Formulation and topical delivery of liposomes and proliposomes containing clofazimine

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It all starts here ™

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This dissertation is presented in the format consisting of four chapters, of which one is in an article format, and appendixes that contains the results and discussions of the experiments. The article for publication has its own authors guidelines for publishing in Appendix F.

"You are never too old to set another goal or to dream a new dream." - **C.S Lewis**

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ABSTRACT

The continual market increase in the transdermal and topical delivery of drugs makes cutaneous drug delivery exploration all the more attractive for scientists (Larrañeta *et al*., 2016:62). The key in the delivery of drugs to the skin is bypassing its natural barrier, i.e. the stratum corneum, which functions as a protective skin layer against exogenous substances and poses as the fundamental obstacle for formulators. Although the stratum corneum constitutes a disadvantage for drug delivery, the skin provides numerous advantageous above the more common administration routes. The large surface of the skin is an appealing advantage by creating a much more accessible point for drug delivery with less patient compliance difficulty (Andrews *et al.,* 2013:1099; Menon, 2002:S4). Although the skin has the barrier function to protect itself, it is still subject to diseases that could potentially damage the skin as seen in the variety of lesions developed form cutaneous tuberculosis (CTB). Tuberculosis (TB) is a bacterial disease of the lungs that originates form the *M. tuberculosis* bacterial organism. Only a small number (1 to 2%) of TB patients develop CTB exogenously or endogenously, the former being the most prevalent (Frankel *et al*., 2009:20-21, Sosnik *et al*., 2010:548). An intimidating challenge has emerged for scientists, as the TB bacterium has begun to generate resistance against the first-line anti-TB drugs (isoniazid and rifampicin), fostering a multidrug resistant-TB (MDR-TB) propagation (Dooley *et al.,* 2013:1352). Recently the use of second-line drugs has been investigated more extensively to possibly alleviate the use of first-line drugs experiencing resistance.

One of these second-line drugs has the characteristic properties to assist the first-line drugs against MDR-TB and forms part of the antibiotic riminophenazine family, namely clofazimine (CLF). Although its mechanism of action is unknown, it has been proven, by Yano *et al*., (2013:10276) that CLF has great potential against resistance from TB isolates, but illustrated issues regarding poor solubility. Naik *et al*. (2000:319) state for a drug to have optimum topical penetration, an aqueous solubility of < 1 mg/ml is required and since CLF has a solubility of 0.000225 mg/ml (Pubchem, 2015), it is presumed to be a highly unlikely candidate for topical delivery.

Extensive studies have been performed in the field of particulate drug carrying systems (Prashar *et al*., 2013:130). These systems, such as liposomes, are capable of delivering drugs and improving the absorption and bioavailability of drugs (Drulis-Kawa & Dorotkiewicz-Jach,

2010:197; Prashar *et al.*, 2013:130). Liposomes also possess an amphiphilic character making them ideal to encapsulate both hydrophobic and hydrophilic drugs (Madni, 2014:401). Hence, encapsulating CLF into the hydrophobic bilayer of liposomes may possibly enhance and improve the solubility of the drug and increase the chances of topical delivery. Liposomes have the added disadvantage of being prone to oxidative and hydrolytic degradation causing stability issues (Çağdaş *et al*. 2014:10), therefore employing proliposomes would safeguard the drug, due to stress caused by liposome instability, without changing the intrinsic character of vesicle (Xu *et al*., 2009:61).

The principal aim of this study was to determine if the two vesicle systems, namely liposomes and proliposomes, would improve the topical diffusion of CLF by improving its solubility. Thus, CLF was encapsulated into liposomes ((**CL2**)) and proliposomes ((**CPL2**)) to evaluate the possible topical delivery that occurred.

The vesicle systems were characterised according to their properties to verify an ideal dispersion for further transdermal/topical studies. A high performance liquid chromatography (HPLC) method for CLF was developed and validated for sample analysis throughout experiments. The (**CL2**) and (**CPL2**) dispersions both showed a successful release of CLF and yielded a similar level of release from both systems. The similar release is atoned to the vesicle systems being equivalent in nature due the same ingredients used during preparation.

