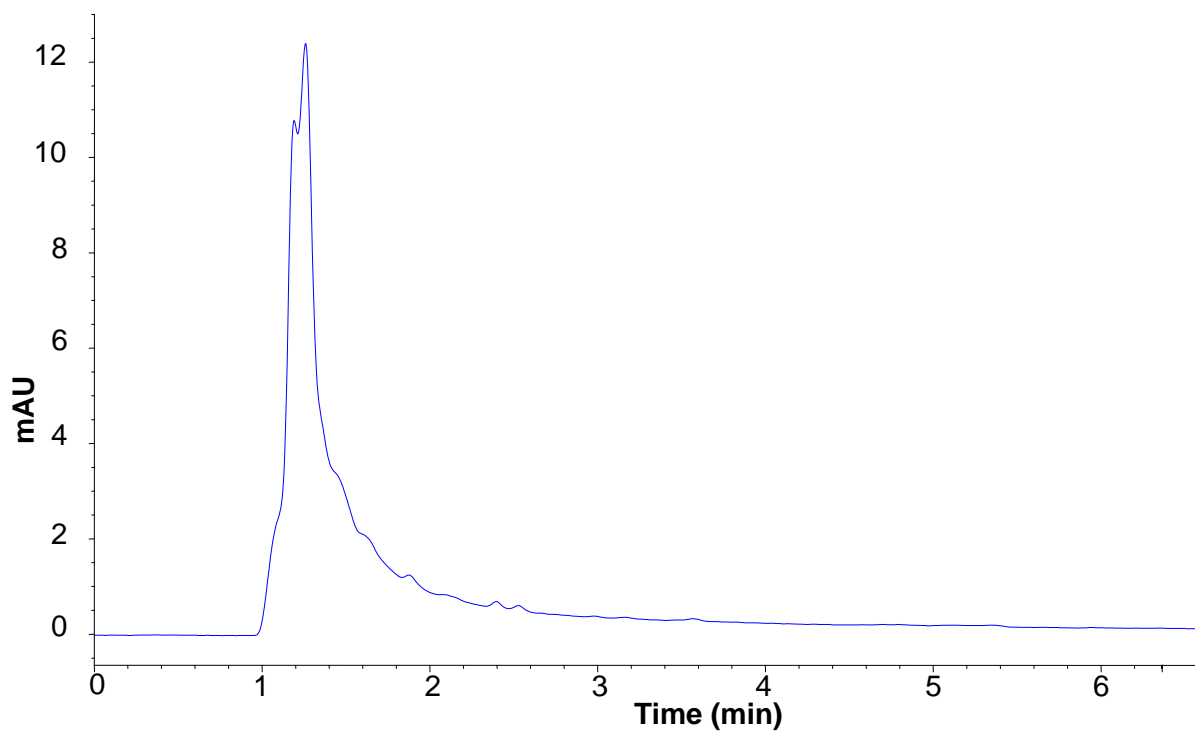
**Figure A.3:** HPLC chromatogram of the clofazimine standard solution pertaining to specificity



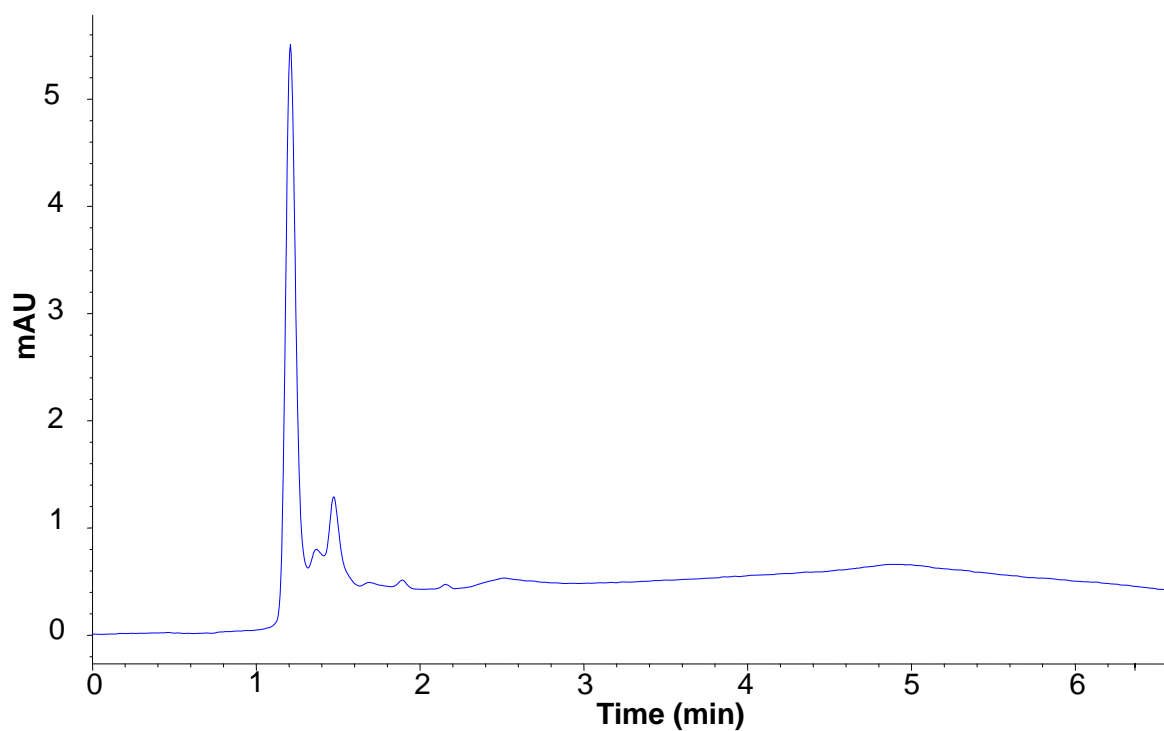
**Figure A.4:** HPLC chromatograph of clofazimine pertaining to specificity with 1 ml of water



**Figure A.5:** HPLC chromatograph of clofazimine pertaining to specificity with 1 ml of hydrochloric acid



**Figure A.6:** HPLC chromatograph of clofazimine pertaining to specificity with 1 ml of sodium hydroxide



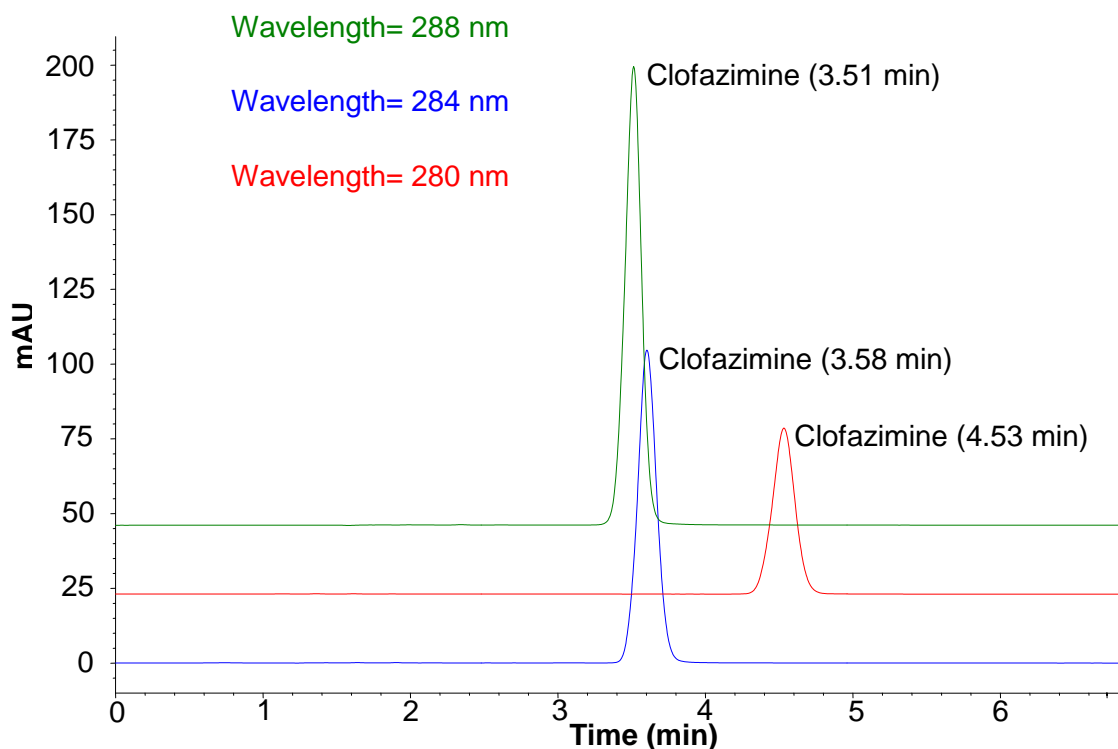
**Figure A.7:** HPLC chromatograph of clofazimine pertaining to specificity with 1 ml of hydrogen peroxide

Figure A.3 demonstrates that the peak of the clofazimine standard solution eluted at 3.58 min. In Figure A.4, the clofazimine standard that had been diluted with 1 ml of water, the peak eluted slightly later than that of the pure standard solution, at a time of 3.61 min. Figure A.5 illustrates the effect of the 1 ml hydrochloric acid on the peak of clofazimine, which eluted earlier than that of the standard solution, at 3.45 min. Despite the small first peak that signalled some decomposition of clofazimine, the clofazimine peak was still clearly detectable, although it had eluted earlier than the peak of the standard solution. In Figures A.6 and A.7 (clofazimine with 1 ml sodium hydroxide and 1 ml hydrogen peroxide, respectively) no clofazimine peaks, nor any signs of decomposition were noted.

For this analysis to be successful, according to the acceptance standards, the degraded samples and the placebo may not contain any peaks whatsoever that could interfere with the determination of the analyte. Where interference does occur, it may not affect the final result 0.5 % (Shabir, 2003:62). The results of these tests illustrated that most of the clofazimine peaks were clear and quantifiable, and although degradation was present in the one sample that had been diluted with 1 ml of hydrochloric acid, it did not interfere with the determination of the clofazimine in that sample. The clofazimine in those samples that had been diluted with the sodium hydroxide and hydrogen peroxide were, however, immeasurable, probably due to the degradation of the clofazimine by these chemicals added to the standard solution.

#### **A.4.8 Robustness**

Robustness is defined as a method's ability to remain unaffected if certain parameters of the method are slightly changed (Shabir, 2003:64). Different wavelengths, flow rates and ratios of the mobile phase components (acetonitrile and octane sulfonic acid) were used to test the robustness parameter of the HPLC method for clofazimine. The initial flow rate was 1.0 ml/min, the mobile phase ratio 65:35 (acetonitrile to octane sulfonic acid) and the wavelength of the method was 284 nm, as summarised in Section A.2. For the purpose of the first robustness test, the wavelength was changed to 280 nm, the flow rate to 1.2 ml/min and the mobile phase ratio to 60:40 (acetonitrile to octane sulfonic acid). In the second robustness test, the parameters were changed to a flow rate of 0.8 ml/min, the wavelength to 288 nm and the mobile phase ratio to 75:25 (acetonitrile to octane sulfonic acid).



**Figure A.8:** HPLC chromatographs of clofazimine standard solutions analysed at different flow rates, mobile phase ratios and wavelengths

Figure A.8 illustrates the results of the robustness parameter tests and although different wavelengths, mobile phase ratios (acetonitrile to orthophosphoric acid) and flow rates were used to analyse the clofazimine standards, all the peaks eluted at more or less the same time. The outcomes from the robustness tests therefore fell within the acceptance criteria set for this parameter, since the chromatographic results were not negatively affected by any of the changes made in wavelength, mobile phase ratios and flow rates of the clofazimine HPLC method.

## A.5 Conclusion

The validation of the HPLC method for clofazimine was found to be adequately sensitive, reliable and reproducible for the accurate quantification of the clofazimine concentrations. All of the parameters used for the validation of this HPLC method had complied with the relevant acceptance criteria set for an HPLC method validation.

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FDA **see** Food and Drug Administration.

Food and Drug Administration. 2001. U.S. Department of Health and Human Services. Guidance for industry: bioanalytical method validation. Rockville. 22p.

ICH **see** International Conference Harmonisation.

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# APPENDIX B

## FORMULATION OF NANO-EMULSIONS CONTAINING CLOFAZIMINE

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### B.1 Introduction

Nano-emulsions are emulsions with droplet sizes ranging between 20 nm and 500 nm. The small droplet sizes of these emulsions make them less prone to instabilities, such as creaming and sedimentation. These kinds of dispersions are watery and translucent (Pey *et al.*, 2006:144). During this study, an o/w nano-emulsion was formulated to attempt the topical delivery of clofazimine. For this purpose nano-emulsions were prepared for investigating the release and diffusion of the API from the formulation, through membrane release and skin diffusion studies, respectively.

To make the application of the watery nano-emulsion to the skin more practical, it was formulated into a more viscous emulgel formulation (see Appendix D).

In this appendix, the physiochemical properties of the nano-emulsions are discussed as part of the pre-formulation process. The formulation of the nano-emulsions is also described.

### B.2 Preparation of nano-emulsions

Two nano-emulsions were prepared for investigating the topical delivery of clofazimine, namely a placebo without the API, and another containing the API. The nano-emulsions were prepared in accordance with a modified combined spontaneous emulsification and high energy method, using both a probe sonicator and a sonicator bath (Bouchemal *et al.*, 2004:243; Solans *et al.*, 2005:103). The formation of nano-droplets was confirmed by transmission electron microscopy (TEM).

#### B.2.1 General preparation method of the nano-emulsions

A combination of a high energy method and a self-emulsification method was used during the preparation of the nano emulsions (Bouchemal *et al.*, 2004:243; Solans *et al.*, 2005:103). The water phase of the nano-emulsion and a non-ionic surfactant (Tween<sup>®</sup> 80) were heated to 60°C on a magnetic hot plate, whilst continuously stirring, until the non-ionic surfactant was completely dissolved in the water. The oil phase (avocado oil) of the nano-emulsion was heated to 60°C on a magnetic hot plate, together with another non-ionic surfactant (Span<sup>®</sup> 60), whilst continuously stirring until the surfactant was completely dissolved in the oil phase. Thereafter the API was added to the oil phase, whilst still being heated and also dissolved into the oil

phase until visibly dissolved. Subsequently the oil phase was drawn up into a 5 ml syringe and added drop-wise to the water phase (still on the magnetic hotplate at a temperature of about 60°C), whilst continuously stirring. This mixture of the oil and water phases was left to auto-stir (magnetically) on the magnetic hot plate at 60°C for another 5 min. The resulting coarse emulsion was then probe sonicated for 3 min at 40 Watt, after which it was placed in a sonicator bath for 15 min.

## B.2.2 Ingredients of the nano-emulsions

The nano-emulsions were formulated using the ingredients listed in Table B.1, along with their purposes, suppliers and batch numbers.

**Table B.1:** Ingredients used during the formulation of the nano-emulsions

| Ingredients           | Purpose                | Supplier           | Batch number   |
|-----------------------|------------------------|--------------------|----------------|
| Avocado oil           | Oil phase              | Flagship programme | 023/09/14      |
| Span <sup>®</sup> 60  | Lipophilic surfactant  | Fluka              | 423065/1 41002 |
| Tween <sup>®</sup> 80 | Hydrophilic surfactant | Merck              | 1043695        |
| Clofazimine           | API                    | Flagship programme | SCF663         |

### B.2.2.1 Avocado oil

Avocado oil is a natural oil that is high in poly-unsaturated fatty acids, such as linoleic acid (Ortiz *et al.*, 2004:11). In this study, avocado oil was included for two reasons, i.e. as the oil phase in both the nano-emulsion and the emulgel, while also serving as a penetration enhancer. The fatty acid content of the avocado oil has penetration enhancing effects that enables stratum corneum penetration of the dispersion and the emulgel formulation (Argenta *et al.*, 2014:4738; Williams & Barry, 2012:129, 132).

### B.2.2.2 Span<sup>®</sup> 60

Span<sup>®</sup> 60 is a lipophilic, non-ionic surfactant that is used to reduce the surface tension between the oil and water phases in emulsions. Span<sup>®</sup> 60 is considered to be non-toxic and relatively non-irritant to the cells of the body. This surfactant also acts as a solubiliser, a permeability enhancer, an emulsifying agent and a wetting agent (Kumar & Rajeshwarrao, 2011:209-210).

### B.2.2.3 Tween<sup>®</sup> 80

Tween<sup>®</sup> 80, also known as polysorbate 80, is a polyoxyethylene sorbitan fatty acid. It is a non-ionic hydrophilic surfactant that is commonly used as an excipient in o/w emulsions (Bouchemal *et al.*, 2004:243). This excipient is regarded as safe for pharmaceutical dispersions, as it is non-toxic and non-irritant (Kumar & Rajeshwarrao, 2011:209). The function

of Tween<sup>®</sup> 80 in nano-emulsions is to act as a wetting agent, as well as an emulsifying agent in the water phase of the formulated emulsions (Dubos & Middlebrook, 1948:521; Mann & Markham, 1998:539).

#### **B.2.2.4 Clofazimine**

Clofazimine, the API being investigated during this study, is a second-line antibiotic that is used in the treatment of MDR-TB (Xu *et al.*, 2012:1104). In this study, clofazimine was formulated into topical nano-emulsions and into nano-emulsion containing formulations, for the potential future treatment of CTB. Topical CTB treatments will never replace the systemic treatment regimes, but are they aimed at possibly shortening the required duration of treatment by applying clofazimine topically, directly to the affected areas. The lipophilic clofazimine was incorporated into the oil phase of the emulsions during the formulation process (TB alliance, 2008:96).

### **B.3 Formulation and physical characterisation of nano-emulsions**

Optimisation of the pre-formulation resulted in the final nano-emulsion containing the API. The physical characteristics (light microscopy, TEM, sizes of the nano-emulsion droplets, the size distributions of the droplets and the entrapment efficacy (EE%)) were determined to ensure an optimal formulation (see Appendix C) (Jadon *et al.*, 2009:1188). Nano-emulsions were initially prepared without any encapsulated API. This was done to determine the ideal lipophilic-hydrophilic surfactant ratio for both the placebo nano-emulsion and the nano-emulsion containing the API.

#### **B.3.1 Preparation of nano-emulsions without the API**

To determine the optimal lipophilic-hydrophilic surfactant ratio, two Span<sup>®</sup> 60 : Tween<sup>®</sup> 80 ratios (3:2 and 1:4) were prepared. Each surfactant ratio was incorporated into a different nano-emulsion and was tested using TEM, light microscopy, droplet size and distribution, aimed at determining the optimal surfactant ratio after probe sonication for 3 minutes and subsequent bath sonication for 15 min. All of the sonicated samples were viewed under the light microscope to establish whether oil droplets had indeed formed and to determine the sizes of any visible droplets. Hereafter, the samples were viewed with the TEM (FEI Tecnai G2 TEM, FEI, Holland) to determine the droplet sizes and also to identify which dispersion had the smallest droplet sizes and which one seemed the most stable. The droplet sizes and droplet size distributions of the nano-emulsion samples were determined, using a Malvern Zetasizer (Nano ZS, Malvern Instruments, UK). By considering all the physical properties of the nano-emulsions, an optimal lipophilic-hydrophilic surfactant ratio could be determined for the nano-emulsion dispersion without the API.

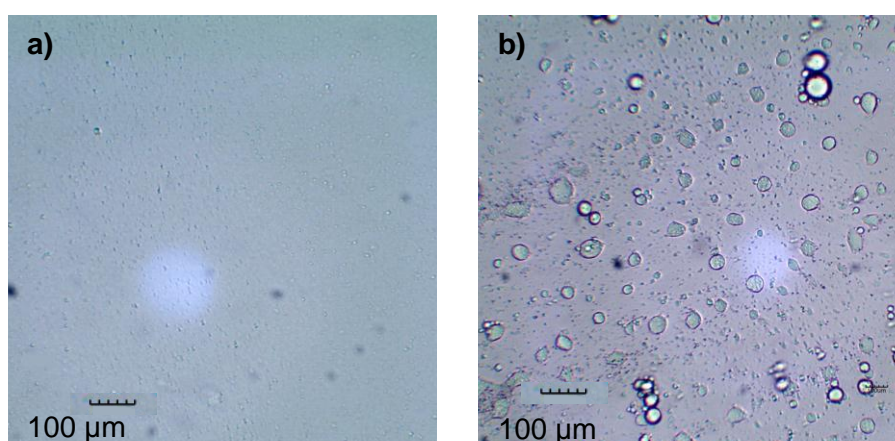
### B.3.1.1 Method of preparation of nano-emulsions without the API

Nano-emulsions without the API were prepared by firstly weighing and measuring all the required amounts of the excipients for both the surfactant ratio nano-emulsions (3:2 and 1:4). The nano-emulsions were each prepared separately. Firstly, the water phase and the Tween<sup>®</sup> 80 were heated at 60°C on a magnetic hot plate during continuous magnetic stirring, until the Tween<sup>®</sup> 80 was completely dissolved in the water. Thereafter, the oil phase and the Span<sup>®</sup> 60 were heated to 60°C, until completely mixed. The heated oil phase was drawn up in a syringe and was added drop-wise to the water phase. The coarse emulsion that formed was heated and magnetically stirred for 5 min at 60°C. The coarse emulsion was then probe sonicated for 3 min and subsequently placed in the sonicator bath for 15 min.

### B.3.2 Physical characterisation of the nano-emulsions

#### B.3.2.1 Light microscopy

Light microscopy was used to establish whether oil droplets had formed after the formulation process. The shapes of the oil droplets, their sizes, as well as their homogeneity were investigated. Light microscopy was used to observe the possible formation of oil droplets during the initial phases of the study, since it is less costly than TEM, more convenient, easier to access and less time consuming. A Nikon Eclipse E4000 microscope (Nikon, Japan, Linkam THMS600), equipped with a Nikon DS-Fi1 camera, was used. A small volume of each of the two dispersions with the different surfactant ratios (3:2 and 1:4) were placed on separate 20 mm microscope slides each. Each dispersion was viewed at a magnification of 40 x.



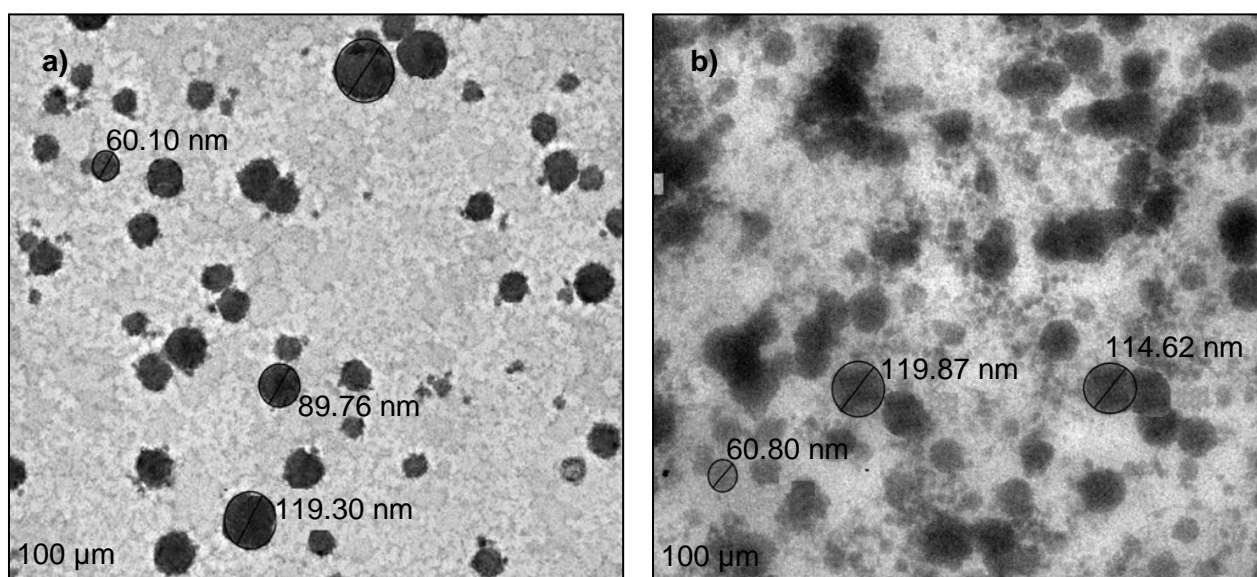
**Figure B.1:** Light microscopic results of a) placebo nano-emulsion (3:2 surfactant ratio) and b) placebo nano-emulsion (1:4 surfactant ratio) with a 100 µm scale indicated in each

Figure B.1 depicts the micrographs of the nano-emulsions with two different surfactant ratios (3:2 and 1:4) at a 40 x magnification. No nano-sized oil droplets could be observed, but only larger oil droplets were visible, due to the limitations of the light microscope.

The 3:2 surfactant ratio nano-emulsions had formed smaller and more uniform oil droplets than the 1:4 surfactant ratio nano-emulsions. The 1:4 surfactant nano-emulsions showed signs of instability, as coagulation of the oil droplets were visible.

### B.3.2.2 Transmission electron microscopy (TEM)

The two nano-emulsions (surfactant ratio dispersions of 3:2 and 1:4) were inspected on a TEM (FEI Technai G2 TEM, FEI, Holland, at 120 kV, fitted with a Gatan bottom mount digital camera) to establish whether nano-emulsions had formed. Dr A. Jordaan (Laboratory for Electron Microscopy, North-West University, Potchefstroom Campus) performed the TEM procedures. After diluting one drop of each sample with 10 ml of distilled water, a drop of sample was placed on a copper carbon-coated 300 mesh grid and allowed to dry for a period of about 15 min. The oil droplets' lipid films were stained, using osmium tetroxide, before inserting the sample into the TEM. The magnifications used were between 5 000 x and 15 000 x.



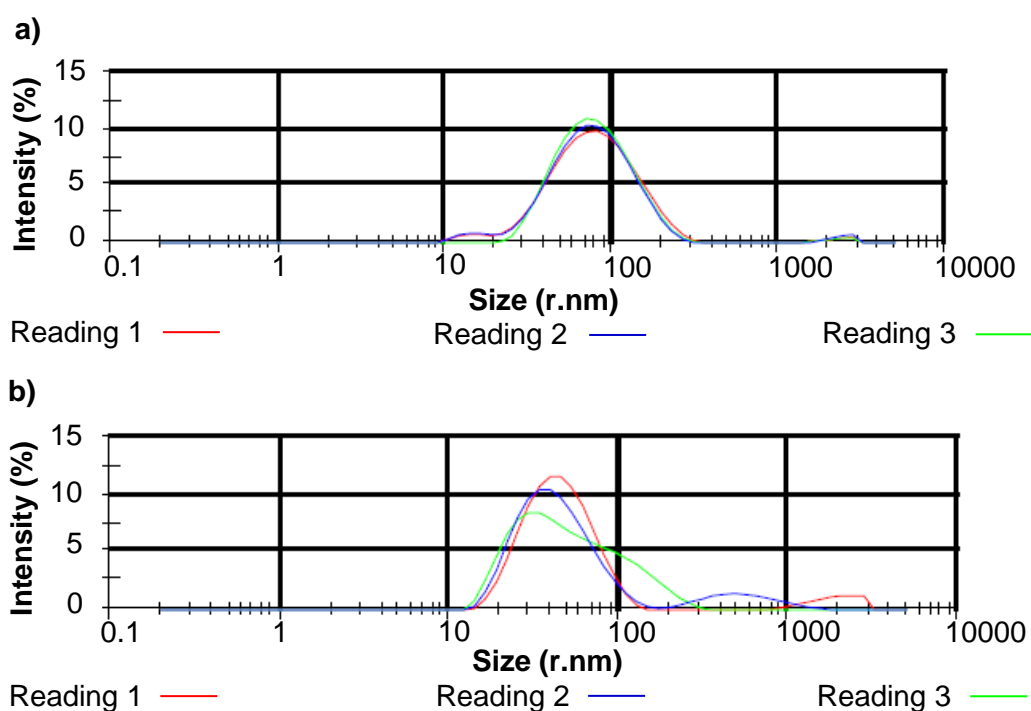
**Figure B.2:** TEM micrographs of a) the 3:2 surfactant ratio placebo nano-emulsion and b) the 1:4 surfactant ratio placebo nano-emulsion

Figure B.2 shows the micrographs obtained from the TEM. Oil droplets had formed in both the nano-emulsions. The darker areas represented the oil droplets of the nano-emulsions. The osmium had attached to the lipid layers of the oil droplets of the nano-emulsions. Some of the oil droplets had stronger lipid layers than others. The droplets with the weaker lipid layers were lightly coloured in the background by the osmium, compared to the darker coloured stronger

lipid layers. Figure B.2. b) illustrates that the 1:4 surfactant dispersion had bigger droplets and that the droplets were closer to each other than those in the 3:2 surfactant dispersion (Figure B.2. a)). The droplets in Figure B.2. b) showed signs of droplet coagulation and instability (Sharma *et al.*, 2010:3).

### B.3.2.3 Droplet size distribution

To determine which of the two dispersions had yielded a uniform and stable dispersion, the droplet sizes and droplet size distributions were determined. Since small droplet sizes and a narrow droplet size distribution in nano-emulsions would be indicative of stable dispersions, it is important to determine the droplet size distribution of the dispersions (Solans *et al.*, 2005:102). The droplet sizes of the dispersions were determined using a Malvern Zetasizer (Nano ZS) (Malvern Instruments, UK). Each of the nano-emulsion samples were diluted by adding 2 drops to 10 ml of distilled water and each sample was transferred into a plastic cuvette for the droplet size determinations. Each sample was analysed in triplicate.



**Figure B.3:** The droplet size distribution of the different surfactant ratio nano-emulsions, i.e. a) the 3:2 ratio placebo nano-emulsion and b) the 1:4 ratio placebo nano-emulsion

### **B.3.3 The ideal surfactant ratio in the placebo nano-emulsion**

The droplet sizes and droplet size distribution results of the two placebo nano-emulsions are depicted in Figure B.3. Both placebo nano-emulsions presented with a narrow droplet size distribution, as well as small average droplet sizes. The average droplet sizes of the 1:4 ratio and the 3:2 ratio placebo nano-emulsions were measured at 41.70 nm and 65.51 nm, respectively, with the 1:4 ratio placebo nano-emulsion showing a smaller average droplet size than the 3:2 ratio placebo nano-emulsion. According to the light microscopic results and the TEM results the 3:2 placebo nano-emulsion was expected to have had a smaller average droplet size than the 1:4 placebo nano-emulsion. However, both placebo nano-emulsions' average particle sizes fell within the preferred droplet size ranges of 20 - 200 nm, as stated by Solans *et al.* (2005:102).

### **B.3.4 The optimal surfactant ratios of the placebo nano-emulsion**

The optimal surfactant ratios were selected based upon the results of the physical characteristics of the nano-emulsions (light microscopy, TEM, droplet sizes and size distributions).

The light microscopic results indicated that the 1:4 surfactant ratio placebo nano-emulsions had larger droplets and signs of droplet coagulation were visible. The 3:2 surfactant ratio placebo nano-emulsion showed much smaller droplets and almost no droplet coagulation was visible. The TEM results showed that the nano-emulsions with the 1:4 surfactant ratio placebo nano-emulsion had demonstrated a higher prevalence of coagulation and the droplets were generally larger than those of the 3:2 surfactant ratio placebo nano-emulsion. Consequently, the droplet sizes and size distributions of the 1:4 ratio placebo nano-emulsion had a smaller average droplet size than the 3:2 surfactant ratio placebo nano-emulsion. Both of these placebo nano-emulsions had a satisfactory droplet size distribution.

Based on the results obtained from the light microscopic analyses, the TEM, and the droplet sizes and droplet size distribution outcomes, the 3:2 surfactant placebo nano-emulsion had proven to be the dispersion of choice for formulating nano-emulsions.

### **B.3.5 Preparation of nano-emulsions containing clofazimine**

#### **B.3.5.1 Determination of the solubility of the API in the oil phase of the nano-emulsion**

After the ideal surfactant ratio was determined, the maximum amount of the API that would dissolve in the oil phase containing the Span<sup>®</sup> 60 was determined, by heating the oil phase (4.6 ml avocado oil) and the Span<sup>®</sup> 60 (1 g) on a magnetic hot plate whilst continuously stirring at 60°C, until completely dissolved. Thereafter an excess amount of API was added to the

heated mixture and magnetically stirred, until completely dissolved. The mixture was left to stir overnight on the magnetic hot plate at a low temperature. On the following day, 200 µl of the mixture was dissolved in tetrahydrofuran and centrifuged at 20 000 rpm for 20 min at a temperature of 25°C. The supernatant was extracted from the centrifuged sample in order to obtain the oil phase of the sample, with the highly lipophilic API entrapped within. The concentration of the API was determined through HPLC analysis.

The maximum amount of API that had dissolved in the heated oil phase (4.6 ml avocado oil and 1 g Span<sup>®</sup> 60), was measured at 7.164 mg.

### **B.3.5.2 Method of preparation of nano-emulsions with entrapped clofazimine**

The nano-emulsion was prepared by using the same method as described in section B.2.1. The quantified amount (7.164 mg) of the API was carefully weighed. The avocado oil and the Span<sup>®</sup> 60 (oil phase) were mixed and heated together in a beaker on a magnetic hot plate at a temperature of 60°C. Thereafter, the API was added to the oil phase of the nano-emulsion. This mixture was subsequently stirred at a temperature of 60°C, until the API had completely dissolved in the oil phase. The oil phase was then added drop-wise to the water phase of the nano-emulsion and magnetically stirred on the magnetic hot plate for 5 min at a temperature of 60°C. The coarse emulsion was then probe sonicated and subsequently bath sonicated.

### **B.3.6 Final preparation of nano-emulsions containing clofazimine**

The final formulation of the nano-emulsions with the entrapped API was decided upon, after the physical characterisation of the pre-formulation had been done (light microscopy, TEM, droplet sizes and size distributions, and the concentration of clofazimine that was entrapped in the oil phase). On grounds of these physical properties, the 3:2 surfactant ratio nano-emulsion was selected as the optimal dispersion formulation. The characterisation of the nano-emulsion containing the API is discussed in Appendix C.

#### **B.3.6.1 Preparation of a nano-emulsion with encapsulated clofazimine**

The final formula for the nano-emulsion containing clofazimine was selected, based upon the physical properties obtained. The 3:2 surfactant ratio nano-emulsion was formulated with the maximum possible quantity of API (7.1 mg) dissolved and encapsulated into the oil phase of the dispersion.

##### **B.3.6.1.1 Method of preparation of nano-emulsions containing clofazimine**

The final method of preparation of the nano-emulsion was as described in Section B.2.1. The only difference being made was that the API was dissolved in the oil phase at a temperature of

60°C, after the Span® 60 had completely dissolved in the avocado oil. Table B.1 lists all the components of the oil phase (A) and the water phase (B) of the final nano-emulsion being formulated for the purpose of investigating the possible topical delivery of clofazimine during this study.

**Table B.2:** Oil phase (A) and water phase (B) of the nano-emulsion containing clofazimine

|          | <b>Ingredients</b> | <b>Required amount</b> |
|----------|--------------------|------------------------|
| <b>A</b> | Span® 60           | 1.0 g                  |
|          | Avocado oil        | 4.6 ml                 |
|          | Clofazimine (API)  | 7.1 mg (0.0142%)       |
| <b>B</b> | Tween® 80          | 3.6 ml                 |
|          | Water              | 42 ml                  |

#### **B.3.6.2.2 Outcome**

The nano-emulsion was watery, light orange in colour and homogenous.

#### **B.4 Conclusion**

The optimum formula of the placebo nano-emulsion was decided upon, based on the outcome of the physical characteristics (light microscopy, TEM, droplet sizes and droplet distributions) of the nano-emulsion. The API was only included in the optimal nano-emulsion formulation. The optimised dispersion contained a surfactant ratio of 3:2 (Span® 60 : Tween® 80) and a maximum API amount of 7.1 mg. The characterisation of the optimised nano-emulsion containing clofazimine is discussed in Appendix C.

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TB alliance **see** Global alliance for TB drug development.

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# APPENDIX C

## CHARACTERISATION OF NANO-EMULSIONS

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### C.1 Introduction

Characterisation was done on the placebo nano-emulsion (**P-NE**) and the nano-emulsion containing the API (**A-NE**), which included a compatibility study between the oil phase of the nano-emulsion and the API (clofazimine), light microscopy, zeta-potential, droplet size, entrapment efficiency percentage (EE%), pH and viscosity. Characterisation of the physical properties of each dispersion (**P-NE** and **A-NE**) was used to evaluate these dispersions' stabilities, physical appearances and homogeneities (Intertek, 2013; Niazi, 2009:61).

### C.2 Optimised o/w nano-emulsion containing clofazimine and avocado oil

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

The optimised nano-emulsion formula and the method of preparation of this particular nano-emulsion were decided upon after the pre-formulation was completed as discussed in Appendix B. The formula of the optimised nano-emulsion containing avocado oil for both the **A-NE** and the **P-NE** formulas with the 3:2 surfactant ratio (Span<sup>®</sup> 60: Tween<sup>®</sup> 80) is given in Table C.1.

**Table C.1:** Formula of the **P-NE** and the **A-NE**

| Phase of nano-emulsion | P-NE                         |           | A-NE                         |           |
|------------------------|------------------------------|-----------|------------------------------|-----------|
|                        | Amount of excipient required |           | Amount of excipient required |           |
| Oil                    | Avocado oil                  | 4.6 ml    | Avocado oil                  | 4.6 ml    |
|                        | Span <sup>®</sup> 60         | 1000.0 mg | Span <sup>®</sup> 60         | 1000.0 mg |
|                        | No API included              | -         | Clofazimine (API)            | 7.2 mg    |
| Water                  | Water                        | 42.0 ml   | Water                        | 42.0 ml   |
|                        | Tween <sup>®</sup> 80        | 3.6 ml    | Tween <sup>®</sup> 80        | 3.6 ml    |

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

The excipients used to formulate the optimised o/w nano-emulsions together with their functions are discussed in Section B.2.2.

## **C.4 Characterisation methods**

Characterisation was done on the **P-NE** and the **A-NE** according to the following parameters: light microscopy, droplet size and droplet size distribution, pH, viscosity, zeta-potential and EE%. Before the characterisation was done, the chemical stability of the API and the oil phase was determined using compatibility studies with microcalorimetry.

### **C.4.1 Clofazimine and avocado oil stability**

#### **C.4.1.1 Compatibility studies of clofazimine and avocado oil**

It is important to evaluate the chemical compatibility and stability of a drug with the excipients of a pharmaceutical dispersion before the development of the dispersion (Selzer *et al.*, 1998:227). A compatibility study was done between avocado oil and clofazimine via microcalorimetry. Calorimetry refers to a technique used for the measuring of the heat flow between a sample and its surroundings as a function of time (Phipps & Mackin, 2000:9).

Microcalorimetry is used to detect incompatibilities, as well as instabilities between an API and/or the excipients (Selzer *et al.*, 1998:227). This is a dependable method that can be used to detect incompatibilities ascribed to the fact that every physical and chemical incompatibility is accompanied with the exchange of heat (Phipps & Mackin, 2000:9). Thus, microcalorimetry is sensitive to all chemical and physical processes that are linked to heat exchange (Selzer *et al.*, 1998:228). The method has a high sensitivity, which allows for the performance of measurements at temperatures close to real conditions and enables it to detect very slow reactions (Schmitt *et al.*, 2001:176). It should be taken into consideration that heat flow can come from either one process or a few processes. It is very important to have sufficient experimental background regarding the sample analysed and therefore, experimental planning must preferably be used in order to detect and isolate specific contributors to the heat data (Selzer *et al.*, 1998:228).

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

In order to perform this study, a 2277 Thermal Activity Monitor (TAM) (TA Instruments, USA) equipped with an oil bath, with a stability of about 100  $\mu$ K (micro-kelvin) over 24 h was used. During the course of this experiment, the temperature of the calorimeter was kept at 32 °C and 60 °C, respectively. The higher temperature was used to speed up or enhance any incompatibility reaction that could possibly present itself. The heat reactions of both avocado oil and clofazimine were tested separately, and in addition to this the heat reactions of a mixture of avocado oil and clofazimine was also tested. All calorimetric outputs observed for these individual samples were added together to give a theoretical response per sample. The

calculated hypothetical response embodies the calorimetric results that would be probable if the two separate samples do not react with each other. If, however, the individual samples do react with each other, the measured calorimetric response would differ from the predicted theoretical calorimetric response.

## **C.4.2 Morphology studies**

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

To prove that oil droplets had indeed been formed in the nano-emulsions, the nano-emulsions was examined with a light microscope. The method used was as described in Section B.3.2.1 and both the sizes and the shapes of the oil droplets of the nano-emulsions were thus observed. Micrographs of both formulations were taken at a magnification of 40 x.

### **C.4.3 Droplet sizes and droplet size distributions**

The sizes and droplet size distributions of the nano-emulsion droplets play a crucial role in the stability of a nano-emulsion. If they are within the acceptable ranges, the nano-emulsion would be stable and free from potential instabilities, such as sedimentation and creaming (Solans *et al.*, 2005:102). The droplet sizes of a nano-emulsion further plays a crucial role in the encapsulation of the API in the dispersion (Kumar & Rajeshwarrao, 2011:213). Smaller droplet sizes and a narrow droplet size distribution in a nano-emulsion hence offer a stable dispersion (Solans *et al.*, 2005:102).

The droplet sizes and distributions of the nano-emulsions were determined, using a Malvern Zetasizer ZS (Malvern Instruments, UK). The **P-NE** and **A-NE** nano-emulsion samples were freshly prepared prior to measurement. 2 ml of each nano-emulsion was diluted separately with 10 ml of distilled water each. Each sample was analysed in triplicate, by injecting a fresh sample into a plastic cuvette prior to each reading without delay, to ensure reliable results.

### **C.4.4 pH**

The pH of the nano-emulsions was measured, using a Mettler Toledo Seven Compact pH meter (Mettler Toledo, Switzerland). The pH meter was calibrated prior to the sample measurements, using three buffered solutions at pH 4, 7 and 10 to ensure accurate sample readings. The measurements were done in triplicate by submerging the probe of the pH meter into three samples each of the freshly prepared **P-NE** and **A-NE** nano-emulsions.

### **C.4.5 Viscosity**

The viscosities of the two **P-NE** and **A-NE** nano-emulsions were determined, using a Brookfield Viscometer (model DV-II+, Stoughton, Massachusetts, USA), equipped with rotating spindles

and Helipath attachments. The viscosity measurements per formulation were done in triplicate, by inserting the rotating spindle, set at a pre-determined rotation speed, into three freshly prepared samples of each formulation, to ensure a reliable average reading. The viscometer data was gathered, using Wingather™ computer software that had been pre-programmed to record 32 readings at 10 second intervals. The collected data of each formulation was then used to calculate the average viscosity of each nano-emulsion.

#### **C.4.6 Zeta-potential**

The zeta-potential of the **P-NE** and **A-NE** nano-emulsions were measured, using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). A sample of each formulation was freshly prepared by diluting 2 ml of each separately in 10 ml of distilled water. On the same day of preparation, a small amount of each nano-emulsion sample was injected separately into a plastic cuvette and the zeta-potential was measured in triplicate to ensure a reliable average reading.

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

Ultra-centrifugation was used to determine the EE% of the oil phase of the nano-emulsions. A Beckman Coulter Optima L-100 XP ultra-centrifuge (Beckman Coulter, South-Africa), equipped with a 50.2Ti fixed angle rotor, was used. The samples were transferred into a suitable tube and centrifuged at a speed of 25 000 rpm for 30 min at a temperature of 25 °C, in order to separate the oil phase, containing the entrapped drug, from the water phase, within the supernatant. The supernatant (1 ml) containing the entrapped drug was removed and diluted with 9 ml of a 99% methanol solvent. 1 ml of the methanol and entrapped drug mixture was filtered through 0.45 µm hydrophilic polyvinylidene difluoride (PVDF) filters and injected into HPLC vials for analysis. A linear curve was generated from clofazimine standard solutions. The EE% of each dispersion was calculated, by employing the equation of Kurakula *et al.* (2012:37):

$$\%EE = \left[ \frac{C_t - C_f}{C_t} \right] \times 100 \quad \text{Equation C.1}$$

Where:

C<sub>f</sub> = the concentration of free un-encapsulated drug (x value);

C<sub>t</sub> = the total API concentration.

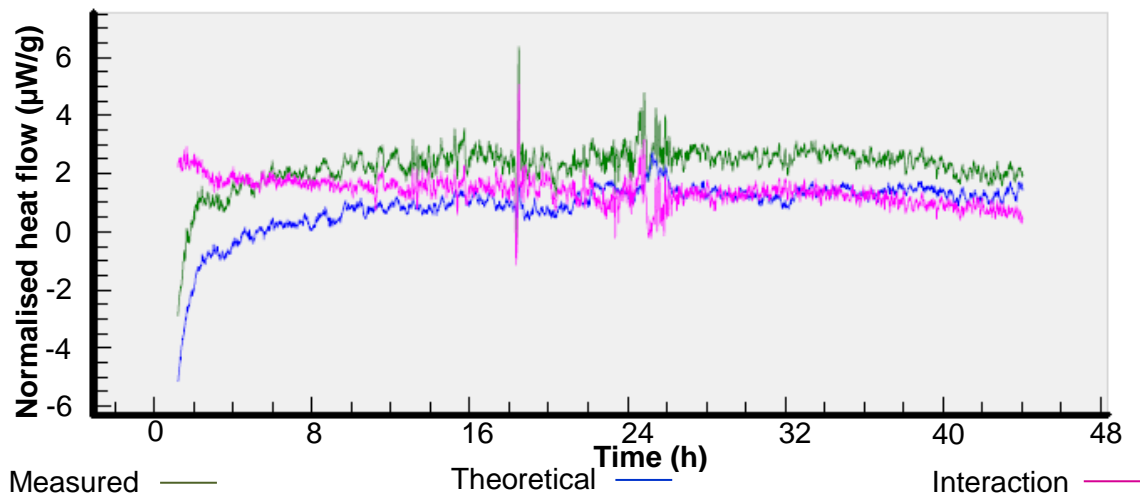
#### **C.4.8 Visual and physical examinations**

Both the **P-NE** and **A-NE** nano-emulsions were inspected visually to investigate their homogeneity and colour.