The skin diffusion studies of the (**CL2**) and (**CPL2**) dispersions showed no presence of the API in the Franz cells receptor phase, which in turn illustrates no systemic absorption. CLF was detected at low levels in the stratum corneum-epidermis and the epidermis-dermis from both vesicle systems, indicating a penetration and permeation of the API into the former and latter layers respectively, therefore supporting topical delivery of the API. It was expected that the lipophilic API would accumulate in the lipophilic stratum corneum. The presence of the lipophilic API in the hydrophilic dermis may be constituted to the use of the vesicle systems, which can theoretically improve the solubility, hence contributing to the permeation into the targeted layer.

The *in vitro* cytotoxicity study on the toxic effect of the free drug (CLF) and vesicle dispersions ((**PL2**) and (**CL2**)) on immortalised human keratinocyte (HaCaT) cells illustrated that the vesicle system had a significant effect on the level of cytotoxicity. The result of the dispersions containing the vesicle system showed similar levels of cytotoxicity compared to the control (non-cytotoxic) samples regardless of their concentration, while the free drug exhibited a proportional increase from weak to strong cytotoxicity as the concentration of the free drug exposed to the cells increased. This highlighted the fact that liposomes provided a protective effect on the

toxicity of the API and correlated to what literature suggested. These results only show the toxicity of CLF on a cell-to-cell basis and do not include the biotransformation and other affecting factors included in the skin's physiology, therefore these results are not relatable to *in vivo* studies and are only deemed as a precursor study for future investigation.

Keywords: Cutaneous Tuberculosis, Topical delivery, Skin diffusion, Clofazimine, Stratum corneum, Epidermis, Dermis, Liposomes, Proliposomes

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UITREKSEL

Die voortdurende toename in die gebruik van transdermale en topikale aflewering van aktiewe farmaseutiese bestanddele maak topikale geneesmiddelafleweringnavorsing al hoe meer aantreklik vir wetenskaplikes (Larrañeta *et al*., 2016:62). Die sleutel tot die aflewering van aktiewe bestanddele in die vel is om die vel se natuurlike versperringslaag; naamlik die stratum korneum te oorkom. Hierdie versperringslaag funksioneer as 'n beskermende vellaag teen eksogene stowwe en dien dus ook as ŉ fundamentele hindernis vir formuleerders. Alhoewel die stratum korneum gesien word as 'n belemmering teen geneesmiddelaflewering, bied die vel talle voordele bo die meer algemeen toedieningsroetes. Die groot oppervlak van die vel skep 'n veel meer toeganklike punt vir geneesmiddelaflewering met aansienlik minder pasiënt-meewerkendheidsprobleme (Andrews *et al.*, 2013:1099; Menon, 2002:S4). Aangesien die vel ŉ versperringsfunksie het om die liggaam te beskerm, is dit steeds onderhewig aan siektes wat potensiëel die vel kan beskadig, soos gesien in die verskeidenheid van letsels wat ontwikkel vanuit ŉ kutane tuberkulose (KTB) infeksie. Tuberkulose (TB) is 'n bakteriële siekte van die longe wat afkomstig is van die *M. tuberculosis* bakterie. Slegs 'n klein aantal (1 tot 2%) van TB-pasiënte ontwikkel KTB en dit kan ontstaan van ŉ bakteriële infeksie van buite- of van binne die liggaam; van waar die eersgenoemde as die mees algemene voorkom (Frankel *et al*., 2009:20-21; Sosnik *et al*., 2010:548). Een van die mees intimiderende uitdagings wat na vore gekom het vir wetenskaplikes is dat die TB-bakterie begin om weerstand te bied teen die eerste-lyn anti-TB middels (isoniasied en rifampisien) wat vervolgens lei tot die bevordering van 'n multi-bestande-TB (MDR-TB) generasie (Doodey *et al*., 2013:1352). Onlangs is die gebruik van tweede-lyn aktiewe farmaseutiese bestanddele meer omvattend ondersoek om moontlik die weerstand teen van die eerste-linie middels te verminder.