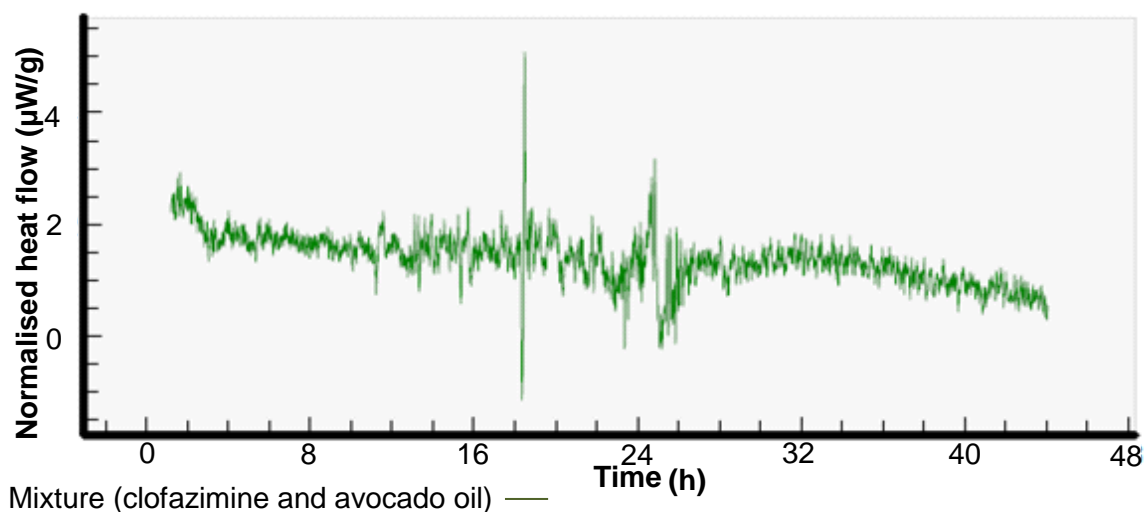
## C.5 Results and discussions of the characterisation of the optimised dispersions

### C.5.1 Clofazimine and avocado oil stability study outcomes (TAM results)

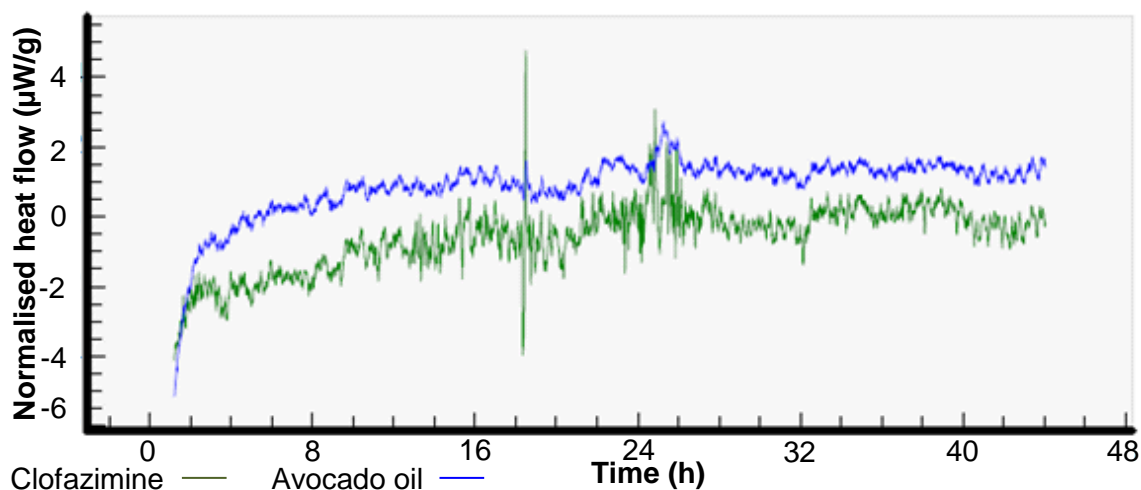
#### C.5.1.1 Clofazimine in combination with avocado oil at 32°C



**Figure C.1:** Combined graphic representation of the measured, theoretical and interaction heat flow for a mixture of clofazimine and avocado oil in a ratio of 0.06:1.00 (%w/w) at 32 °C



**Figure C.2:** Graphic representation of the heat flow *versus* time data for a mixture of clofazimine and avocado oil in a ratio of 0.06:1.00 (%w/w) at 32 °C



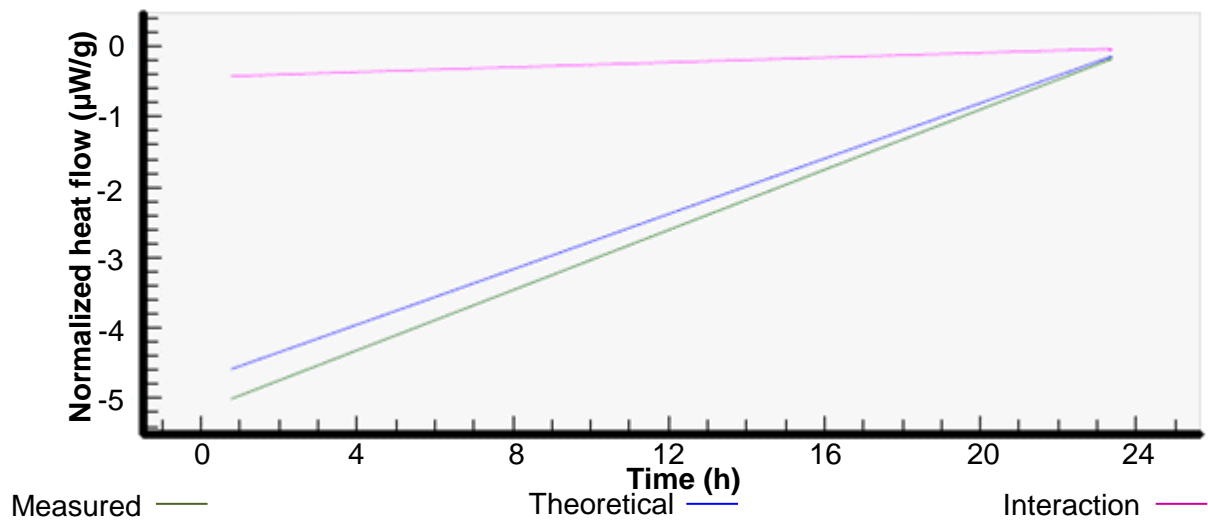
**Figure C.3:** Graphic representation of the heat flow *versus* time data for the clofazimine and avocado oil separately at 32 °C

Figure C.1 illustrates the heat flow *versus* time data that was obtained for the clofazimine and avocado oil combination. The weight ratio used was 0.06:1.00 (%w/w) (clofazimine : avocado oil), in accordance with the pre-determined solubility concentrations. No incompatibility; nor interaction had occurred between the clofazimine and the avocado oil at a temperature of 32 °C. The results of the interaction heat flow were calculated as  $1.3815 \pm 1.4545 \mu\text{W/g}$  as represented by the almost straight magenta curve in Figure C.1. If any incompatibility had occurred, the slope of the magenta curve would have shown an unexpected increase or decrease, and the interaction heat flow would not have been close to 0  $\mu\text{W/g}$ .

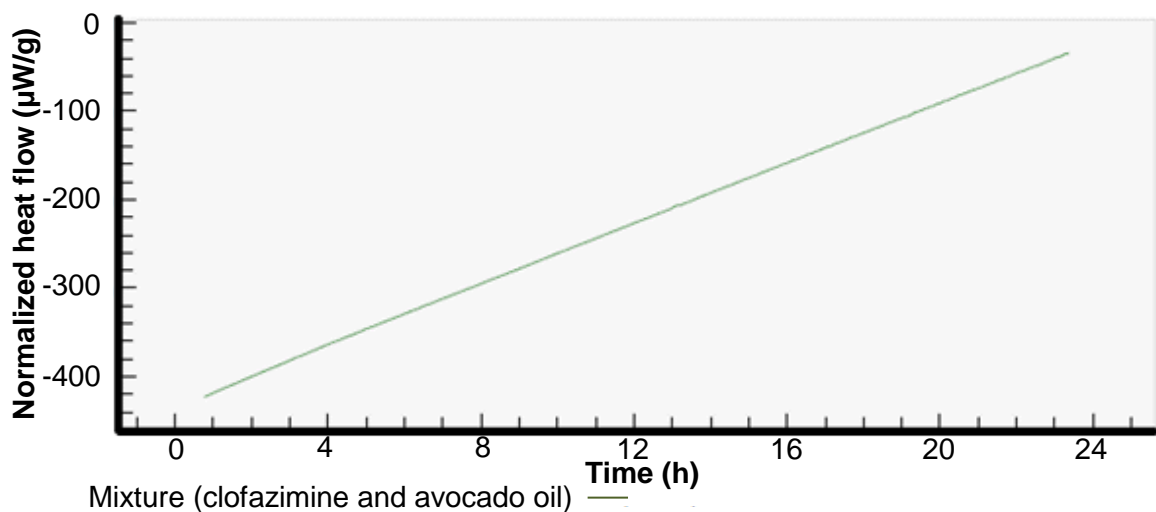
#### **C.5.1.2 Clofazimine in combination with avocado oil at 60°C**

The clofazimine avocado oil combination was also investigated at 60°C in a ratio of 0.06 : 1.00 (%w/w). Figure C.4 illustrates the heat flow data obtained for the clofazimine avocado oil combination over a period of 48 h. No interaction was identified during this analysis. Based upon the calculated average interaction heat flow of  $252.25 \pm 226.20 \mu\text{W/g}$ , it was concluded that no interaction had occurred between the clofazimine and avocado oil at a temperature of 60 °C. The almost straight magenta curve in Figure C.4 illustrates that no interaction had occurred between the clofazimine and avocado oil at a temperature of 60°C, when maintained at that condition for a period of 24 h.

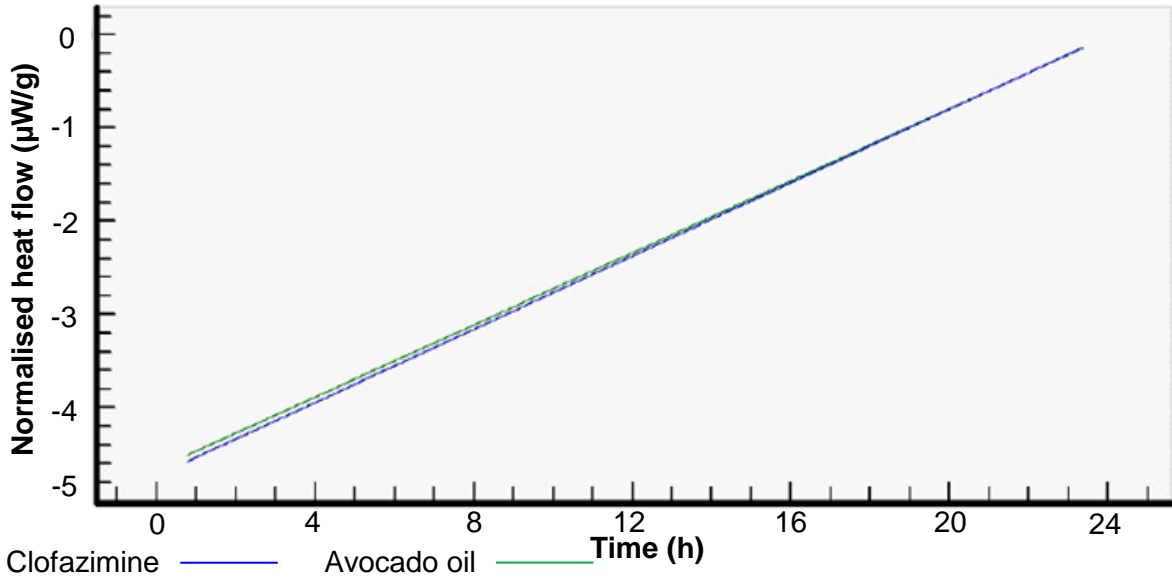
As can be observed in Figure C.6, both components had followed exact similar heat flow behaviours over the 24 h period. This was indicative of the favourable compatibility of clofazimine and avocado oil.



**Figure C.4:** Combined graphic representation of the measured, theoretical and interaction heat flow for a mixture of clofazimine and avocado oil in a ratio of 0.06 : 1.00 (%w/w) at 60 °C



**Figure C.5:** Graphic representation of the heat flow *versus* time data for a mixture of clofazimine and avocado oil in a ratio of 0.06 : 1.00 (%w/w) at 60 °C



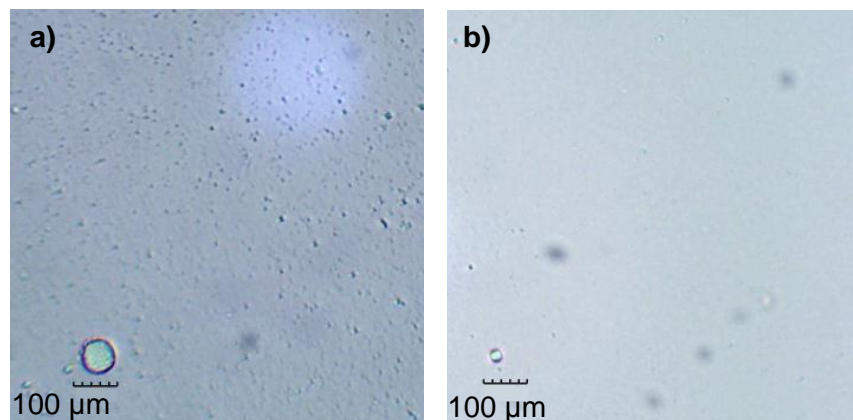
**Figure C.6:** Graphic representation of the heat flow *versus* time data of the clofazimine and avocado oil separately at 60 °C

### C.5.1.3 Conclusion of compatibility study outcomes

The compatibility of clofazimine and avocado oil in combination were tested at both 32 °C and 60 °C. Since no incompatibilities between these two components had been identified, it was concluded that the clofazimine and avocado oil were compatible with each other at the tested temperature range of 32 °C – 60 °C and that they could be used in combination in a nano-emulsion.

## C.5.2 Morphology study outcomes

### C.5.2.1 Light microscopy

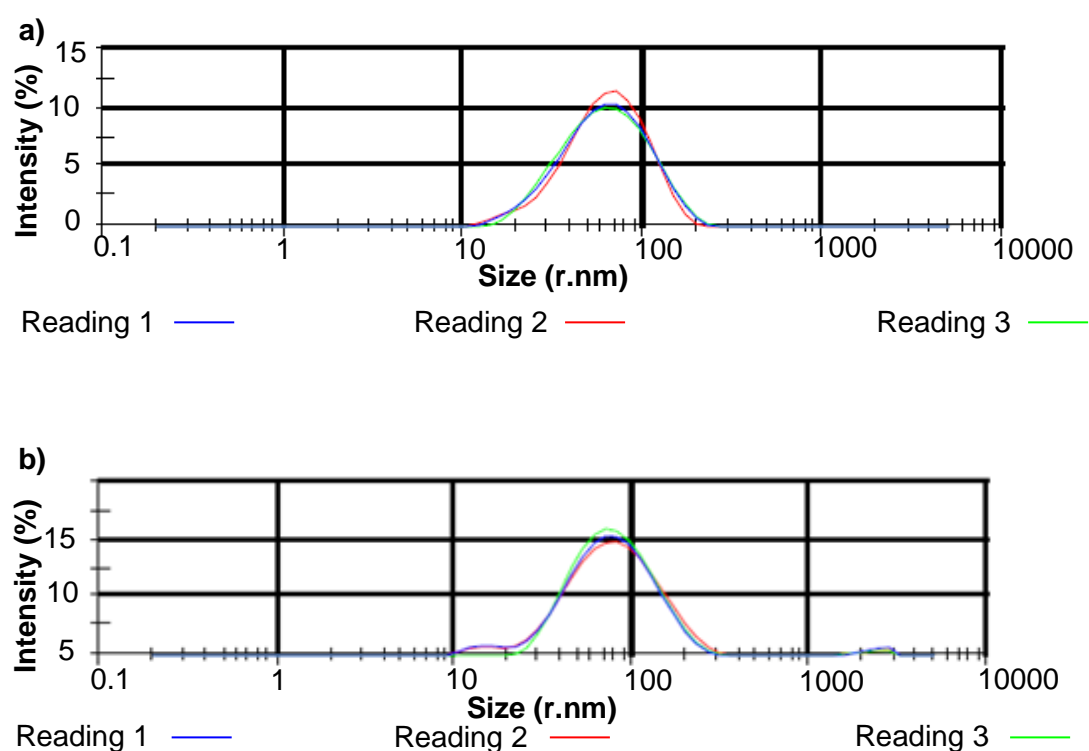


**Figure C.7:** Micrographs of a) the **P-NE** and b) the **A-NE** nano-emulsions at a scale of 100 µm

Micrographs were taken of the **P-NE** (Figure C.7.a)) and the **A-NE** (Figure C.7.b)). The micrographs were taken at a magnification of 40 x. Most of the droplets were too small to be visible by light microscope. However, the micrographs indicated that spherical droplets had formed, ascribed to the very small droplets that can be observed as small dark dots, concluding that a nano-emulsion had been formed.

### C.5.3 Droplet sizes and droplet size distributions

The droplet sizes of the two nano-emulsions (**P-NE** and **A-NE**) were each measured in triplicate from freshly prepared samples, in order to deliver reliable average droplet size results. Figure C.8 represents the average droplet size of each nano-emulsion.

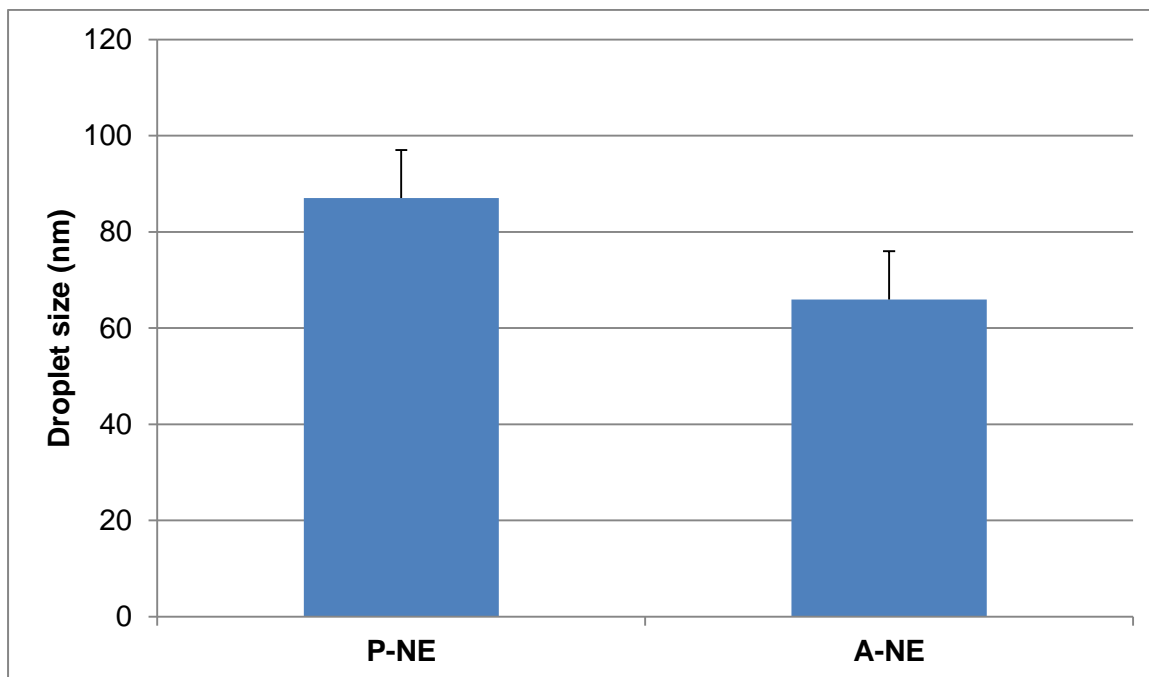


**Figure C.8:** Droplet size and droplet size distribution curves of a) the **P-NE** and b) the **A-NE** nano-emulsions

The average droplet sizes of the final **P-NE** and **A-NE** dispersions were  $87.06 \pm 2.2$  nm and  $65.97 \pm 0.4$  nm, respectively. The **P-NE** had a droplet size distribution (PDI) of 0.262 and the **A - NE** had a PDI of 0.209. The **A-NE** had showed a smaller PDI than the **P-NE**. However, the PDI of both the dispersions indicated on a narrow particle size distribution (Patel *et al.*, 2013:34). Although the **A-NE** had smaller droplet sizes than the **P-NE**, both dispersions' droplet sizes complied with the acceptable nano-emulsion droplet sizes (Pey *et al.*, 2006:144). The

smaller droplet sizes of the **A-NE** may contribute towards better membrane and skin diffusion outcomes, as the droplets were all in the nano-meter range of 10 - 1000 nm, as illustrated in Figure C.8 (Kumar & Rajeshwarrao, 2011:209).

The smaller droplet sizes of the final dispersions may have been due to the usage of higher volumes of the non-ionic surfactants (Tween<sup>®</sup> 80 and Span<sup>®</sup> 60) and hence their more optimal percentages, than what had been used during pre-formulation.



**Figure C.9:** Graphic representation of the average droplet sizes (nm) of the **P-NE** and **A-NE** nano-emulsions

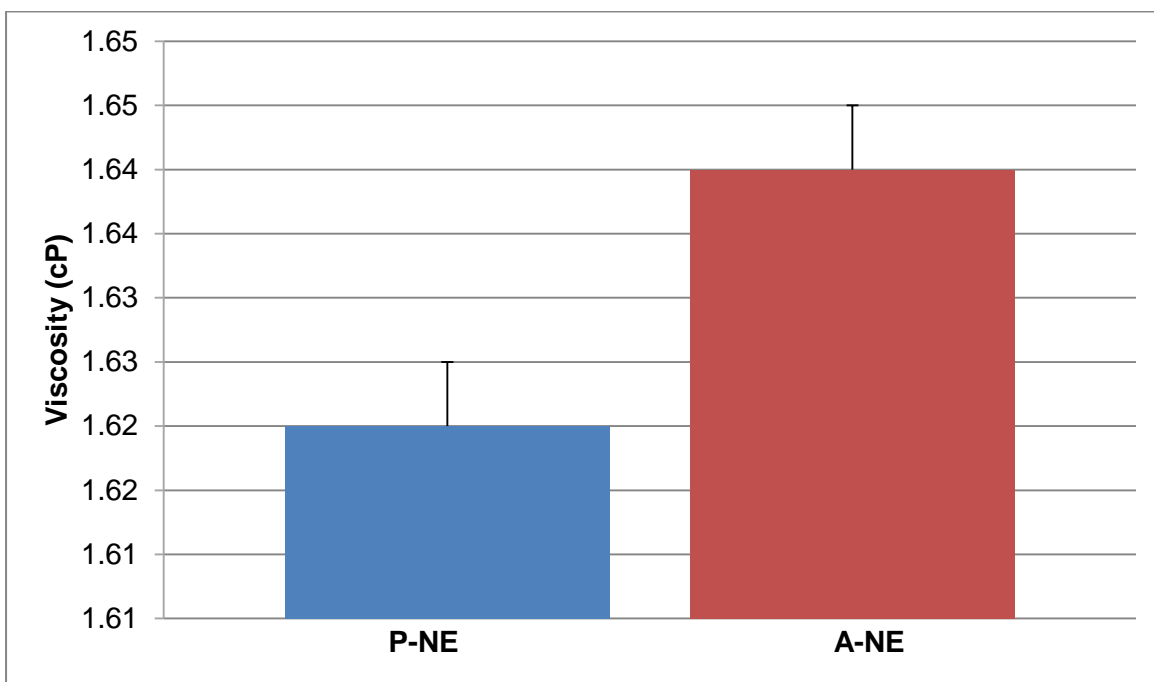
#### C.5.4 pH

The **A-NE** had an average pH value of  $6.61 \pm 0.02$  and the **P-NE** an average of  $6.44 \pm 0.03$ . The **A-NE** therefore was more basic than the **P-NE**. The presence of the API could have possibly resulted in the rise in the pH of the **A-NE**. The pH values of both the nano-emulsions were within the safe range for skin applications of pH 5 - 7 (Paudel *et al.*, 2010:118).

#### C.5.5 Viscosity

The average viscosities of the two nano-emulsions are displayed in Figure C.10. Although the **A-NE** ( $1.64 \pm 0.03$  cP) had a slightly higher viscosity than the **P-NE** ( $1.62 \pm 0.03$  cP), the average viscosity values of the two dispersions were very close to each other. The **A-NE** probably had a higher average viscosity reading, because of the API that had been added. Both dispersions had very low viscosities, as were expected for nano-emulsions (Solans *et al.*,

2005:107). The low viscosities of the nano-emulsions may benefit membrane and skin permeation (Li *et al.*, 2011:1008).

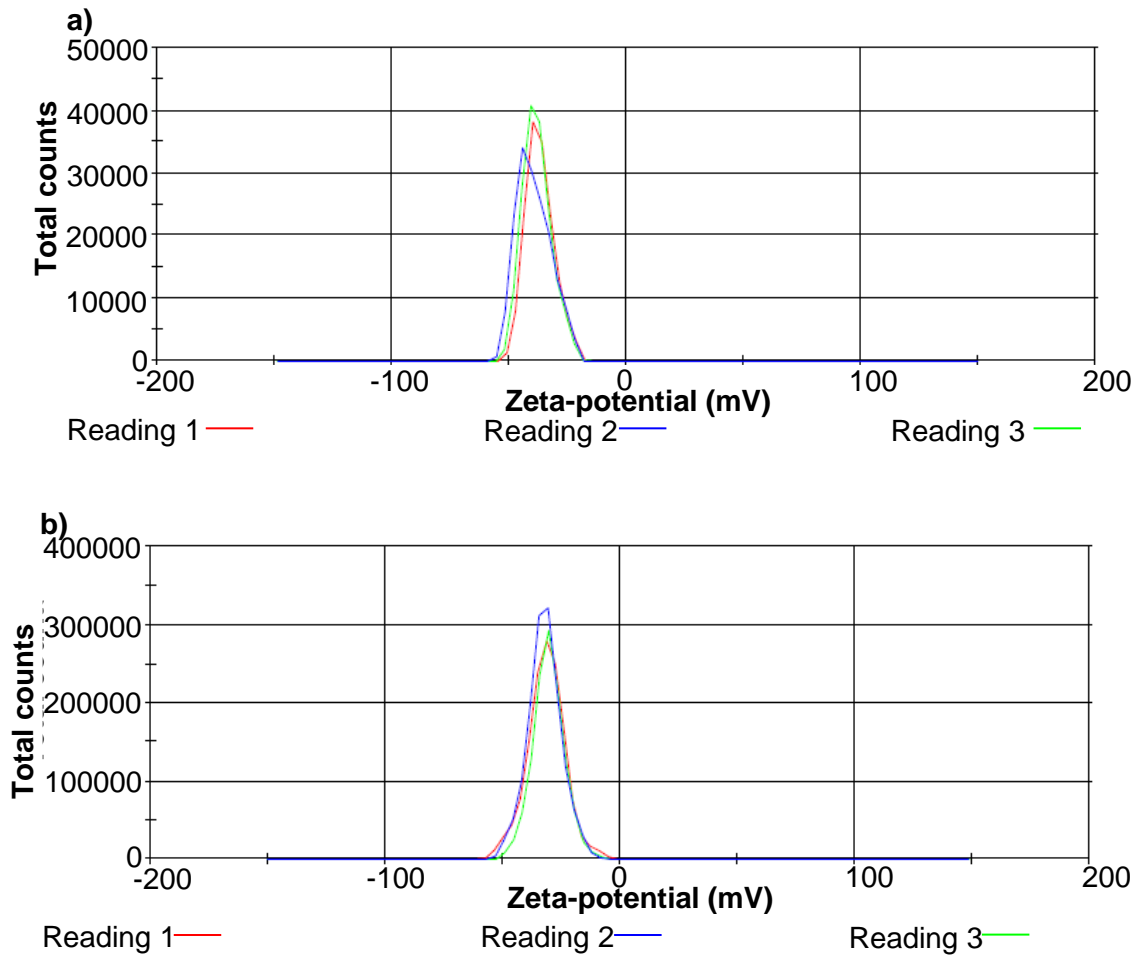


**Figure C.10:** Graphic representation of the average viscosity (cP) values of the **P-NE** and **A-NE** nano-emulsions

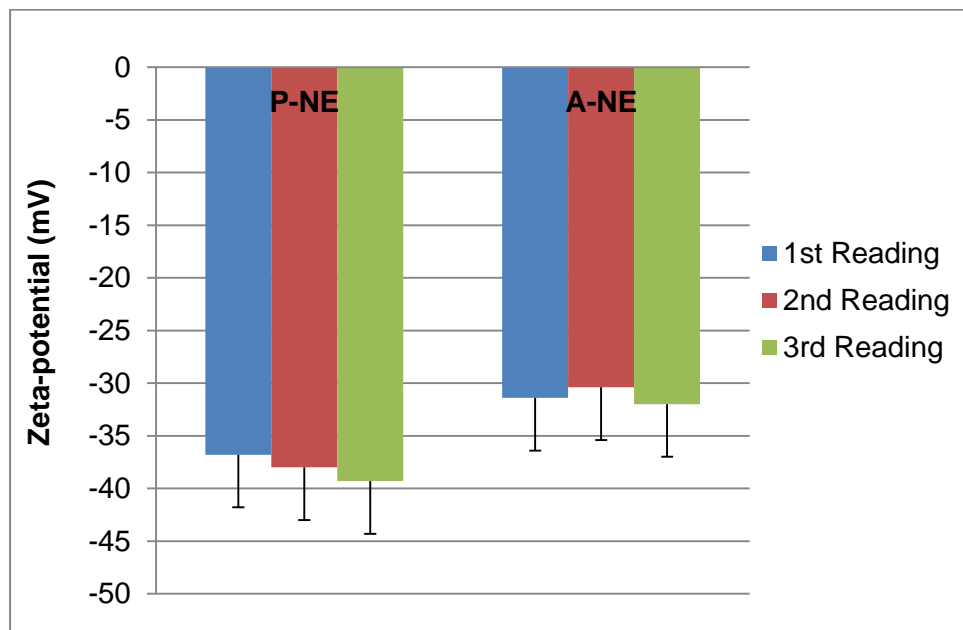
### C.5.6 Zeta-potential

Zeta-potential is defined as the potential at a solid liquid interface (Kirby & Hasselbrink, 2004:184). Zeta-potential can be used to determine the electrostatic repulsion between droplets in an emulsion (De Morias *et al.*, 2006:110). The zeta-potential of a vesicle surface, such as a nano-emulsion oil droplet, plays a very important role in the stability of the nano-emulsion (Kumar & Rajeshwarrao, 2011:213; Pather *et al.*, 1995:1283-1284). A highly charged surface keeps droplets from aggregating and thus prevents the formation of bigger droplets to enhance the stability of nano-emulsions. The zeta-potential, or the negative charge on a droplet's surface also safeguards droplet size stability and uniformity (Agarwal *et al.*, 2001:44).

Figure C.11 illustrates the zeta-potential distribution curves of the **P-NE** and the **A-NE**. The **P-NE** had an average zeta-potential value of  $-38.03 \pm 1.25$  mV and the **A-NE** an average value of  $-31.27 \pm 0.81$  mV. As observed, the average zeta-potential values were highly negatively charged, which were indicative of satisfactory emulsion stabilities. The zeta-potential value of the **P-NE** was slightly more negative than that of the **A-NE**.



**Figure C.11:** Graphic representation of the zeta-potential (mV) curves of a) the **P-NE** and b) the **A-NE** nano-emulsions



**Figure C.12:** Graphic representation of the zeta-potential (mV) readings of the **P-NE** and **A-NE** nano-emulsions

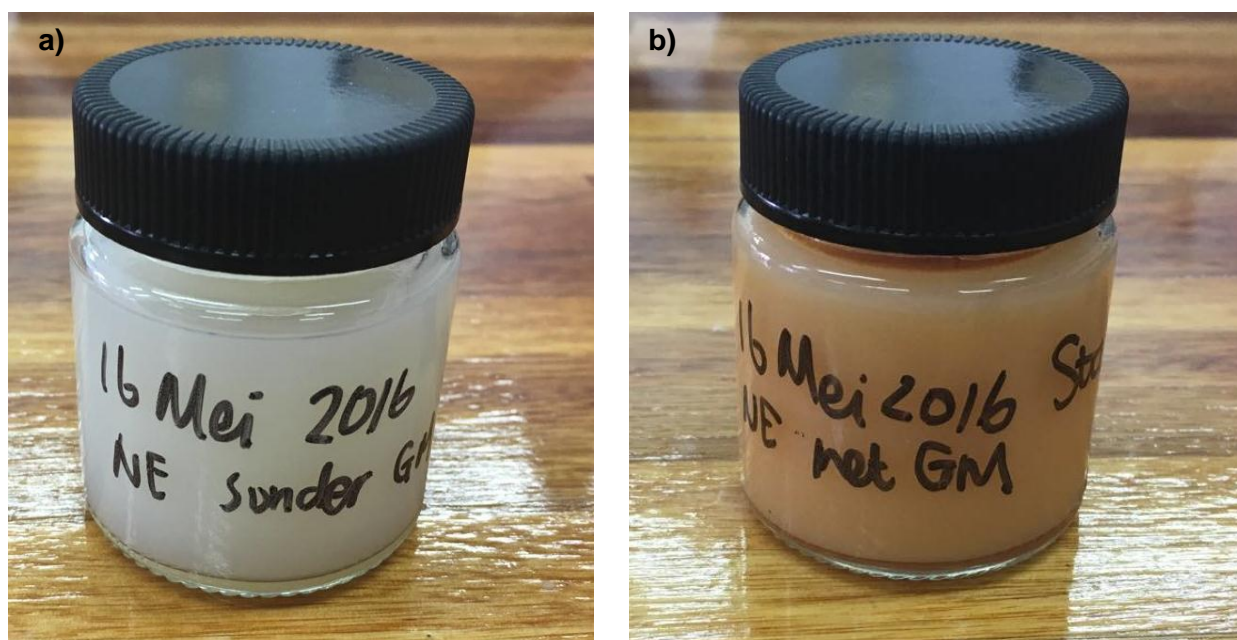
In Figure C.12, the triplicate zeta-potential values of each of the two nano-emulsions are illustrated. The zeta-potential values of the **P-NE** ranged from - 38.0 to - 39.3 mV and those of the **A-NE** were slightly less negative, ranging from - 30.4 to - 32.0 mV. A highly negatively charged droplet, or a highly negative zeta-potential ensures a nano-emulsion's stability, since a negative charge on a droplet repels it from other droplets and thereby keeps droplets from aggregating together (De Morias *et al.*, 2006:110). The zeta-potential values being measured for both the nano-emulsions were indicative of good nano-emulsion stability with regards to droplet size growth. It would seem that the presence of the API had decreased the zeta-potential of the **A-NE**, which had increased the chances of instability in the dispersion. However, the change in the zeta-potential values was so small, that it could be regarded as irrelevant.

### C.5.7 Entrapment efficiency

The drug entrapment of clofazimine in the oil phase of the **A-NE** was calculated at a satisfactory, quite high 95.62%.

### C.5.8 Visual and physical examinations

The **P-NE** and **A-NE** dispersions were inspected visually. The **P-NE** was translucent in colour and homogenous. The **A-NE** was pumpkin yellow in colour and homogenous. Photographs of these dispersions are illustrated in Figure C.13.



**Figure C.13:** Photographs illustrating the colours and consistencies of a) the **P-NE** and b) the **A-NE** nano-emulsions

## C.6 Conclusion

It is very important to characterise nano-emulsions in order to determine all their properties, which can in turn be used to optimise their topical delivery. It was found that clofazimine was compatible with the oil phase of the nano-emulsion (as illustrated by the microcalorimetric results). No unwanted reactions were therefore expected to occur between this API and oil combination. Additionally, the light microscopic results illustrated that very small and uniform spherically shaped oil droplets, dispersed within the water phase, had formed. The droplet size and droplet size distribution results indicated that the nano-emulsions could be regarded as stable with homogenous droplet sizes and a narrow particle size distribution. Since the **A-NE** had smaller droplet sizes than the **P-NE**, it was expected that the **A-NE** would probably show better stability. The zeta-potential values of the nano-emulsions were all relatively negative, which were indicative thereof that these nano-emulsions were stable, due to the repulsive behaviour of the emulsion droplets associated with this type of zeta-potential charge, which would prevent the droplets from aggregating. The pH results of the nano-emulsions indicated that quite a large amount of unionised species was present that would enhance skin diffusion, while the favourable pH values would not cause skin irritation. The EE% demonstrated that a large amount of the API was favourably entrapped in the oil phase of the **A-NE**. The viscosity results showed that the nano-emulsions each had a very low viscosity, which would possibly aid skin diffusion. Lastly, the visual inspections of the nano-emulsions showed that the **P-NE** was translucent, while the **A-NE** was pumpkin yellow. Both dispersions were homogenous. All of these characterisation study outcomes were applied in the assessment of the subsequent experimental results.

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## APPENDIX D

# FORMULATION OF EMULGELS CONTAINING CLOFAZIMINE

### D.1 Introduction

Since the o/w nano-emulsions had such low viscosity (as seen in Appendix C); it was decided that two emulgel formulas containing the API would be formulated to aid in the topical application of the nano-emulsions (Valenta & Schultz, 2004:257). The two emulgel formulas were prepared for comparison purposes and consisted of an emulgel containing a nano-emulsion (**N-EM**) and the other emulgel containing a coarse emulsion (**C-EM**).

An emulgel is defined as a semi-solid pharmaceutical formula containing the combination of an emulsion (w/o or o/w) with a gelling agent added in order to form a gel. Emulgels typically improve patient compliance and aid in topical diffusion of hydrophobic drugs due to the fact that they consist of a combination of hydrophobic and hydrophilic parts (Supriya *et al.*, 2014:1; Vats *et al.*, 2014:650).

The formulation and physicochemical properties of the emulgels without API will be discussed in this appendix, while the characterisation of the emulgels containing the API will be discussed in Appendix E.

### D.2 Excipients used to formulate the emulgel formulas

Emulgels were formulated using the excipients listed in Table D.1.

**Table D.1:** Excipients used for the formulation of emulgels

| Ingredient                         | Purpose  | Supplier                 | Batch number   |
|------------------------------------|--|--------------------------|----------------|
| Emulsion (coarse or nano-emulsion) | Water phase to contain API                           | -                        | -              |
| Span <sup>®</sup> 60               | Non-ionic surfactant incorporated into the oil phase | Fluka                    | 423065/1 41002 |
| Tween <sup>®</sup> 80              | Non-ionic surfactant incorporated into the oil phase | Merck                    | 1043695        |
| Liquid paraffin                    | Emollient incorporated into the oil phase            | Merck                    | 1034378        |
| Xanthan gum                        | Gelling agent  | Warren Chem Specialities | 4450902790     |

|                            |         |          |                |
|----------------------------|---------|----------|----------------|
| Milli-Q <sup>®</sup> water | Solvent | In house | Direct Pure UP |
|----------------------------|---------|----------|----------------|

### D.2.1 Nano-emulsion

Fresh nano-emulsions without the API were prepared as described in Section B.3.1.1 for the purpose of incorporating it into the emulgel formula.

### D.2.2 Coarse emulsion

Freshly prepared coarse emulsions without the API were prepared for the purpose of incorporating it into an emulgel formula. Coarse emulsions were prepared as per the method described in Section B.3.1.1, but without involving the probe and bath sonication processes.

### D.2.3 Span<sup>®</sup> 60

Span<sup>®</sup> 60 is classified as a lipophilic non-ionic surfactant and makes up part of the oil phase of the emulgel (Monosroi *et al.*, 2003:133).

### D.2.4 Tween<sup>®</sup> 80

Tween<sup>®</sup> 80 is also classified as a hydrophilic, non-ionic surfactant, which is incorporated into the oil phase of the emulgel (Patrolecco *et al.*, 2004:6).

### D.2.5 Liquid paraffin

Liquid paraffin is an emollient used as part of the oil phase of the emulgel (Dogra *et al.*, 1994:72).

### D.2.6 Xanthan gum

Xanthan gum is classified as an anionic polysaccharide and is the gelling agent used for the formulation of emulgels of previously prepared nano-emulsions and coarse emulsions (Sun *et al.*, 2007:556).

### D.2.7 Water

Milli-Q<sup>®</sup> water was used as solvent as part of the water phase in the emulgel formulas.

## D.3 Formulation of the selected emulgel formulas

### D.3.1 Method for the preparation of the emulgel containing a nano-emulsion

Table D.2 illustrates the excipients with the volumes and weights used in the **N-EM**. Firstly, the nano-emulsion without the API was freshly pre-formulated for incorporation into the emulgel.

Milli-Q<sup>®</sup> water (50 ml) was heated to about  $\pm 40$  °C. Thereafter, the gelling agent (1.5 g) was mixed with the heated water using the Heidolph<sup>®</sup> RZR 2041 homogeniser (Germany) at 340 W and a frequency of 50 Hz with a speed of 777 rpm until a homogenous mixture was formed. While mixing the gelling agent and water; the oil phase counterpart of the emulgel was heated to  $\pm 80$  °C on a magnetic stirring plate until homogenous. The pre-formulated nano-emulsion was added to the gelling agent and water mixture and mixed at a speed of 777 rpm with the homogeniser until homogenous. After that, the oil phase was added to the water phase and homogenised on a speed of 13 500 rpm until it cooled down to a temperature of  $\pm 25$  °C.

**Table D.2:** The excipients, oil phase, water phase and the measured amounts of the excipients for use in the **N-EM** formulation

| Excipient                     | Oil phase/ water phase counterparts | Amounts weighed or measured |
|-------------------------------|-------------------------------------|-----------------------------|
| Nano-emulsion without the API | Water phase                         | 50 ml                       |
| Milli-Q <sup>®</sup> water    | Water phase                         | 50 ml                       |
| Xanthan gum                   | Gelling agent for the water phase   | 1.5 g                       |
| Span <sup>®</sup> 60          | Oil phase                           | 0.5 g                       |
| Tween <sup>®</sup> 80         | Oil phase                           | 4.5 g                       |
| Liquid paraffin               | Oil phase                           | 20.0 g                      |

### D.3.2 Method for the preparation of the emulgel containing a coarse emulsion

The coarse emulsion without the API was prepared as described in Section B.3.1.1. However, the nano-emulsion was not probe- or bath sonicated in order to form a coarse emulsion (50 ml, water phase). For the formulation of the emulgel, the same formulation technique was used as described in Section D.3.1, except that the coarse emulsion instead of the nano-emulsion was added to the emulgel formula.

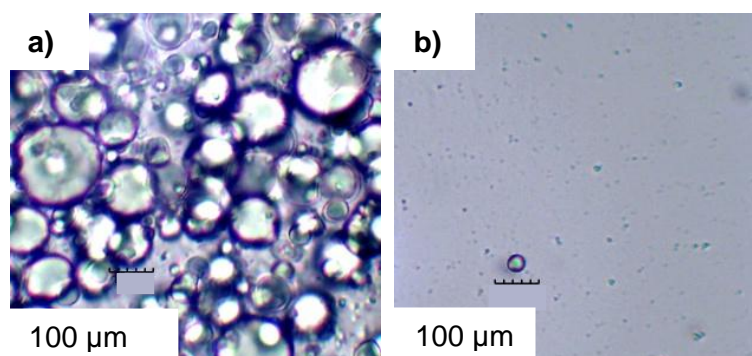
### D.4. Tests performed in order to determine the ideal formula

Both the emulgel formulas were evaluated by using the following tests: light microscopy, pH, viscosity, zeta-potential, as well as droplet size and distribution. These tests were used to determine which of the emulgel formulas would be best suited for topical application.

#### D.4.1 Light microscopy

Light microscopy was performed on both the emulgel formulas without the API using a Nikon Eclipse E4000 microscope (Nikon, Japan Linkam THMS600) equipped with a Nikon DS-Fi1 camera. Light microscopy was performed on the samples to see if any oil droplets were present

in the emulgel formulas. A small amount of each formula was placed on a 20 mm microscope slide. Both of the emulgel formulas were viewed separately at a 40 x magnification on the microscope.



**Figure D.1:** Micrographs of the emulgel formulations, a) the **C-EM** and b) the **N-EM** at a 100 µm scale, as indicated

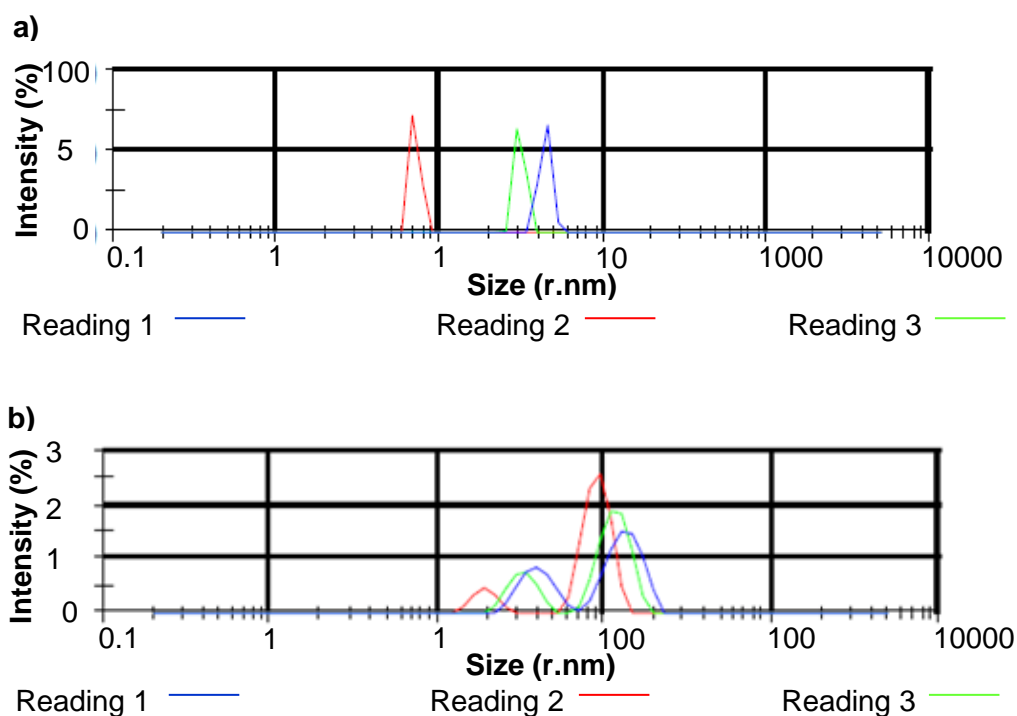
Micrographs were taken in duplicate from each sample, however only one micrograph from each sample was chosen to represent the light microscopy results. Oil droplets were visible in both of the emulgel formulas, but unfortunately the low magnification of the light microscope only allowed for the visibility of the larger oil droplets. The oil droplets in the **C-EM** (Figure D.1.a)) were larger than those of the **N-EM** (Figure D.1.b)) due to the fact that the nano-emulsion droplets are smaller than those of the coarse emulsion; the larger droplets can lead to possible aggregation of the oil droplets in the emulgel formula. The **N-EM** has smaller and larger droplets probably due to droplet coagulation during the emulgel formulation. The emulgel containing the nano-emulsion would probably be more stable and be a more ideal formula, which might yield better membrane and skin diffusion results.

#### **D.4.2 Droplet size and -distribution**

For each of the emulgel formulas the average droplet size and distribution was measured to see which of the two provided the smallest average droplet size in order to determine which emulgel formula would be more stable against droplet aggregation; since droplet size generally plays an important role in emulsion stability (Fontenot & Schork, 1993:373). The droplet size and distribution were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK).

The average droplet size and distribution results obtained for both the emulgel formulas are depicted in Figure D.2. The average droplet size of the **C-EM** was measured as  $2\,390.0 \pm 0.03$  nm and the polydispersity index (Pdl) was measured as 0.9823. The average droplet size of the **N-EM** was measured as  $287.6 \pm 0.08$  nm and the Pdl was measured as 0.9516. The average droplet size of the **N-EM** complies with the droplet size range of nano-emulsions (Pey *et al.*, 2006:144). The average droplet sizes of the emulgels are much larger

than the droplet sizes in the results of the nano-emulsions listed in Appendix C. However, as illustrated, the average droplet size of the **C-EM** is much larger than that of the **N-EM**. The **N-EM** therefore has the more ideal droplet size and -distribution results and is more likely to be the more stable emulgel formula of the two emulgel formulas. The PDI results of both emulgels have illustrated that the **N-EM** has a better droplet distribution than the **C-EM** (Patel *et al.*, 2013:34).



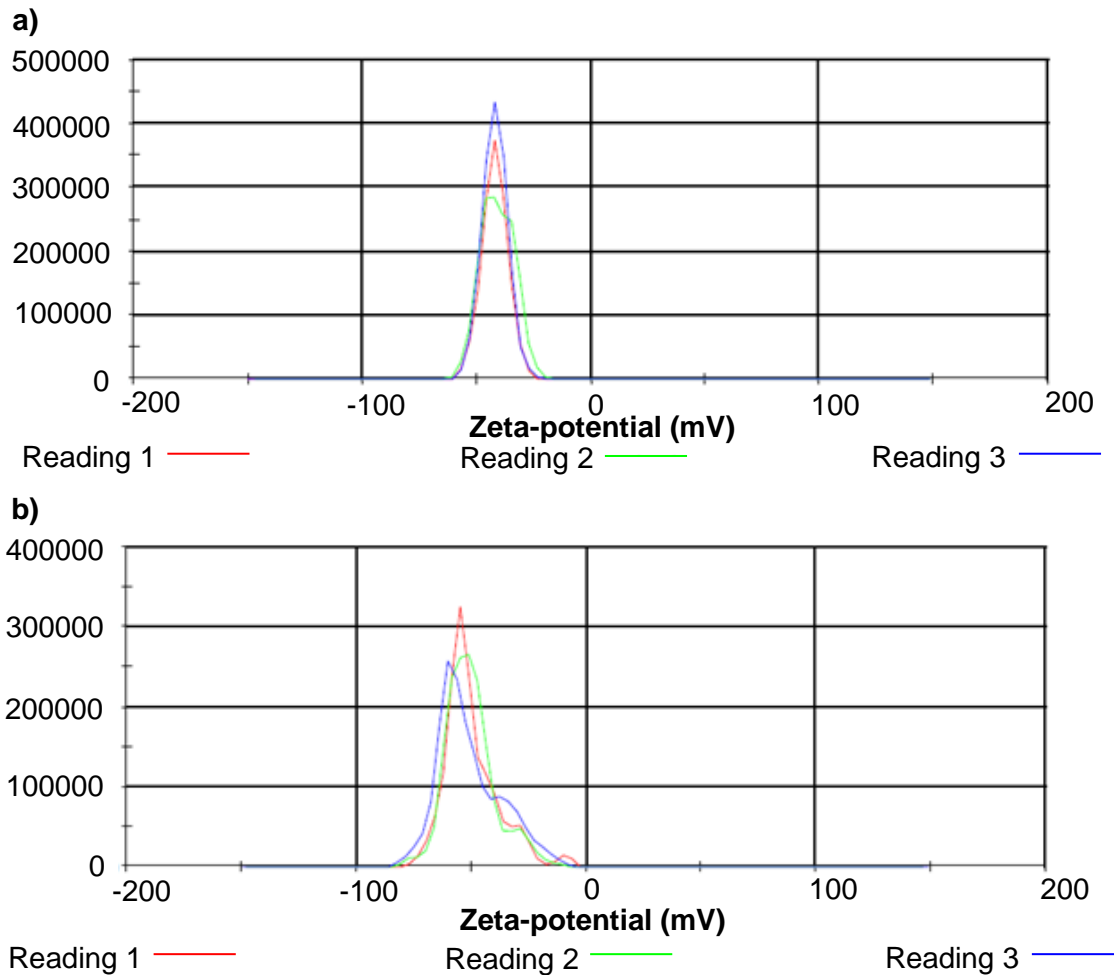
**Figure D.2:** The droplet size and droplet size distribution curves of a) the **C-EM** and b) the **N-EM**

#### D.4.3 Zeta-potential

The zeta-potential of both emulgel samples were determined using the Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The zeta-potential was measured to determine the stability of the emulgel formulas (Patel *et al.*, 2013:34). Each of the samples were diluted separately, namely two drops of each emulgel formula in 10 ml of distilled water. A small amount of each sample was injected into a plastic cuvette before the zeta-potential was measured. Each sample was measured in triplicate to ensure a good average reading.