Een van hierdie tweede-lyn aktiewes het kenmerkende eienskappe wat kan bydrae tot die eerste-linie middels se bevegting teen MDR-TB en is afkomstig van die antibiotika riminofenasien familie naamlik, klofasimien (KLF). Hoewel die meganisme van aksie onbekend is, is dit deur Yano *et al*. (2013:10276) bewys dat KLF groot potensiaal toon teen die weerstand van TB isolate, maar dit het ook sekere probleme; veral swak oplosbaarheid. Naik *et al*. (2000:319) stel voor dat 'n aktiewe farmaseutiese bestanddeel 'n wateroplosbaarheid van 1 mg/ml nodig het om optimale topikale penetrasie te hê. Aangesien KLF 'n oplosbaarheid van 0.000225 mg/ml (Pubchem 2015) het, kan daar gespekuleer word dat hierdie geneesmiddel 'n hoogs onwaarskynlike kandidaat vir topikale aflewering kan wees.

Verdere studies was ook uitgevoer op die gebied van die afleweringstelsels vir aktiewe farmaseutiese bestanddele wat nie ideale karaktereienskappe besit nie (Prashar *et al*., 2013:130). Liposome is in staat om aktiewe farmaseutiese bestanddele af te lewer en die biobeskikbaarheid van geneesmiddels te verbeter (Drulis-Kawa & Dorotkiewicz-Jach, 2010:197; Prashar *et al*., 2013:130). Liposome besit 'n amfifiliese karakter wat dit ideaal maak om beide hidrofobiese en hidrofiliese geneesmiddels te enkapsuleer (Madni, 2014:401). KLF sal dus in die hidrofobiese dubbellaag van die liposome opgevang kan word en in die proses moontlik die oplosbaarheid van die geneesmiddel verbeter en ’n groter kans bied vir topikale aflewering. Liposome het ook ’n bykomende nadeel dat hulle geneig is om oksidatiewe en hidrolitiese degradasie te ondergaan wat stabiliteitsprobleme kan veroorsaak (Çağdaş *et al*., 2014:10). Die gebruik van proliposome kan dus moontlik die aktiewe farmaseutiese bestanddeel beskerm sonder om die integriteit van die liposome te beïnvloed (Xu *et al*., 2009:61).

Die doel van hierdie studie was om te bepaal of die twee vesikelsisteme naamlik, liposome en proliposome, die topikale aflewering van KLF sal verbeter deur onder andere die swak oplosbaarheid te oorkom. KLF is dus in liposome ((**CL2**)) en proliposome ((**CPL2**)) geënkapsuleer om die moontlike topikale aflewering te evalueer.

Die vesikelsisteme is albei gekarakteriseer volgens geselekteerde fisiese eienskappe om sodoende 'n ideale dispersie te kon identifiseer vir verdere transdermale/topikale studies. 'n Hoë druk vloeistofchromatografie (HDVC) metode vir KLF was ontwikkel en gevalideer vir die analise van monsters gedurende die eksperimente. Die (**CL2**) en (**CPL2**) dispersies het suksesvolle vrystelling van KLF getoon en lewer soortgelyke vrystelling van die geneesmiddel vanuit beide stelsels. Die amper identiese vrystelling van die geneesmiddel vanuit die twee vesikelsisteme kan toegeskryf word aan die soortgelyke fisiese eienskappe van die vesikels; wat dien ooreenkomstig toegeskryf kan word aan hul ooreenstemmende bestanddele wat gebruik was tydens die voorbereiding daarvan.