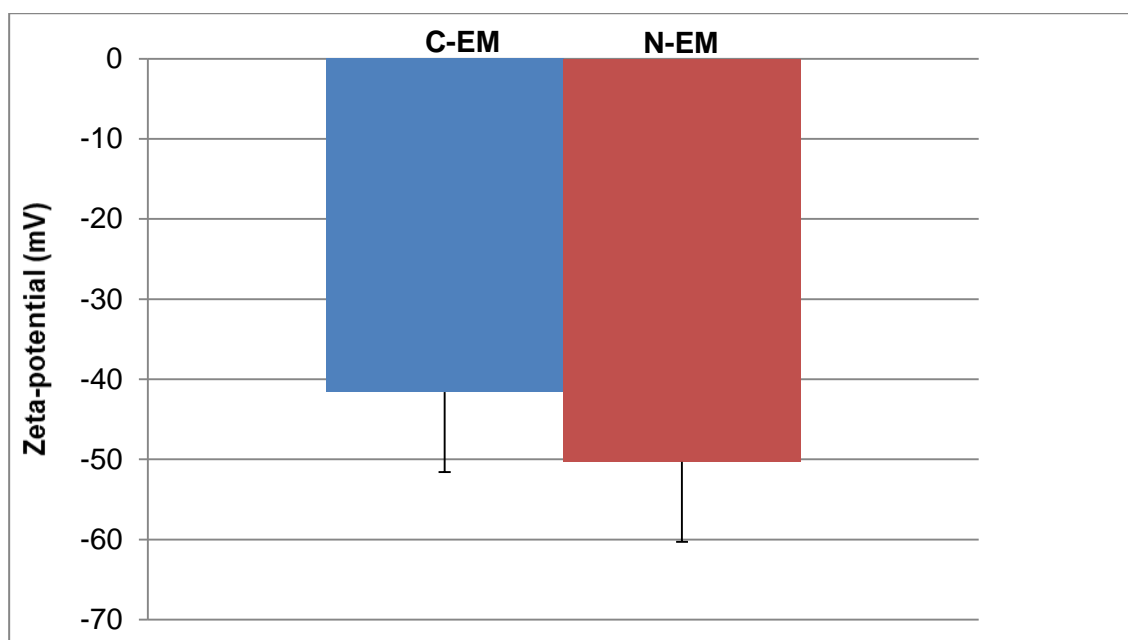
Figure D.3 illustrates the zeta-potential curves of the two separate emulgel formulas in which the zeta-potential was taken in triplicate, each time using fresh samples. The average zeta-potential reading of the **C-EM** was measured as  $-41.6 \pm 0.69$  mV and the average zeta-potential of the **N-EM** had higher zeta-potential readings than the **C-EM**, which was measured

as  $-50.3 \pm 0.46$  mV. The more negative or positive the zeta-potential of the emulgel formula is, the more stable the emulgel formula will be; since the particles do not coagulate due to the charge on the oil droplets. If an emulgel shows a charge of more than + 30 mV or more negative than - 30 mV, it is considered as a stable emulgel formula (Patel *et al.*, 2013:34). Thus the **C-EM** and the **N-EM** are considered stable.



**Figure D.3:** Zeta-potential distribution curves of a) the **C-EM** and b) the **N-EM**

Figure D.4 displays the average zeta-potential values of the two emulgel formulas. It is observed that the **N-EM** has a greater zeta-potential than the **C-EM**. Both emulsion formulas seem to be stable in the emulgel formulas; however since the **N-EM** has a higher zeta-potential value it is deemed the more stable formula of the two.



**Figure D.4:** Graphic representation of the average zeta-potential values of the **C-EM** and the **N-EM**

#### D.4.4 pH

The pH of the two different emulgel formulas was determined using a Mettler Toledo Seven Compact pH meter (Mettler Toledo, Switzerland). Before measuring the pH of the two different emulgel formulas, the pH meter was calibrated at a pH of 4, 7 and 10 by inserting the probe into buffered solutions. The pH readings were taken in triplicate for each of the different formulas, with fresh emulgel samples each time to ensure reliable average readings.

The **C-EM** had an average pH of  $5.84 \pm 0.04$  and the **N-EM** had an average pH of  $6.04 \pm 0.10$ . The pH of the **N-EM** was higher than that of the **C-EM**, but the pH values of both the emulgels are very close to each other. The pH values of both the emulgels fall within the ranges of safe skin application of 5 – 7 (Paudel *et al.*, 2010:118).

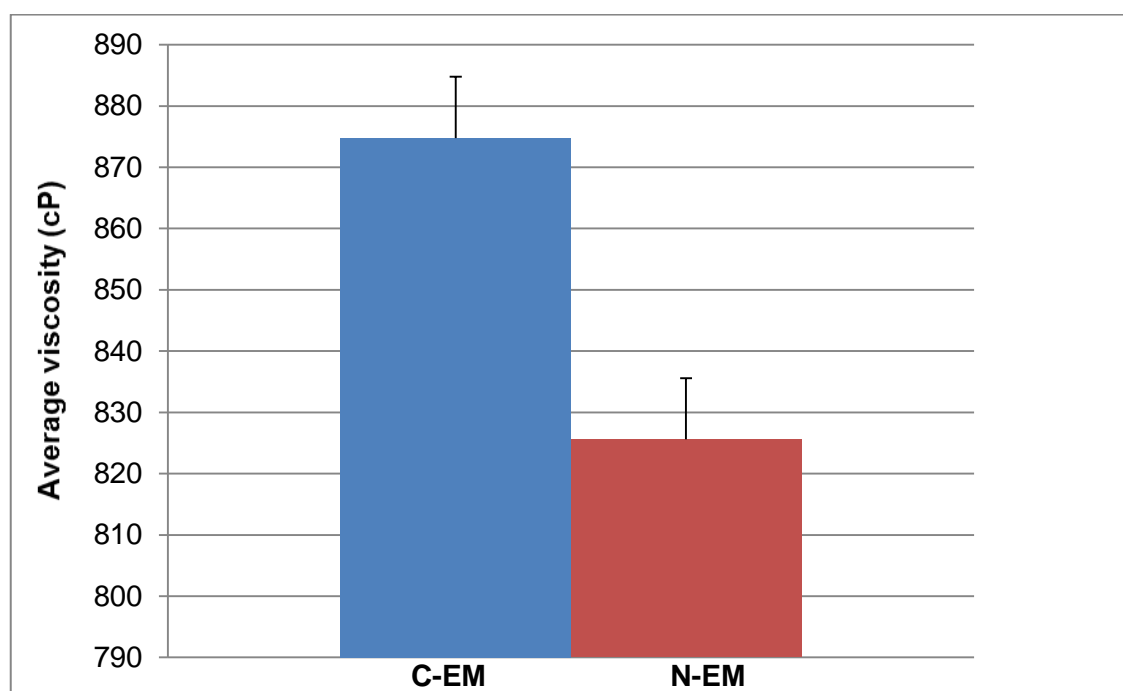
#### D.4.5 Viscosity

As nano-emulsions are relatively isotropic formulas with a very low viscosity, their usage are limited (Solè *et al.*, 2006:140; Valenta & Schultz, 2004:257). The **N-EM** was designed to overcome this problem and the **C-EM** was designed for the purpose of comparison during the membrane and skin diffusion studies. Prior to performing the membrane and skin diffusion studies the viscosity of the two emulgel formulas was measured to determine which of the two had the best average viscosity value for drug delivery.

The viscosity of the two different emulgel formulas was measured using a Brookfield Viscometer (model DV-II+, Stoughton, Massachusetts, USA), equipped with rotating spindles and Helipath

attachments. To measure the viscosity, the rotating spindle, set at a pre-determined speed, was inserted into the two sample emulgel formulas. The viscometer data was collected by Wingather<sup>®</sup> software, which was programmed to record 32 readings at 10 sec intervals. The data that was thus collected was used to calculate the average viscosity. The viscosity of each of the two different emulgel formulas was taken separately in triplicate, each time using a fresh sample of each of the different emulgel formulas.

The average viscosity results for each of the two different emulgel formulas are depicted in Figure D.5. The **C-EM** had a higher average viscosity ( $874.77 \pm 4.95$  cP) than the **N-EM** ( $825.60 \pm 4.54$  cP). The emulgel with the lower viscosity value is suspected to show better drug release in membrane and skin diffusion studies, as found in literature (Li *et al.*, 2011:1008; Valenta & Schultz, 2004:264). Consequently, the **N-EM** is deemed the more ideal emulgel formula for the purpose of membrane and skin diffusion studies.



**Figure D.5:** Graphic representation of the average viscosity readings (cP) of the two emulgel formulations.

## D.5 Conclusion

After performing the physical characterization of the formulations, it was possible to conclude which of the emulgel formulas would be the more ideal for the membrane and skin diffusion studies. As seen from the light microscopy results, the **N-EM** had the smallest droplets. From the droplet size and distribution results, the **N-EM** had the smaller average droplet size, as well as better Pdl results. The zeta-potential results showed that the **N-EM** had the best stability. The pH of the two systems were nearly identical; however, the **N-EM** had a slightly more basic

pH, but will not cause skin irritation as it falls in a pH range of 5 – 7 (Paudel *et al.*, 2010:118). The viscosity results indicated that the **N-EM** had a lower viscosity than the **C-EM** and according to literature, the lower viscosity formula would probably show better membrane and skin diffusion results (Li *et al.*, 2011:1008; Valenta & Schultz, 2004:264).

Consequently, the **N-EM** is deemed the ideal formula, with more optimal properties for the purpose of membrane and skin diffusion studies and would probably show better results in these studies than the **C-EM**.

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# APPENDIX E

## CHARACTERISATION OF EMULGELS

---

### E.1 Introduction

Characterisation studies were performed on the two final, optimised emulgel formulations, both containing clofazimine, i.e. the emulgel containing the coarse emulsion (**AC-EM**) and the emulgel containing the nano-emulsion (**AN-EM**). The parameters included light microscopy, zeta-potential, droplet size and droplet size distribution, pH and viscosity determinations in order to compare the two final formulations with each other. Characterisation was done to assess the physical properties of the emulgels, i.e. their stabilities, physical appearances and homogeneities (Intertek, 2013; Niazi, 2009:61).

### E.2 Optimised emulgel formulations

The ingredients included in the two optimised **AN-EM** and **AC-EM** emulgel formulations are summarised in Table E.1.

**Table E.1:** The optimised emulgel formulations, both containing clofazimine

| Emulgel phase  | Ingredient                         | Required amount |
|--|------------------------------------|-----------------|
| Water phase that incorporates the API                      | Emulsion (coarse or nano-emulsion) | 50.0 ml         |
| Non-ionic surfactant being incorporated into the oil phase | Span <sup>®</sup> 60               | 1.0 g           |
| Non-ionic surfactant being incorporated into the oil phase | Tween <sup>®</sup> 80              | 4.5 g           |
| Emollient being incorporated into the oil phase            | Liquid paraffin                    | 20.0 g          |
| Gelling agent  | Xanthan gum                        | 1.5 g           |
| Solvent  | Milli-Q <sup>®</sup> water         | 50.0 ml         |
| API  | Clofazimine                        | 7.1 mg          |

### E.3 Excipients used in the optimised emulgel formulations

The excipients used in the emulgel formulations and their purposes were discussed in Section D.2.

## **E.4 Characterisation methods**

### **E.4.1 Light microscopy**

Light microscopy was conducted on the **AC-EM** and the **AN-EM** to observe whether some of the emulsion droplets were still visible in the formulations after being formulated into gels, and what the sizes and shapes of those droplets were.

Micrographs were taken at a magnification of 40 x of the emulgel containing the coarse emulsion, and of the emulgel containing the nano-emulsion.

### **E.4.2 Droplet sizes and droplet size distributions**

Droplet size and distribution play a very important role in the stability of emulsions. It is of vital importance that the droplet sizes are within the desired parameters to keep the nano-emulsion formulations stable (Solans *et al.*, 2005:102).

The droplet sizes and distributions were determined by using a Malvern Zetasizer ZS (Malvern Instruments, UK). The **AC-EM** and the **AN-EM** formulations were freshly prepared and a sample of each was freshly prepared by diluting 2 ml of each formulation separately in 10 ml of distilled water. Each sample was injected into clean plastic cuvettes in triplicate and measured without delay.

### **E.4.3 pH**

The pH values of the emulgels were determined for comparison purposes and also to ensure that the values of both formulations were safe for topical application.

The pH of the two emulgels (**AC-EM** and **AN-EM**) was determined, using a Mettler Toledo Seven Compact pH meter (Mettler Toledo, Switzerland). The pH meter was calibrated prior to the pH measurements of the emulgels, by using buffered solutions at pH 4, 7 and 10. The pH of each emulgel formulation was subsequently measured in triplicate by submerging the meter's probe into it. Fresh samples were used for every measurement to ensure a reliable average pH outcome.

### **E.4.4 Viscosity**

The viscosities of both emulgel formulations containing the API were measured, using a Brookfield Viscometer (model DV-II+, Stoughton, Massachusetts, USA), equipped with rotating spindles and Helipath attachments. The viscosity measurements per formulation were done in triplicate, by inserting the rotating spindle, set at a pre-determined speed, into three freshly prepared samples of each. The viscometer data was collected by means of Wingather™

software that had been pre-programmed to record 32 readings at 10 second intervals. This collected data was used to calculate the average viscosity of each formulation.

#### **E.4.5 Zeta-potential**

The negative charge on droplets in an emulsion (zeta-potential) prevents droplets from coagulating, thereby yielding emulsion stability (McClements 2007:616; Patel *et al.*, 2013:34).

The zeta-potentials of the **AC-EM** and the **AN-EM** emulgel formulations that both contained clofazimine were determined. Samples of each emulgel were freshly prepared by separately diluting two drops of each in 10 ml of distilled water. The zeta-potential of both emulgels were determined on the same day, using a Malvern Zetasizer ZS (Malvern Instruments, UK). Each sample was separately injected into a clean plastic cuvette in triplicate to ensure reliable average values and the zeta-potential was measured in mV.

#### **E.4.6 Visual and physical examinations**

Visual and physical examinations were performed on the **AC-EM** and the **AN-EM** to observe their colours and to investigate their homogeneities.

### **E.5 Results and discussions of the characterisation of the optimised emulgels**

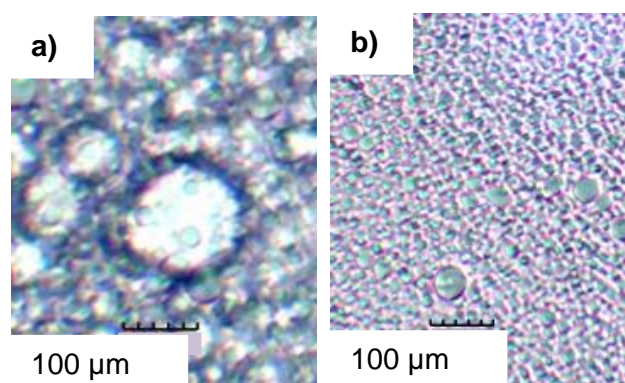
#### **E.5.2 Morphology study outcomes**

##### **E.5.2.1 Light microscopy**

The micrographic results revealed that oil droplets were still visible in the two different emulsions that had been incorporated into the separate emulgel formulations. The oil droplets, as illustrated in Figure E.1, were quite large, as a result of the occurrence of coagulation of the emulsion droplets during the incorporation of the emulsions into the emulgel formulations. As can be observed from the micrograph below, the **AC-EM** (Figure E.1.a)) had much larger droplets than the **AN-EM** (Figure E.1.b)), as had been expected of a coarse o/w emulsion, compared to a nano-emulsion, as reported in the literature (El-Aasser *et al.*, 1988:103; Fernandez *et al.*, 2004:53). Coarse emulsions typically have a droplet size range above 1  $\mu\text{m}$ , while nano-emulsions have droplet sizes between 200 nm and 500 nm (El-Aasser *et al.*, 1988:103; Fernandez *et al.*, 2004:53).

Therefore the micrograph outcomes of this study confirmed the literature reports. The larger droplets of the **AC-EM** probably resulted from coagulation. The droplets of the **AC-EM** were furthermore packed more densely than the smaller droplets of the **AN-EM**. This was indicative of the emulsion's instability, due to the larger droplets coagulating under the force of gravity (El-Aasser *et al.*, 1988:103; Fernandez *et al.*, 2004:53). Both the emulgel formulations displayed

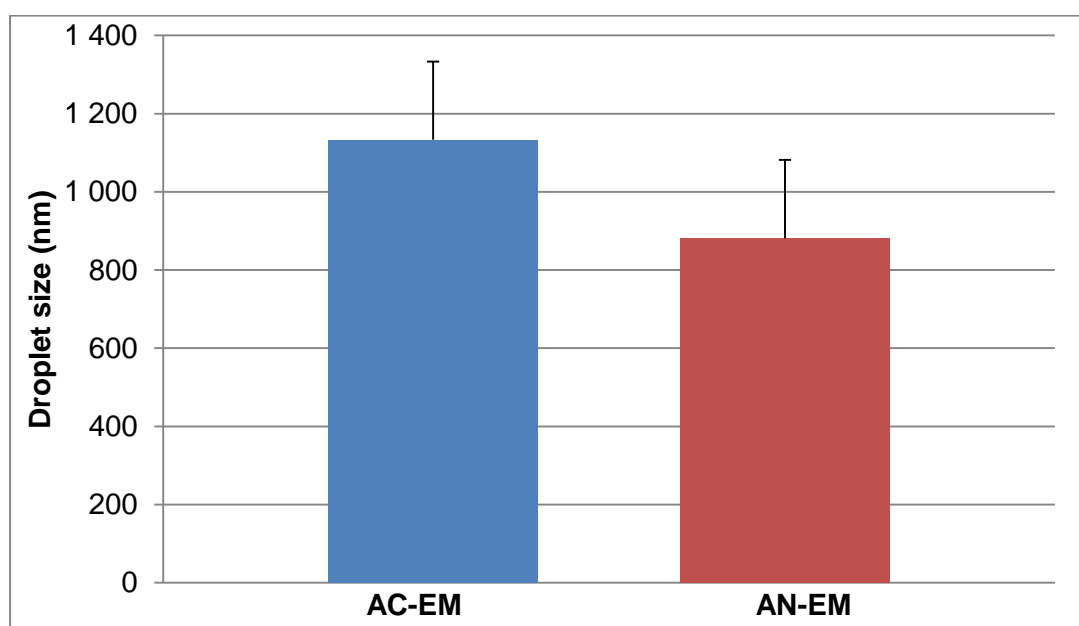
variations in their droplet sizes and they also had larger droplets than the emulgel without the API (**N-EM** and **C-EM**), as described in Appendix D. It appeared that the addition of the API to the formulations led to an increase in the emulsions' droplet sizes.



**Figure E.1:** Micrographs of the two emulgel formulations containing clofazimine, a) the **AC-EM** and b) the **AN-EM** at a 100 µm scale, as indicated

### E.5.3 Droplet sizes and droplet size distributions

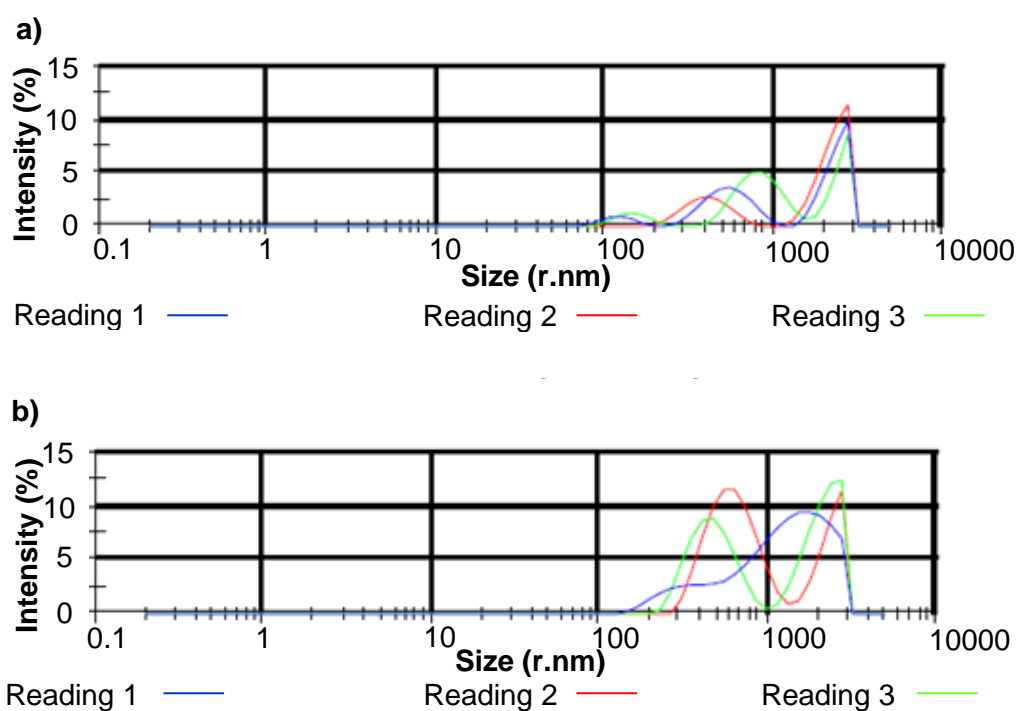
The droplet sizes and droplet size distributions were determined for each formulation, by freshly preparing samples of the **AC-EM** and the **AN-EM** emulgels. Each sample was tested in triplicate to assure reliable average droplet size and distribution measurement results. Figure E.2 graphically represents the average droplet sizes of the **AC-EM** and the **AN-EM**, respectively.



**Figure E.2:** Graphic representation of the average droplet sizes (nm) of the two emulgel formulations (**AC-EM** and **AN-EM**), containing clofazimine

Figure E.2 illustrates that the average droplet size of the **AC-EM** was larger than that of the **AN-EM**. This could be ascribed to the overall larger droplet sizes of the coarse emulsion, compared to those of the nano-emulsion, as discussed earlier. The **AC-EM** had an average droplet size of  $1133 \pm 0.04$  nm and the **AN-EM** an average of  $881 \pm 0.08$  nm. Both were within the acceptable ranges for droplet sizes of coarse emulsions and nano-emulsions, respectively (Gupta *et al.*, 2016:2828; Pey *et al.*, 2006:144).

Figure E.3 illustrates the droplet size distribution curves in triplicate of the **AC-EM** (Figure E.3.a) and the **AN-EM** (Figure E.3.b)). Figure E.3 demonstrates that the **AN-EM** had a better PDI than the **AC-EM**, being 0.463 and 0.585, respectively. The PDI is an indication of particle homogeneity. The better PDI that had been shown by the **AN-EM** was indicative thereof that it had better particle homogeneity and narrower droplet size distribution than the **AC-EM** (Patel *et al.*, 2013:34).



**Figure E.3:** Graphic representation of the droplet size distribution curves of a) the **AC-EM** and b) the **AN-EM** emulgel formulations

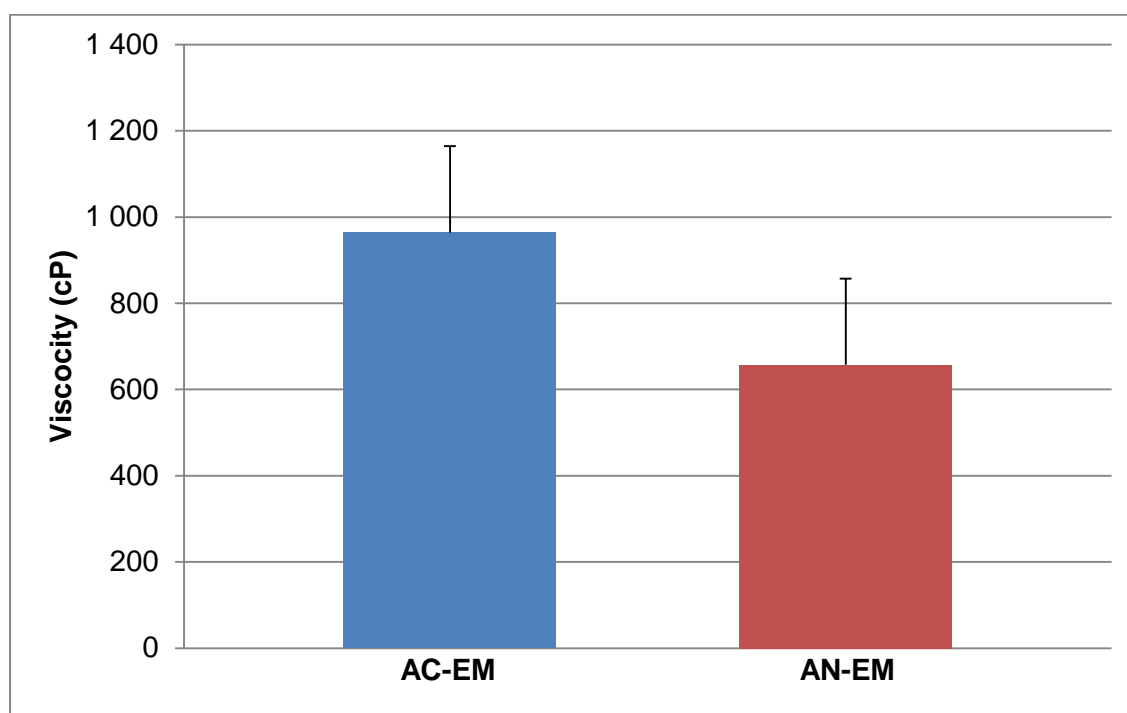
#### E.5.4 pH

The average pH of each sample was determined from the triplicate measurements of each individual emulgel formulation. The pH of the **AC-EM** ( $5.75 \pm 0.01$ ) was slightly lower and more acidic than that of the **AN-EM** ( $5.96 \pm 0.01$ ). However, the average pH values were so close to each other that the difference was considered irrelevant, since both emulgels consisted of the

same ingredients, while only different preparation methods were employed. The clofazimine had lowered the pH values in both formulations slightly. The pH values of both emulgels were deemed acceptable for safe skin use, because both were within the normal physiological pH range of the skin of pH 4.0 - 7.4 (Hadgraft & Valenta, 2000:243).

### E.5.5 Viscosity

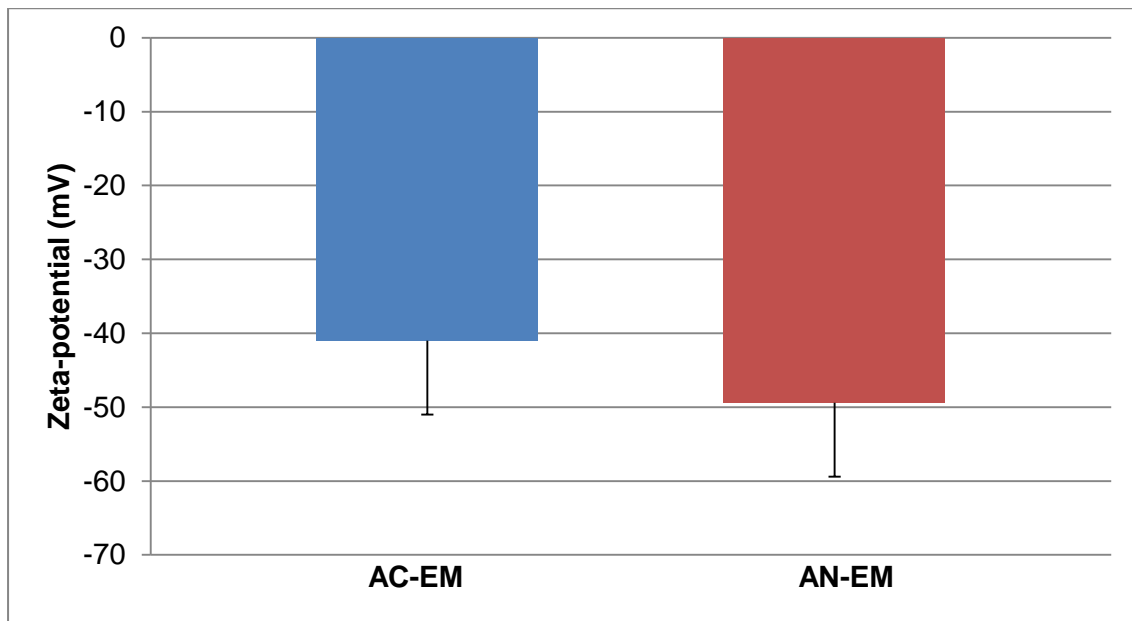
The viscosity results are graphically represented in Figure E.4. The **AN-EM** had an average viscosity of  $657.47 \pm 5.14$  cP, while the **AC-EM** had a higher viscosity of  $964.4 \pm 14.84$  cP. From these outcomes, it was expected that the **AN-EM** with the lower viscosity would show better membrane and skin diffusion results (Li *et al.*, 2011:1008; Valenta & Schultz, 2004:264).



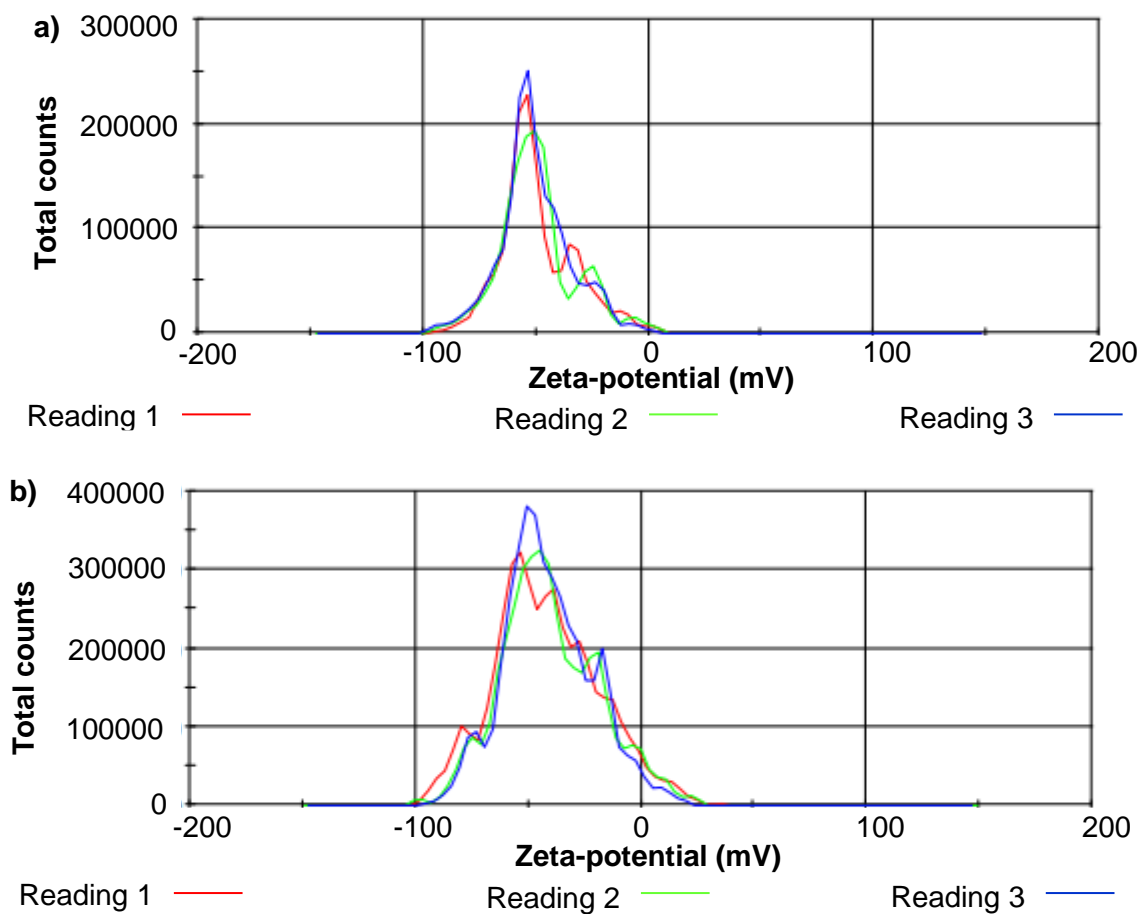
**Figure E.4:** Graphic representation of the viscosity (cP) results of the two emulgel formulations, containing clofazimine

### E.5.6 Zeta-potential

The average zeta-potential values were all negatively charged, which were indicative thereof that both emulgel formulations were stable (Patel *et al.*, 2013:34). The average zeta-potential of the **AC-EM** was  $-41 \pm 0.70$  mV and that of the **AN-EM** was slightly more negative at  $-49.4 \pm 0.85$  mV. Both formulations were therefore deemed stable, because the more negative zeta-potential values would prevent the droplets in both emulsions from aggregating (McClements, 2007:616; Patel *et al.*, 2013:34).



**Figure E.5:** Graphic representation of the zeta-potential (mV) results of the two emulgel formulations, containing clofazimine



**Figure E.6:** Graphic representation of the zeta-potential curves of a) the **AC-EM** and b) the **AN-EM** emulgels

### E.5.7 Visual and physical examination

The emulgels were inspected visually. Both formulations were homogenous with a relatively high viscosity. The **AN-EM** and the **AC-EM** were both light orange in colour, as illustrated in the images of the Franz cells below in Figure E.7.



**Figure E.7:** Photograph of Franz cells, containing the light orange coloured **AN-EM** and **AC-EM** formulations

### E.6 Conclusion

Physical characterisation of each emulgel formulation, containing clofazimine, was performed. These properties may affect the outcomes of the emulgels' membrane diffusion and topical skin delivery studies. The light microscopic results showed that both emulgels had formed favourable, spherical droplets. The droplet size and distribution results were indicative of the uniformity and stability of both emulgel formulations. The **AN-EM** had smaller droplet sizes than the **AC-EM**. The negative zeta-potential values of both formulations were indicative thereof that no aggregation had occurred in the emulgels. The pH values of both formulations were within the safe ranges for topical application. The viscosity results showed that the **AN-EM** had the lower viscosity of the two and would thus probably demonstrate better skin and membrane diffusion study results than the **AC-EM**.

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# APPENDIX F

## DIFFUSION STUDIES OF CLOFAZIMINE FROM A NANO-EMULSION AND TWO EMULGEL FORMULATIONS

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### F.1 Introduction

CTB is a form of extra-pulmonary TB that is often associated with MDR-TB (Barbagallo *et al.*, 2002:319), which causes unsightly lesions on the skin. Currently, no topical treatments are available on the market for CTB, but only systemic dosage forms (DermNet New Zealand, 2015). During this study, three topical (target site) preparations, containing clofazimine, were formulated to investigate their possible topical delivery of the API, aimed at finding a potential skin treatment for CTB. If successful, the topical preparation would be used in combination with a systemic treatment to achieve a synergistic effect, as it takes quite a while for the systemic treatment to reach the affected skin areas (Frankel *et al.*, 2009:24).

A topical preparation is typically applied to the affected area on the skin during treatment (Tadwee *et al.*, 2011:14). Topical preparations of drugs are easily applied to the affected areas and are therefore patient friendly (Kute & Saudagar, 2013:371). Clofazimine was formulated into a natural oil nano-emulsion (**A-NE**) and two emulgel formulations (**AN-EM** and **AC-EM**), aimed at improving the topical delivery of clofazimine to the affected areas. During this study, the **A-NE** was compared to the **AN-EM**, while the **AN-EM** was compared to the **AC-EM**, to identify the formulation that would yield the best membrane release, transdermal and topical results. The membrane release, skin diffusion and tape stripping studies that were performed during this research and their outcomes are discussed in this appendix.

### F.2 Methods

#### F.2.1 Sample analyses through high performance liquid chromatography

HPLC analyses were performed to quantify the concentrations of clofazimine found in the receptor phases of the Franz cells, in the stratum corneum-epidermis (SCE) and in the epidermis-dermis (ED), in accordance with the methods, as described in Sections A.2 and A.3.

#### F.2.2 Donor phase preparation for the membrane release and skin diffusion studies

The three formulations, i.e. the **A-NE**, the **AN-EM** and the **AC-EM** were used as the donor phases. The nano-emulsion (**A-NE**) was prepared in accordance with the method, as outlined

in Section B.3.6.1.1, while the **AN-EM** and **AC-EM** emulgel formulations were formulated, as discussed in Section D.3.1 and D.3.2, respectively. The three formulations were prepared for comparison purposes during the membrane release and skin diffusion studies, i.e. the **A-NE** and the **AN-EM** results were compared, while the results of the **AN-EM** and **AC-EM** were compared.

### **F.2.3 Receptor phase preparation for the membrane release and skin diffusion studies**

For the membrane diffusion studies, a receptor phase of analytical grade ethanol (99.9%) at pH 7.0 was used, because clofazimine does not dissolve in a phosphate buffer solution (PBS) (pH 7.4). During the skin diffusion studies, PBS (pH 7.4) and analytical grade ethanol were used as the receptor phase in a 9:1 ratio (PBS: ethanol). PBS was prepared by dissolving 0.013 M of  $\text{KH}_2\text{PO}_4$  in 800 ml of distilled water and 0.003 M of sodium hydroxide (NaOH) in 500 ml of distilled water. The  $\text{KH}_2\text{PO}_4$  was added to the NaOH and made up to a volume of 2 000 ml with distilled water and the pH adjusted to 7.4.

### **F.2.4 Aqueous solubility of clofazimine**

A water bath was pre-heated to 32 °C, representative of the temperature of the human skin. A magnetic stirring rod and PBS (pH 7.4) were transferred into a polytop. An excess of clofazimine was added to the solvent in the polytop and magnetically stirred for a period of 24 h. Care was taken to ensure that this solution remained saturated at all times through regular checking. After mixing, the solution was filtered and the concentration of the clofazimine determined was through HPLC analysis. This experiment was performed in triplicate.

### **F.2.5 Determination of the *n*-octanol-buffer distribution coefficient of clofazimine**

In order for the hydrophilic PBS (pH 7.4) and lipophilic *n*-octanol phases to co-saturate with clofazimine, equal volumes of each were added together into test tubes, sealed with screw caps, and equilibrated against each other with an excess of clofazimine over a period of 24 h. The experiment was done in triplicate. The excess of clofazimine was initially added to the pre-saturated buffer phase in attempts to try and dissolve some of the lipophilic API in the hydrophilic solvent. An equal volume each of pre-saturated *n*-octanol was then added to each clofazimine-buffer mixture, the test tubes sealed and were the solutions placed into a pre-heated water bath at 32 °C and mechanically shaken overnight. The solutions were centrifuged to split the two phases and HPLC analyses were performed on the clear solutions from each sample to determine the concentrations of the API in each of the separate phases. Finally, the log D value was calculated as a ratio of the API concentration in the *n*-octanol phase to the buffer phase.

## F.2.6 Membrane release studies

Membrane diffusion studies were performed, using the **A-NE**, **AN-EM** and **AC-EM** formulations as donor phases. These studies were performed to establish whether the API would be released from each of the three preparations. A placebo dispersion was prepared of each formulation to serve as the control during each experiment. Twelve vertical Franz cells were used per formulation for testing, of which two were used as controls during each experiment.

The method used during the membrane diffusion studies was as follows:

- The donor and receptor phases were prepared first and then allowed to warm in pre-heated water baths.
- The three donor phases (formulations) were placed in a water bath at a temperature of 32 °C, representative of the temperature on the skin's surface.
- The receptor phase was placed in a water bath at a temperature of 37 °C, representative of the temperature of blood in the human body.
- A magnetic stirring rod was placed in the receptor compartment of each vertical Franz cell.
- A Porafil cellulose nitrate filter, with a pore size of 20 µm (Separations, Johannesburg, South Africa), was placed on each receptor compartment.
- The two Franz cell compartments were mounted to each other after applying vacuum grease (Dow Corning<sup>®</sup>, Sigma-Aldrich, Germany) to the openings of both to seal them.
- Each vertical Franz cell was securely fastened with a horseshoe clamp to prevent the two compartments from shifting and to prevent leakage.
- Analytical grade ethanol (pH 7.0; 2 ml) was carefully injected into each receptor phase, taking care not to introduce any air bubbles.
- 1 ml of each preparation, containing the clofazimine, was placed into the donor compartments of the first ten vertical Franz cells.
- 1 ml each of the related placebo formulation was placed into the donor compartments of the two remaining vertical Franz cells per experiment.
- Each donor compartment was then covered with a double layer of Parafilm<sup>®</sup> to prevent any leakage.
- All twelve vertical Franz cells were placed into a Grant<sup>®</sup> water bath (Grant Instruments, UK) on a Variomag<sup>®</sup> magnetic hot plate (Variomag, USA) at a temperature of 37 °C.

- The receptor compartments of the twelve vertical Franz cells were also placed in the water bath to maintain the temperature at 37 °C, which led to the donor compartments reaching the skin's surface temperature of 32 °C (Williams, 2003:62).
- The magnetic hot plate was set at a stirring speed of 750 rpm throughout the duration of the experiment.
- The duration of the experiment was 6 h. The contents of each receptor phase was extracted at hourly intervals and refilled with fresh analytical grade ethanol (pH 7.0, pre-heated at 37 °C).
- HPLC analysis was performed on each of the samples from the membrane diffusion study to determine the concentration of the clofazimine that had been released from the donor phases through the membranes into the receptor phases.

### **F.2.7 Skin preparation for the skin diffusion studies**

Female, Caucasian skin was obtained following abdomino-plastic surgery. Prior ethical approval (reference number NWU-00114-11-A5) had been granted by the North-West University's Ethics Committee for collecting and using such biological material. After collection, the skin was frozen at - 20 °C, but not for a period longer than 6 months. The skin was thawed and carefully examined for stretch marks and other malformations and such sections were not used during these studies. The skin was dermatomed to a thickness of 400 µm (including the stratum corneum, the epidermis-dermis and the dermis), using a Zimmer™ electronic dermatome, model 8821 (Germany). The dermatomed skin was placed, stratum corneum facing upwards, on Whatman® filter paper to dry. The skin samples were cut into circles on the filter paper. Each skin circle on filter paper was wrapped in foil and frozen at - 20 °C, until use during the skin diffusion studies. Any remaining skin was kept frozen at - 20 °C and was discarded later, after all tests were completed.

### **F.2.8 Franz cell skin diffusion studies**

Glass vertical Franz cells were used to perform the skin diffusion studies in a pre-heated water bath, at a temperature of 37 °C. A circle of dermatomed skin, with the stratum corneum facing upwards, was placed on each receptor phase. The donor and receptor phases were assembled, sealed with vacuum grease and securely clamped together. The receptor phase was filled with PBS (pH 7.4). The donor compartment of the Franz cells contained either the natural oil nano-emulsion (**A-NE**), or one of the two emulgels (**AN-EM** or **AC-EM**). Each donor compartment was covered with Parafilm® to prevent evaporation of the solvent. Samples were taken at two-hourly intervals from the Franz cells and carefully transferred into HPLC vials. After sampling, the removed volumes were replaced with freshly prepared, pre-heated PBS

(pH 7.4):ethanol (99%, pH 7.0) (9:1, v/v). The samples were analysed on HPLC to determine whether diffusion of the API had occurred during the skin diffusion study and to quantify any such diffusion.

### **F.2.9 Tape stripping**

The Franz cells were disassembled after removing the buffer solution from the receptor phase. Each circle of skin was removed and carefully dabbed dry on clean laboratory paper to remove the excess formulation. The twelve skin samples were each mounted onto Parafilm® on a solid wooden surface with pins. 3M Scotch® Magic™ tape was cut into sixteen small strips per skin sample to cover the skin circles. The first tape strip per skin sample was discarded after being placed on the diffusion area, since it was contaminated with the donor phase. The fifteen remaining tape strips were then successively placed on the skin, and pulled off. These fifteen tape strips per skin sample, containing the SCE, were transferred into a polytop that contained 5 ml of extraction solution (99% analytical grade ethanol). The remaining pieces of skin circles, representing the ED, were each cut into small pieces and transferred into a polytop per skin sample, which also contained 5 ml of the extraction solution. The polytops were covered with Parafilm® and stored in the refrigerator overnight at 4 °C, to ensure that the clofazimine would dissolve in the extraction solution. The contents of both polytops per skin sample were analysed on HPLC.

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

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

The aqueous solubility of clofazimine in the literature is differently reported as either  $0.104 \times 10^{-2}$  mmol/l, or 0.225 mg/l and is it also stated as being practically insoluble in water (Drugbank, 2015; O'Driscoll & Griffin, 2008:618). During this study, clofazimine was indeed found insoluble in PBS (pH 7.4) at a temperature of 32 °C and no HPLC peaks were yielded during HPLC analysis of the prepared samples.

### **F.3.2 *n*-Octanol-buffer distribution coefficient of clofazimine**

The log P value of clofazimine is 7.132 (Drugbank, 2015). During this study, however, the log D value of clofazimine was determined at 4.6. It was concluded that the larger part of the clofazimine was found in the *n*-octanol phase, rather than in the PBS (pH 7.4) phase, which therefore proved that clofazimine was a lipophilic API. Since the ideal log P value for topical delivery is between 1 and 3 (Hadgraft, 2004:292; Drugbank, 2015), the API was not within that range and it was expected that the topical delivery of clofazimine would be challenging.

### F.3.3 Membrane diffusion studies

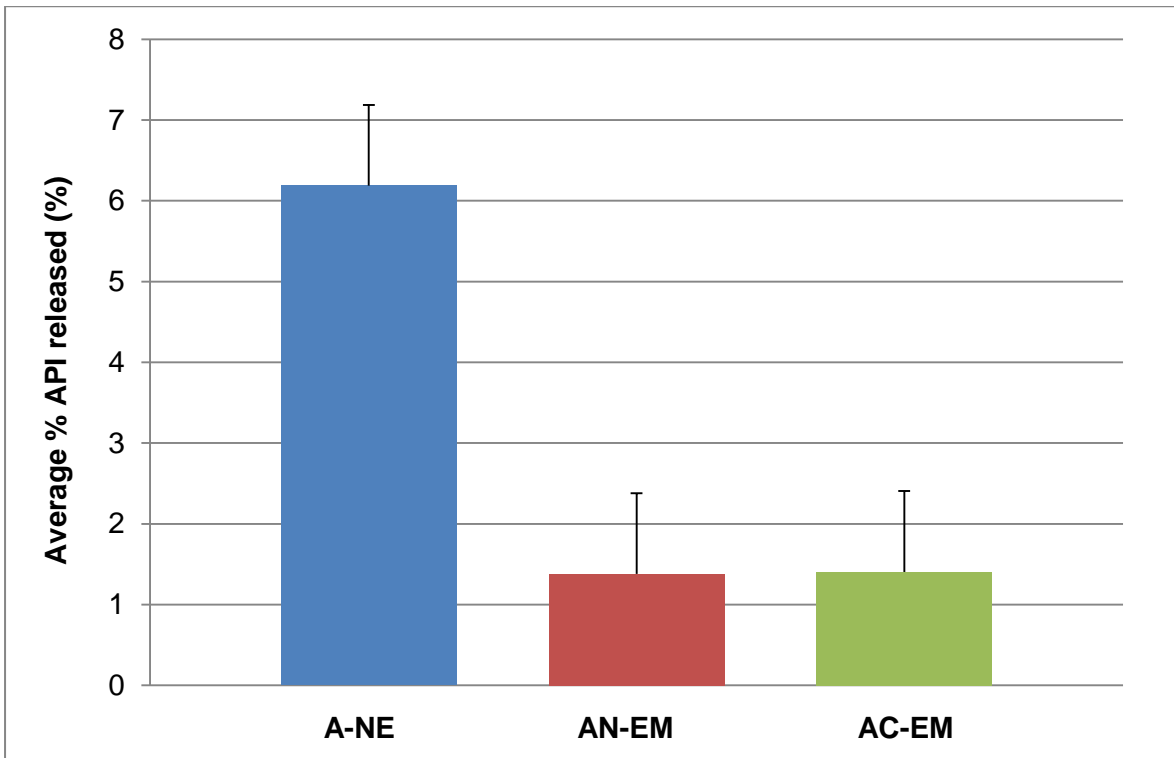
Three membrane diffusion studies were conducted during this study, one for each of the three formulations, i.e. for the nano-emulsion (**A-NE**) and for the two emulgel formulations (**AN-EM** and **AC-EM**). The results showed that clofazimine had been released from all three preparations during these studies. The number of Franz cells (FCs) for which the results are reported below vary, because some of the Franz cells showed signs of leakage and they were therefore excluded from the results to prevent inaccurate data.

The average flux values ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of clofazimine and the average percentages of clofazimine that had been released from each of the three preparations during the membrane release studies after a period of 6 h are summarised in Table F.1. The **A-NE** ( $2.71 \pm 0.66 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ) had the highest average flux, followed by the **AC-EM** ( $0.56 \pm 0.04 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ) and lastly, the **AN-EM** ( $0.31 \pm 0.04 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ). It should be noted that the nano-emulsion (**A-NE**) had significantly higher values than both the emulgel formulations (**AN-EM** and **AC-EM**).

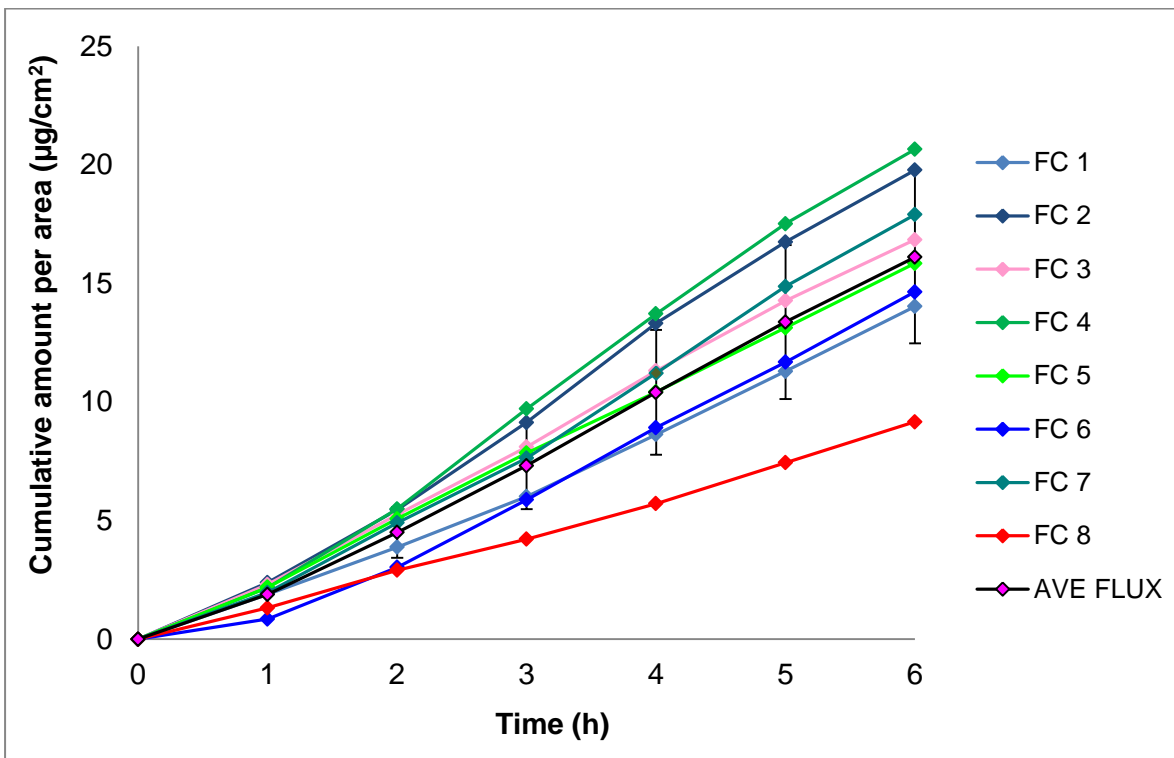
**Table F.1:** Average flux values and average percentages of released clofazimine during the membrane release studies of the three preparations after a period of 6 h

| Formulation type | Preparation  | Average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) | Average released (%) |
|------------------|--------------|---|----------------------|
| Nano-emulsion    | <b>A-NE</b>  | $2.71 \pm 0.66$   | $6.18 \pm 1.40$      |
| Emulgel          | <b>AN-EM</b> | $0.31 \pm 0.04$   | $1.38 \pm 0.17$      |
|                  | <b>AC-EM</b> | $0.56 \pm 0.04$   | $1.41 \pm 0.67$      |

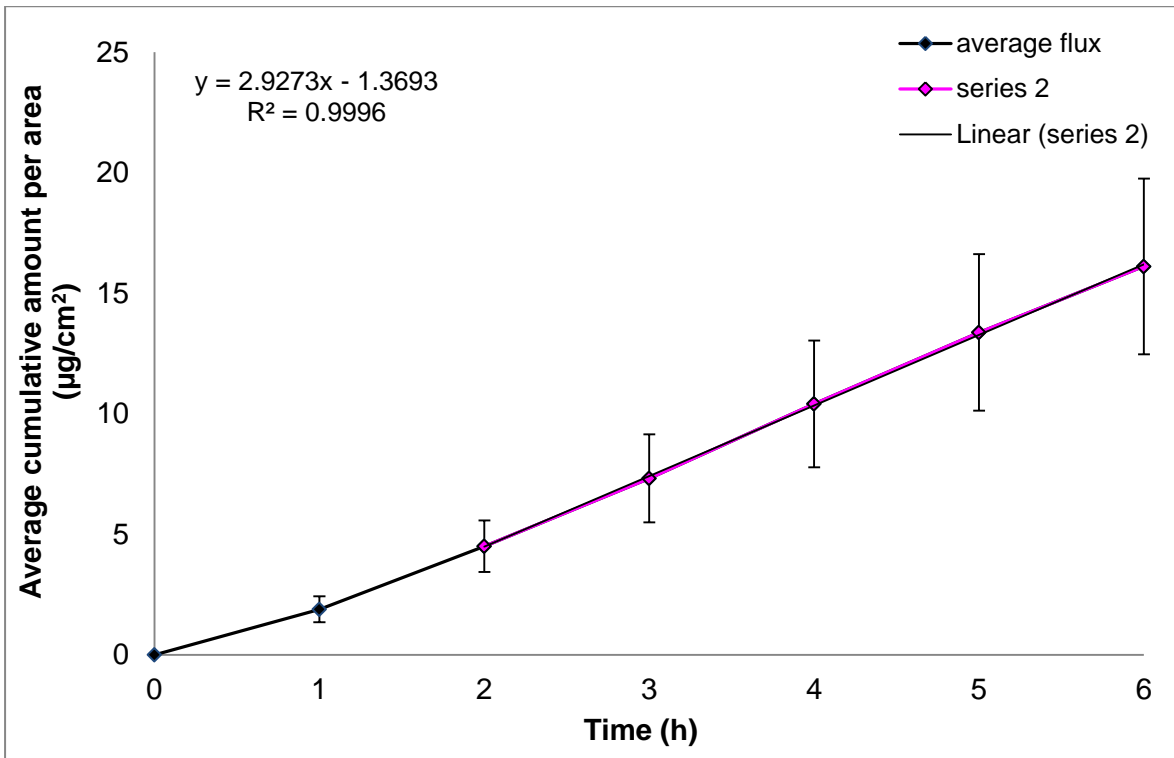
Figure F.1 illustrates the average percentages of clofazimine being released during the membrane diffusion studies from the natural oil nano-emulsion (**A-NE**) and from the two emulgels, the **AN-EM** and **AC-EM**. The average percentage of clofazimine being released from the **A-NE** ( $6.187 \pm 1.40\%$ ) was significantly the highest, followed by the much lower releases from the **AC-EM** ( $1.406 \pm 0.67\%$ ) and lastly, from the **AN-EM** ( $1.380 \pm 0.17\%$ ). The **A-NE** had by far shown the best drug release of clofazimine during the membrane diffusion studies, compared to the emulgels that had released very similar, but much lower concentrations of the API.



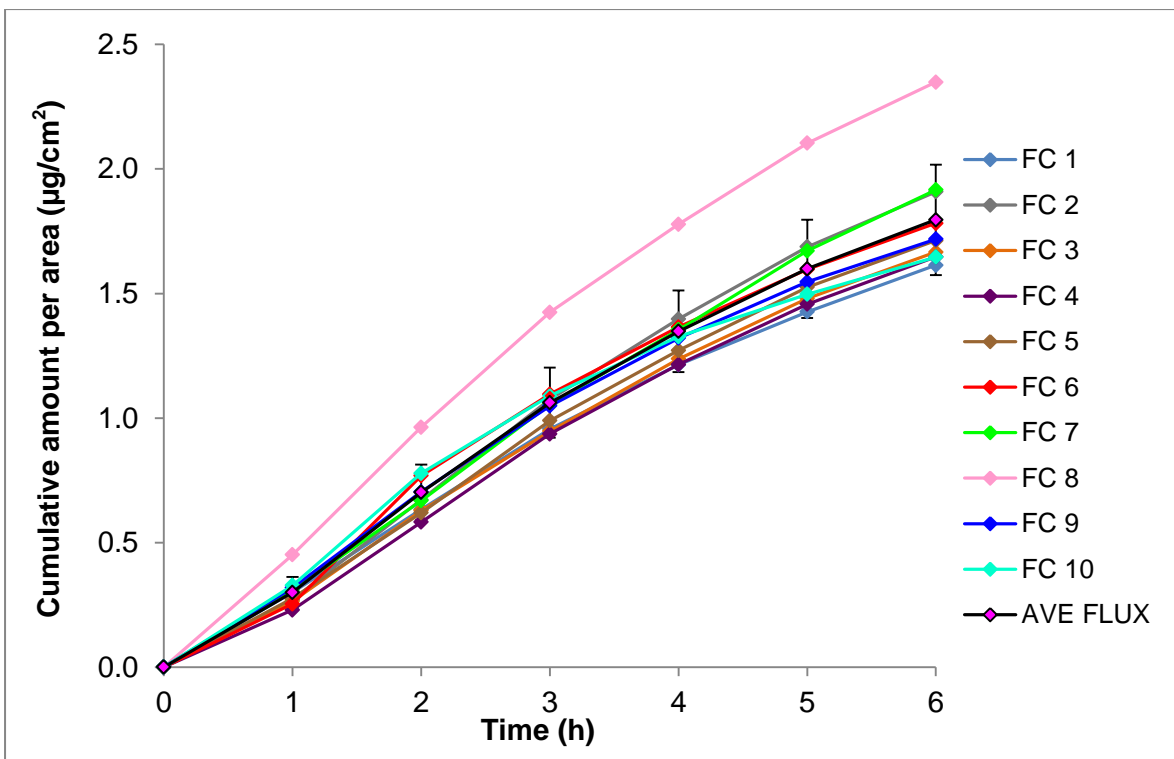
**Figure F.1:** Average percentages of clofazimine being released from the three formulations during the membrane diffusion studies



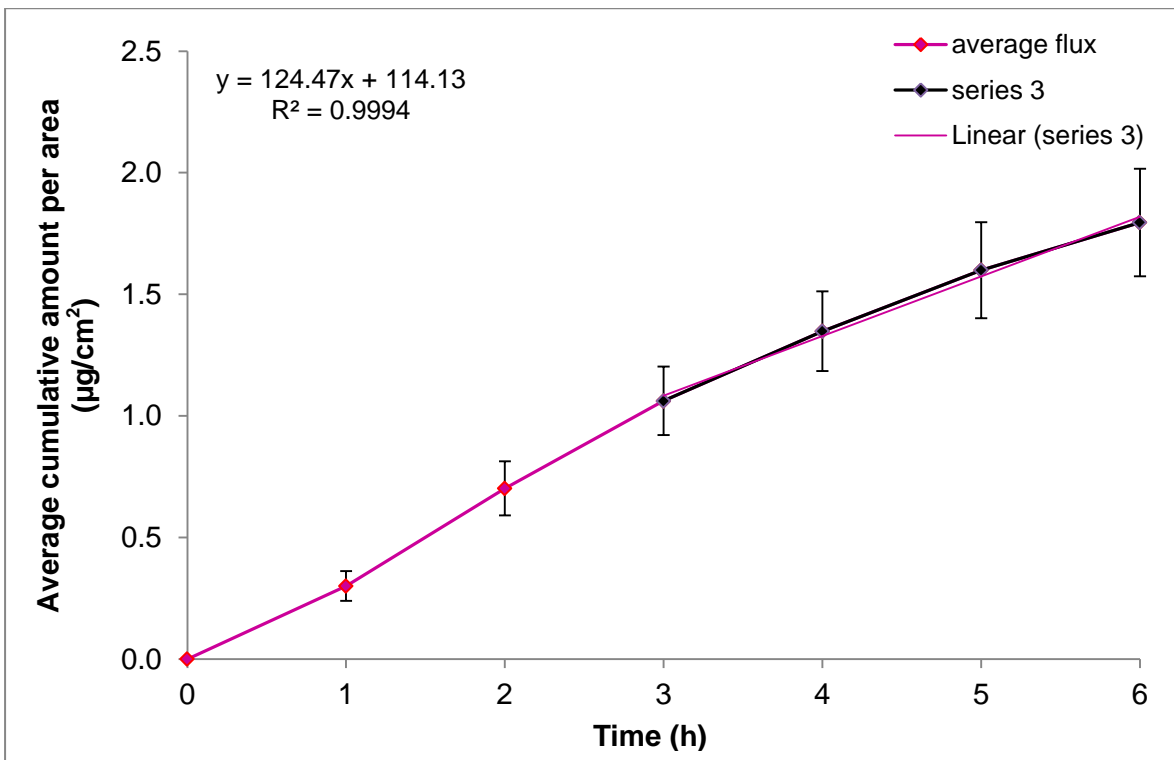
**Figure F.2:** Cumulative amounts per area ( $\mu\text{g}/\text{cm}^2$ ) of clofazimine that had permeated from the **A-NE** formulation through the membrane of each Franz cell over the period of 6 h ( $n = 8$ )



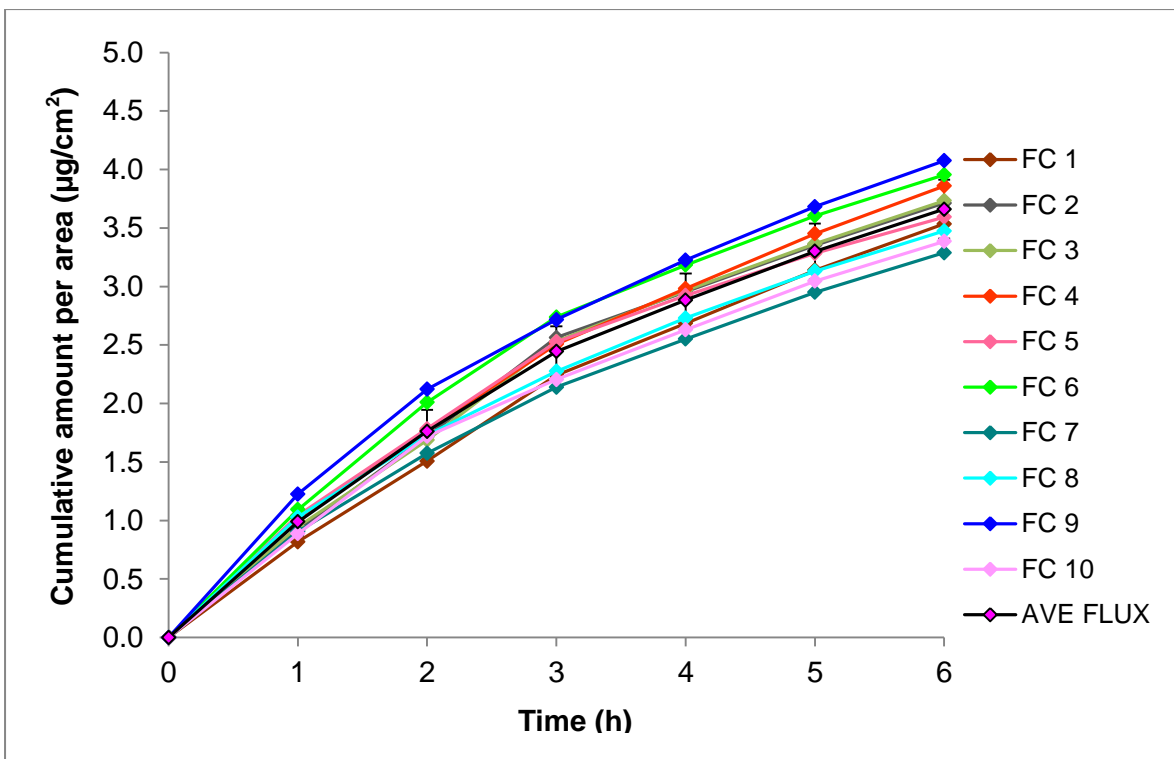
**Figure F.3:** Average cumulative amounts per area ( $\mu\text{g}/\text{cm}^2$ ) of clofazimine that had permeated from the **A-NE** formulation through the Franz cell membranes as a function of time ( $n = 8$ )



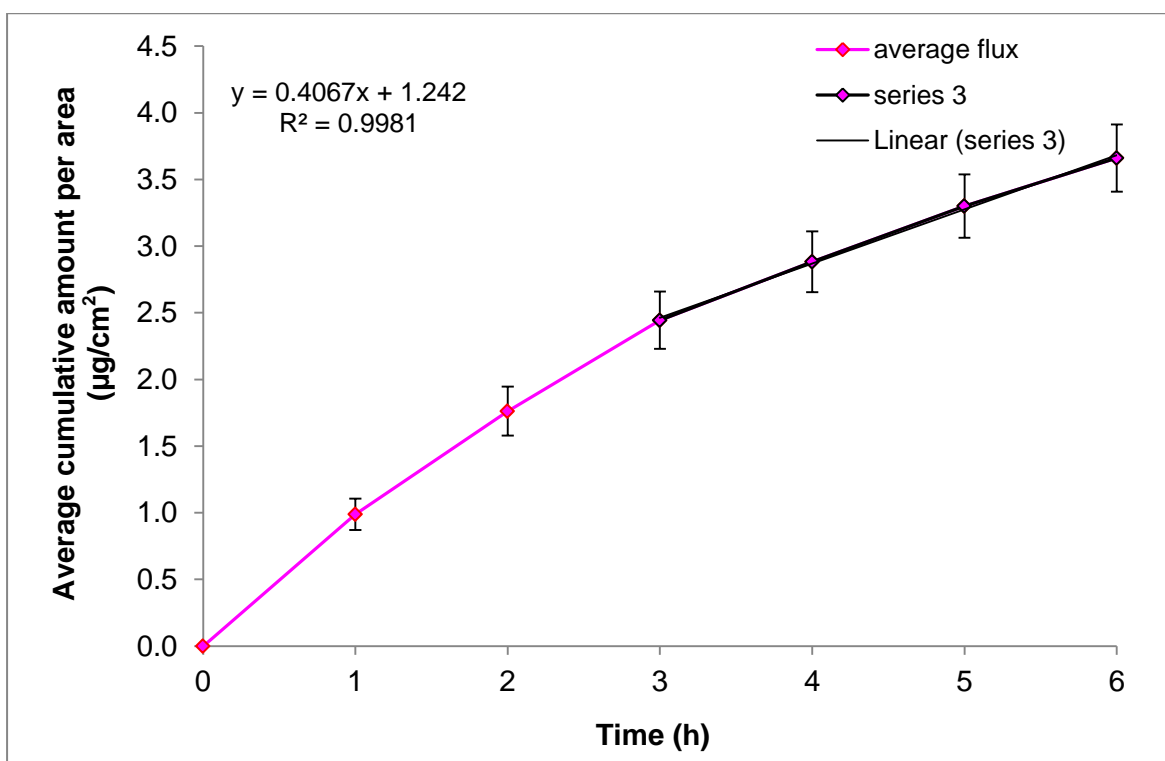
**Figure F.4:** Cumulative amounts per area ( $\mu\text{g}/\text{cm}^2$ ) of clofazimine that had permeated from the **AN-EM** formulation through the membrane of each Franz cell over the period of 6 h ( $n = 10$ )



**Figure F.5:** Average cumulative amounts per area ( $\mu\text{g}/\text{cm}^2$ ) of clofazimine that had permeated from the **AN-EM** formulation through the Franz cell membranes as a function of time ( $n = 10$ )



**Figure F.6:** Cumulative amounts per area ( $\mu\text{g}/\text{cm}^2$ ) of clofazimine that had permeated from the **AC-EM** formulation through the membrane of each Franz cell over the period of 6 h ( $n = 10$ )



**Figure F.7:** Average cumulative amounts per area ( $\mu\text{g}/\text{cm}^2$ ) of clofazimine that had permeated from the **AC-EM** formulation through the Franz cell membranes as a function of time ( $n = 10$ )

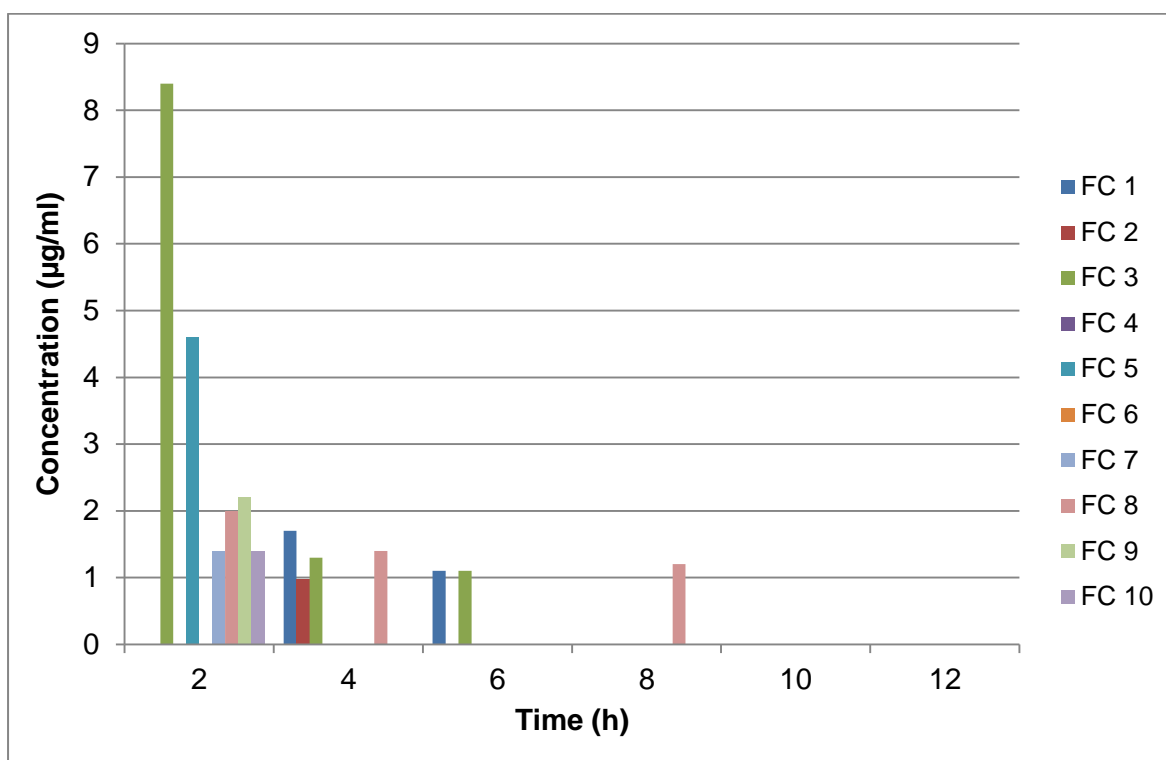
Figures F.2, F.4 and F.6 illustrate the cumulative amounts of clofazimine per area ( $\mu\text{g}/\text{cm}^2$ ) as a function of time that had permeated through each individual Franz cell membrane over a period of 6 h from each of the three formulations, while Figures F.3, F.5 and F.7 illustrate the average cumulative amounts of clofazimine per area ( $\mu\text{g}/\text{cm}^2$ ) as a function of time that had permeated through the Franz cell membranes over the 6 h period from the three formulations.

In conclusion, the **A-NE** formulation had released the highest concentration of clofazimine during the membrane diffusion studies, followed by the **AC-EM** and lastly, the **AN-EM** formulation. Clofazimine had therefore been successfully released from all three preparations during the membrane release studies.

The results being obtained from the membrane release studies, using the Porafil cellulose nitrate filters were, however, not indicative of the results from the Franz cell skin diffusion studies. There was no correlation between the results obtained from the membrane release studies and those from the skin diffusion studies. The membrane release studies had only aimed at determining whether the API would be released from the formulations. Therefore, if no positive results were obtained from the skin diffusion studies, the possibility that the API had not been released from the formulation, could at least be eliminated.

### F.3.4 Transdermal diffusion

Franz cell diffusion studies were performed on the nano-emulsion (**A-NE**) and the two emulgel formulations (**AN-EM** and **AC-EM**) to establish whether the API had been delivered transdermally. HPLC analyses of the samples, collected during the three transdermal diffusion studies, only detected clofazimine in the samples from the **A-NE** study. The peaks were very small and non-quantifiable, ascribing to the fact they did not comply with the validated limit of detection (LOD) and lower limit of quantification (LLOQ) ranges, as established during the HPLC method validation of clofazimine (Section A.4). The results could not be quantified and were unreliable due to noise (ICH, 2005:5).



**Figure F.8:** Clofazimine concentrations in the receptor phases of the Franz cells during the diffusion study performed on the **A-NE** formulation

Figure F.8 represents the concentrations ( $\mu\text{g/ml}$ ) of clofazimine being obtained from the receptor phases of Franz cells 1 - 10 (FCs 1 - 10) for all sampling done at two-hourly intervals. FC 3 showed the highest clofazimine concentration, with a value of  $8.4 \mu\text{g/ml}$  after 2 h, while FC 2 showed the lowest concentration at  $0.98 \mu\text{g/ml}$  of clofazimine after 4 h. Some of the Franz cells had offered no results, with variations among the outcomes from samples taken at specific sampling intervals. From the sampling done at the 6 h interval, for example, values were only detected in the samples from FC 1 and FC 3. With regards to the samples taken at the 8 h interval, only FC 8 showed results, while no clofazimine traces were detected in samples taken from any of the Franz cells at the 10 h and 12 h sampling intervals. Consequently, it was

concluded that no clofazimine from the **A-NE** formulation had been present in the receptor phase after 12 h. Clofazimine was therefore scarcely present in the receptor phases of all of the Franz cells throughout this 12 h study. Most of the results were unreliable and hence excluded, as they had noise and were non-quantifiable (ICH, 2005:5).

It was, however, concluded that no clofazimine had diffused transdermally into the receptor phase that represents the blood in the human body. These results were thus favourable, since the aim of the study had been the topical delivery of clofazimine to the targeted skin surface only and not its transdermal diffusion.

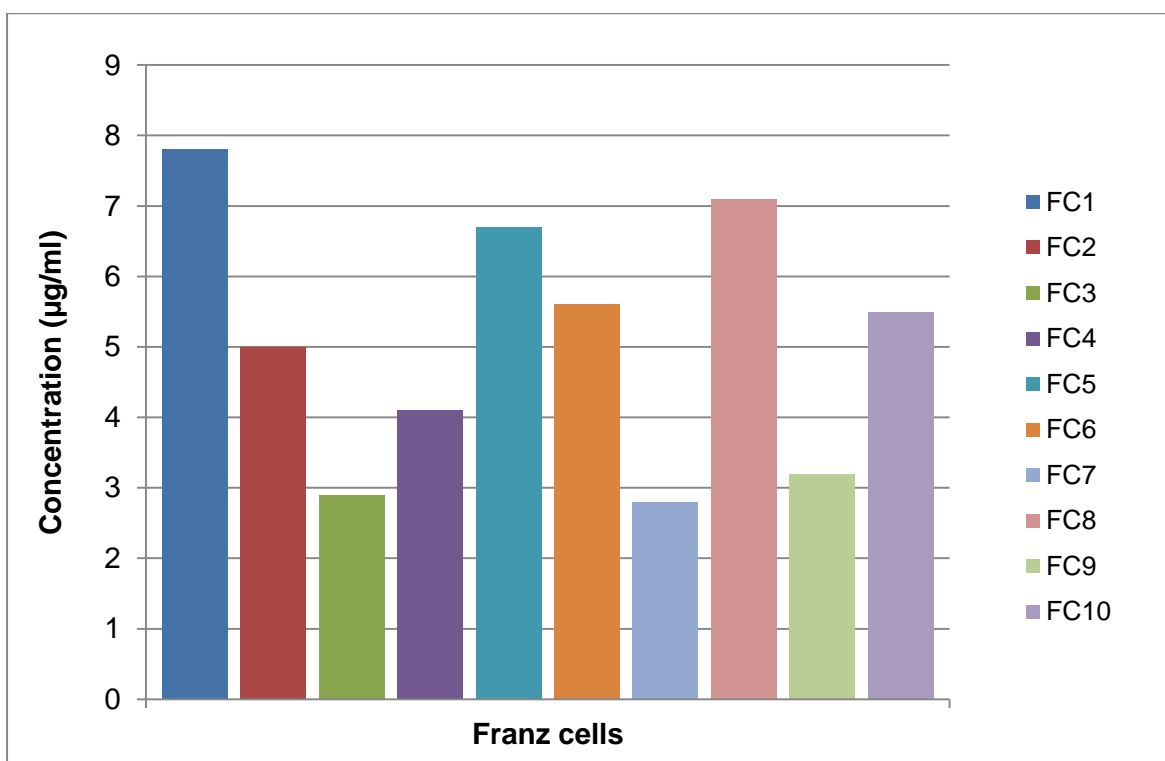
### **F.3.5 Tape stripping**

Tape stripping was used to investigate whether clofazimine had been delivered topically from the **A-NE**, the **AN-EM** and the **AC-EM** formulations. The targeted site during this study was the skin, where CTB causes skin lesions (Barbagallo *et al.*, 2002:320). It was expected that because clofazimine is very lipophilic as per the literature review (Chapter 2), and as proven in Sections F.3.1 and F.3.2 of this study, that the API would rather stay in the lipophilic SCE than in the hydrophilic ED.

#### **F.3.5.1 Stratum corneum-epidermis**

HPLC analyses of the samples obtained from tape stripping showed very small peaks for the samples obtained from the **A-NE** formulation only. The results were, however, non-quantifiable, as they did not fall within the validated LOD and LLOQ parameters, as had been established during the HPLC method validation of clofazimine. The results obtained from the tape stripping samples of the three preparations' membrane studies were all ruled out, due to noise and unreliable results (ICH, 2005:5). There was therefore no API present in the SCE after application of the emulgel formulations (**AN-EM** or **AC-EM**), whereas a very small amount was present in the SCE where the nano-emulsion had been applied (**A-NE**).

Figure F.9 represents the results from the **A-NE** formulation after tape stripping of the skin samples from the individual Franz cells. The tape stripped skin sample from FC 1 showed the best results, with a clofazimine concentration of 7.8 µg/ml, while FC 3 showed the lowest diffusion results of 2.9 µg/ml. Although most of these concentrations were too low to be quantified, a small amount of the drug had indeed diffused through the SCE, while a small amount of topical delivery had taken place during the skin diffusion study of the **A-NE** formulation.

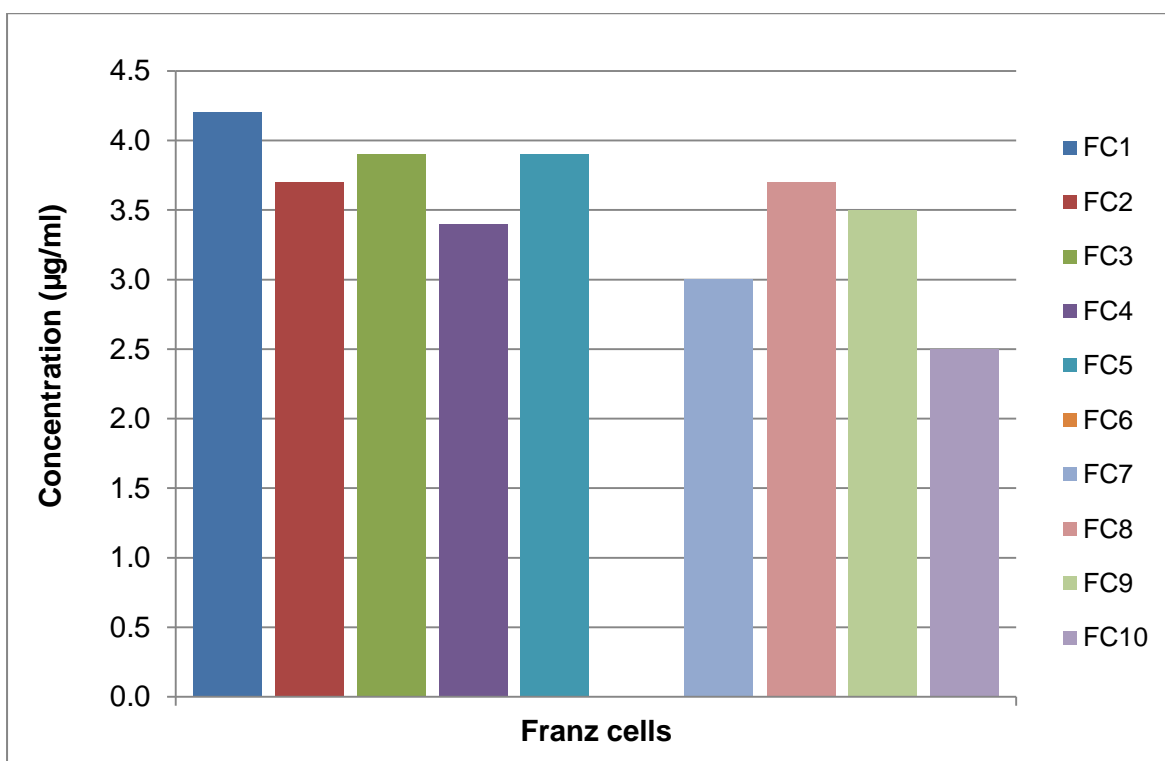


**Figure F.9:** Clofazimine concentrations ( $\mu\text{g/ml}$ ) from the **A-NE** formulation in the stratum corneum-epidermis after tape stripping

#### F.3.5.2 Epidermis-dermis

The ED unfortunately did not offer any quantifiable results during HPLC analysis either. Peaks were only detected in the **A-NE** samples, but they were also non-quantifiable and were ruled out due to noise (ICH, 2005:5). Consequently, it was concluded that there had been very little of the API present in the ED, treated with the **A-NE** formulation, while no topical diffusion had occurred from the two emulgel formulations (**AN-EM** and **AC-EM**).

Figure F.10 represents the ED results from the **A-NE** formulation. Low and non-quantifiable clofazimine concentrations were detected and therefore only a very small amount of clofazimine from the **A-NE** formulation had penetrated through to the ED. FC 1 showed the highest results, with a concentration of  $4.2 \mu\text{g/ml}$ , while FC 6 showed no results. It was therefore concluded that, since a very small amount of the API had been delivered topically, the aim of the topical delivery of clofazimine had indeed been achieved, although in non-quantifiable amounts.



**Figure F.10:** Clofazimine concentrations ( $\mu\text{g/ml}$ ) from the **A-NE** formulation in the ED after tape stripping

#### F.4 Conclusion

The aim of this study was to deliver clofazimine topically in the SCE and possibly the ED, for the possible targeted treatment of the unsightly lesions being caused by CTB, in combination with the existing systemic dosage form (Barbagallo *et al.*, 2002:320).

Three clofazimine containing formulations had been prepared as donor phases during this study, i.e. a natural oil nano-emulsion (**A-NE**) and two emulgel formulations (**AN-EM** and **AC-EM**). The membrane diffusion study results showed that clofazimine had been released from all three preparations. The **A-NE** formulation showed the best API release, followed by the **AC-EM** and lastly, the **AN-EM**. The two emulgel formulations (**AC-EM** and **AN-EM**) were formulated for comparison purposes. The **AN-EM** formulation, within which the **A-NE** nano-emulsion had been incorporated during the preparation of this emulgel, showed less API release during the membrane diffusion studies, compared to the **AC-EM**. This could have been due to the oil phase of the **A-NE** formulation having ascended to the top of the **AN-EM** emulgel, which could have led to a higher clofazimine concentration in the upper part of the formulation. Consequently, because the API was only released from the lower part of the emulgel in the donor phase, where less API had been encapsulated in the oil phase of the **A-NE**, a very low API concentration was measured.

The diffusion study results showed that no clofazimine had been released into the receptor phases of both the emulgel formulations (**AC-EM** and **AN-EM**), but small API peaks were detected in the receptor phases of the **A-NE** formulation. It was therefore concluded that no transdermal delivery of the API had taken place during the two emulgel skin diffusion studies, while very little, non-quantifiable amounts of clofazimine had diffused into the receptor phases during the **A-NE** skin diffusion study, whereas only a small amount of transdermal diffusion had occurred. It had been expected, prior to conducting these studies, that very little or no transdermal permeation would occur, due to the API being very lipophilic, whereas for a substance to penetrate transdermally into the blood stream of the skin, it should possess both lipophilic and hydrophilic characteristics, because of the lipophilic and hydrophilic characteristics of the different layers of the skin (Hadgraft, 2004:292; TB alliance, 2008:96).

Very low, non-quantifiable amounts of clofazimine were present in the tape strips from the **A-NE** formulation, which was indicative thereof that only small amounts of clofazimine were present in the SCE and that topical diffusion had occurred. Contrary, HPLC analyses of the samples from the tape stripping studies from the emulgels (**AC-EM** and **AN-EM**) detected no traces of the API and no API had been present in the SCE. Lastly, no clofazimine had been present in the ED from the two emulgel formulations, which was also indicative thereof that no topical diffusion had occurred. The **A-NE** showed very small, non-quantifiable peaks in the ED samples which means that very little topical diffusion occurred. It was concluded that the **A-NE** formulation had delivered the API better topically than both the emulgel formulations (**AC-EM** and **AN-EM**) and that the topical diffusion study results of the **AN-EM** and the **AC-EM** were very similar.

The log D value of clofazimine was measured as 4.6, which confirmed that the API was very lipophilic. The corresponding lack of aqueous solubility of clofazimine was also highlighted in Section F.3.1. The ideal log P value that a drug should have in order to diffuse topically is between 1 and 3 (Hadgraft, 2004:292). The unsuitably high log P value of clofazimine was determined at 7.132, which had indicated that skin permeation would not occur. The skin diffusion study results confirmed that clofazimine had not been delivered transdermally (Drugbank, 2015).

The aims and objectives of this study were partially met, since clofazimine had only been delivered topically and transdermally from the **A-NE** formulation, but only in small and non-quantifiable concentrations, while the two emulgel formulations (**AC-EM** and **AN-EM**) had not succeeded in delivering the API topically, possibly due to the lipophilic nature of the API, or due to the small amounts of the API that had dissolved in the avocado oil phase of the preparations (Section B.3.5.1). In order to try and enhance the topical delivery of clofazimine from the **AN-EM** formulation, another oil phase, in which clofazimine would be more soluble, could be considered in future studies.

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## APPENDIX G

# AUTHOR'S GUIDE FOR THE EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

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### G.1 Introduction

Manuscripts submitted to the journal are accepted on the understanding that: (1) they are subject to editorial review, (2) they have not been and will not be published in whole or in part in any other journal and (3) the recommendations of the most recent version of the Declaration of Helsinki, for humans, and the European Community guidelines as accepted principles for the use of experimental animals, have been adhered to. *The European Journal of Pharmaceutical Sciences* will, therefore, only consider manuscripts that describe experiments which have been carried out under approval of an institutional or local ethics committee.

### G.2 Types of Paper

#### G.2.1 Research articles

The *European Journal of Pharmaceutical Sciences* publishes research articles in the multidisciplinary field of pharmaceutical sciences, with a focus on topics relevant for drug discovery and development.

More specifically, the Journal publishes reports on medicinal chemistry, pharmacology, drug absorption and metabolism, pharmacokinetics and pharmacodynamics, pharmaceutical and biomedical analysis, drug delivery (including gene delivery), drug targeting, pharmaceutical technology, pharmaceutical biotechnology and clinical drug evaluation.

The journal will typically not give priority to manuscripts focusing primarily on organic synthesis, natural products, adaptation of analytical approaches, or discussions pertaining to drug policy making.

Important other criteria for manuscript acceptance are conceptual novelty, scientific rigorousness of the experiments, relevance for a broad readership beyond the specific topic of the manuscript, and adherence to high ethics standards of experimentation. Research articles should comply with the format requirements set forth in the section "Article Structure below".

## **G.2.2 Review articles**

The manuscript of a review article should be arranged as described for research articles but according to the following sections: title page, abstract and keywords (indexing terms, normally 3-6 items), Introduction, Specific sections determined by the author, Conclusions, Acknowledgements, References, Figure legends and Figures, Tables. Sections ranging from the Introduction to the Conclusions should be numbered. Subdivisions within a section should also be numbered within that section: 2.1., 2.2., 2.3. etc. All pages should be numbered consecutively, the title page being p.1.

## **G.2.3 Commentaries and Mini-reviews**

One page suggestions for comprehensive reviews, commentaries or mini-reviews should be sent to the Editor-in-Chief at [ejps@sdu.dk](mailto:ejps@sdu.dk) for consideration. Please see detailed information on commentaries and mini-reviews below.

## **G.2.4 Commentaries (Guidance)**

The definition of a Commentary for EJPS is three-fold. Firstly, it can be an argued piece of provocative scientific writing purporting to take a balanced position on a controversial pharmaceutical science topic. A second option is for the author to approach the topic from a particular viewpoint on one side of an argument. A third option is to provide a topical update on a hot topic in Pharmaceutical Sciences and this can be more informative than controversial.

Commentaries will be commissioned by the editors in advance or invited from non-commissioned authors if they wish to initially submit a one page summary of the intended Commentary to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The journal is looking for a stimulating and provoking essays, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to preferably recent peer-reviewed material. The word count should be approximately 2,000 words maximum. The commentary should have a short abstract summary of 150 to 200 words and 4-5 key words should be included, The text should be broken down into 4-5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19,1-11 by R.D. Combes.

### **G.2.5 Mini-review (Guidance)**

Mini-reviews are thought provoking reviews of contemporary pharmaceutical research. Themes are as described in the Scope of the Journal section.

Mini-reviews will usually be commissioned by the editors in advance, but contributions are invited from non-commissioned authors if they wish to initially submit a one page summary of the intended review to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The structure of the mini-review is as follows: a title page followed by a 200-300 word abstract with 4-5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-reviewed material. There is a combined maximum of 5 figures / tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

### **G.3 Submission checklist**

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

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### **G.5.2 Declaration of interest**

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### **G.5.13 Language (usage and editing services)**

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Please submit, with the manuscript, the names, postal addresses and e-mail addresses of at least four potential reviewers. Good suggestions lead to faster processing of your paper. Please note:

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## **G.6 Preparation**

### **G.6.1 Use of word processing software**

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the [Guide to Publishing with Elsevier](#)). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

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### **G.6.3 Article structure**

#### **G.6.3.1 Subdivision numbered sections**

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

#### **G.6.3.2 Introduction**

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

#### **G.6.3.3 Material and methods**

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

#### **G.6.3.4 Results**

Results should be clear and concise. Text, tables and figures must show minimal overlap, and must be internally consistent.

#### **G.6.3.5 Discussion**

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

#### **G.6.3.6 Conclusions**

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

#### **G.6.3.7 Appendices**

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

### G.6.3.8 Essential title page information

- **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- **Corresponding author.** The manuscript must be submitted by the person who is in charge of correspondence at all stages of the editorial process, production, and post-publication. Ensure that phone numbers (with country and area codes) are provided, in addition to the e-mail address (preferably an institutional e-mail address) and the complete postal address. Contact details of the other authors must be kept up to date by the corresponding author.
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### G.6.3.9 Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

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A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more,

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#### **G.6.3.11 Keywords**

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

#### **G.6.3.12 Chemical compounds**

You can enrich your article by providing a list of chemical compounds studied in the article. The list of compounds will be used to extract relevant information from the NCBI PubChem Compound database and display it next to the online version of the article on ScienceDirect. You can include up to 10 names of chemical compounds in the article. For each compound, please provide the PubChem CID of the most relevant record as in the following example: Glutamic acid (PubChem CID:611). Please position the list of compounds immediately below the 'Keywords' section. It is strongly recommended to follow the exact text formatting as in the example below:

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#### **G.6.3.13 Abbreviations**

Abbreviations are a hindrance for the reader. Use as few abbreviations as possible and write out names of compounds, receptors, etc., in full throughout the text of the manuscript, with the exceptions given below. Unnecessary and nonsense abbreviations are not allowed. Generic names should not be abbreviated. As an example, AMP, HAL, HIST, RAMH, TAM, SST, for amphetamine, haloperidol, histamine, (R)- $\alpha$ -methylhistamine, tamoxifen, somatostatin, are not accepted. Abbreviations which have come to replace the full term (e.g., GABA, DOPA, PDGF, 5-HT, for  $\gamma$ -aminobutyric acid, 3,4-dihydroxyphenylalanine, PDGF, 5-hydroxytryptamine) may be used, provided the term is spelled out in the abstract and in the body of the manuscript the first time the abbreviation is used. Unwieldy chemical names may be abbreviated. As an example, 8-OH-DPAT, DOI, DTG, BAPTA, for 8-hydroxy-2-(di-*n*-propylamino)tetralin, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, 1,3-di(2-tolyl)-guanidine, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, are acceptable; however, the full

chemical name should be given once in the body of the manuscript and in the abstract, followed in both cases by the abbreviation. Code names may be used, but the full chemical name should be given in the text and in the abstract. *Authors not conforming to these demands may have their manuscripts returned for correction with delayed publication as a result.*

Some abbreviations may be used without definition:

|                                  |   |
|----------------------------------|---|
| ADP, CDP, GDP, IDP               | 5'-pyrophosphates of adenosine                                    |
| UDP                              | cytidine, guanosine, inosine, uridine                             |
| AMP etc.                         | adenosine 5'-monophosphate etc.                                   |
| ADP etc.                         | adenosine 5'-diphosphate etc.                                     |
| ATP etc.                         | adenosine 5'-triphosphate etc.                                    |
| CM-cellulose                     | carboxymethylcellulose  |
| CoA and acetyl-CoA               | coenzyme A and its acyl derivatives                               |
| DEAE-cellulose                   | O-(diethylaminoethyl)-cellulose                                   |
| DNA                              | deoxyribonucleic acid   |
| EGTA                             | ethylene glycol-bis(2-aminoethyl ether)N,N,N',N'-tetraacetic acid |
| FAD                              | flavin-adenine dinucleotide                                       |
| FMN                              | flavin mononucleotide   |
| GSH, GSSG                        | glutathione, reduced and oxidized                                 |
| Hepes                            | 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid              |
| NAD                              | nicotinamide-adenine dinucleotide                                 |
| NADP                             | nicotinamide-adenine dinucleotide phosphate                       |
| NMN                              | nicotinamide mononucleotide                                       |
| P <sub>i</sub> , PP <sub>i</sub> | orthophosphate, pyrophosphate                                     |
| RNA                              | ribonucleic acid  |
| Tris                             | 2-amino-2-hydroxymethylpropane-1,3-diol                           |

Two alternative conventions are currently in use in some cases. For example, for the phosphoinositides there are both the abbreviations recommended by the IUPAC-IUB and those of the Chilton Convention (e.g., PtdIns(4,5)P<sub>2</sub> vs. PIP<sub>2</sub> for phosphatidylinositol 4,5-bisphosphate). The journal will accept either of these forms but not their combination.

Abbreviations of units of measurements and other terms are as follows:

*Units of mass*

|                      |               |            |                 |
|----------------------|---------------|------------|-----------------|
| kilogram             | kg            | millimole  | mmol            |
| gram                 | g             | micromole  | $\mu\text{mol}$ |
| milligram            | mg            | nanomole   | nmol            |
| microgram            | $\mu\text{g}$ | picomole   | pmol            |
| nanogram             | ng            | femtomole  | fmol            |
| mole (gram-molecule) | mol           | equivalent | eq              |

*Units of time*

|        |     |             |               |
|--------|-----|-------------|---------------|
| hour   | h   | millisecond | ms            |
| minute | min | microsecond | $\mu\text{s}$ |
| second | s   |             |               |

*Units of volume*

|            |               |  |  |
|------------|---------------|--|--|
| litre      | l             |  |  |
| millilitre | ml            |  |  |
| microlitre | $\mu\text{l}$ |  |  |

*Units of length*

|            |    |            |               |
|------------|----|------------|---------------|
| metre      | m  | micrometre | $\mu\text{m}$ |
| centimetre | cm | nanometre  | nm            |
| millimetre | mm |            |               |

*Units of concentration*

|               |               |           |    |
|---------------|---------------|-----------|----|
| molar (mol/l) | M             | nanomolar | nM |
| millimolar    | mM            | picomolar | pM |
| micromolar    | $\mu\text{M}$ |           |    |

*Units of heat, energy, electricity*

|                             |                    |         |          |
|-----------------------------|--------------------|---------|----------|
| joule                       | J                  | volt    | V        |
| degree Celsius (centigrade) | $^{\circ}\text{C}$ | ohm     | $\Omega$ |
| coulomb                     | C                  | siemens | S        |
| ampere                      | A                  |         |          |

### *Units of radiation*

|                   |     |                            |     |
|-------------------|-----|----------------------------|-----|
| curie             | Ci  | disintegrations per minute | dpm |
| counts per minute | cpm | becquerel                  | Bq  |

### *Miscellaneous*

|  |                                     |
|--|-------------------------------------|
| gravity                                    | $g$                                 |
| dissociation constant                      | $K_d$                               |
| median doses                               | LD <sub>50</sub> , ED <sub>50</sub> |
| probability                                | $P$                                 |
| routes of drug administration              | i.v., i.p., s.c., i.m.              |
| square centimetre                          | cm <sup>2</sup>                     |
| standard deviation                         | S.D.                                |
| standard error of the mean                 | S.E.M.                              |
| Svedberg unit of sedimentation coefficient | S                                   |
| Hill coefficient                           | $n_H$                               |

The isotope mass number should appear before the atomic symbol, e.g., [<sup>3</sup>H]noradrenaline, [<sup>14</sup>C]choline. Ions should be written: Fe<sup>3+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>. The term absorbance (A) is preferred to extinction or optical density. For abbreviations not included in this list consult: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Authors and Editors*, 1994 (The Royal Society of Medicine, London), ISBN 0-905958-78-0, or *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 6th edn. (Cambridge University Press, Cambridge), ISBN 0-521-47154-0.

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