Tydens die veldiffusiestudies van die (**CL2**) en (**CPL2**) dispersies was geen aktiewe bestanddeel in die Franz selle se reseptor fase teenwoordig nie; wat dus illustreer dat geen sistemiese absorpsie plaasgevind het nie. Lae konsentrasies van KLF was opgespoor in die stratum korneum-epidermis en die epidermis-dermis van beide vesikelstelsels, wat daarop dui dat daar wel penetrasie van die aktief deur die stratum korneum in die voormalige en laasgenoemde lae onderskeidelik gevind is en topikale aflewering het dus plaasgevind. Daar was verwag dat die lipofiele aktiewe farmaseutiese bestanddele sal ophoop in die lipofiele stratum korneum. Die teenwoordigheid van die lipofiele KLF in die hidrofiliese dermis kan

moontlik toegeskryf word aan die gebruik van vesikelsisteme, wat teoreties die oplosbaarheid kan verbeter en dus bydra tot die diffusie van KLF tot in die teikenlaag.

Die *in vitro* studies van die aktief (KLF), (**PL2**) en (**CL2**) dispersies toon die vlak van sitotoksisiteit op die HaCaT selle. Die resultate illustreer dat die vesikelstelsel wel 'n invloed op die vlak van sitotoksisiteit het. Die resultate van die dispersies toon dat die vesikelsisteem soortgelyke vlakke van sitotoksisiteit het wanneer dit vergelyk word met die kontrole monsters, ongeag die konsentrasie, terwyl die aktief (KLF) 'n proporsionele toename van swak na sterk sitotoksisiteit toon soos die konsentrasie van die aktiewe bestanddeel verhoog. Hierdie resultate toon slegs die toksisiteit van KLF op 'n sel tot sel basis en sluit nie die biotransformasie en ander faktore van velfisiologie in nie. Dus is hierdie resultate nie vergelykbaar met *in vivo* studies nie en word net as 'n voorloperstudie gebruik vir toekomstige navorsing.

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$$J = - D \frac{\partial C}{\partial x}$$ **Equation 2.1**

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial^2 x}$$ **Equation 2.2**

CHAPTER 3

Log D = concentration in *n*-octanol / concentration in PBS **Equation 1**

APPENDIX B

$$EE\% = \frac{\text{Drug (total) - Drug (supernatant)}}{\text{Drug (total)}} \times 100$$ **Equation B.1**

APPENDIX D

$$\text{Log D} = \frac{\text{concentration in n-octanol}}{\text{concentration in PBS}}$$ **Equation D.1**

APPENDIX E

$$\frac{\text{Live cells in squares (n = 10)}}{10} \times (5 \times 10^4) \times \text{Total volume of cell suspension}$$ **Equation E.1**

$$\%\text{viable cells} = \frac{\text{Unstained cells (n)}}{\text{Total number of cells}} \times 100$$ **Equation E.2**

$$\%\text{Cytotoxicity} = \frac{\text{Experimental LDH release (OD490)}}{\text{Maximum LDH release (OD490)}} \times 100$$ **Equation E.3**

LIST OF ABBREVIATIONS

API	Active Pharmaceutical Ingredient
CL1	Liposomes containing 1% clofazimine
CL2	Liposomes containing 2% clofazimine
CL3	Liposomes containing 3% clofazimine
CLF	Clofazimine
cP	Average Viscosity
CPL1	Proliposomes containing 1% clofazimine
CPL2	Proliposomes containing 2% clofazimine
CPL3	Proliposomes containing 3% clofazimine
CTB	Cutaneous Tuberculosis
DNA	Deoxyribonucleic Acid
ED.D	Epidermis-Dermis
EE%	Percentage Drug Entrapment Efficiency
GIT	Gastrointestinal Tract
HaCaT	Immortal Human Keratinocyte Cell Line
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatographic
LDH	Lactate Dehydrogenase-Release
LLOQ	Lower Limit of Quantification

LOD	Limit of Detection
Log D	Distribution Coefficient
Log P	Partition Coefficient
LUV	Large Unilamellar Vesicles
MDR-TB	Multidrug-Resistance Tuberculosis
MIC	Minimum Inhibitory Concentration
MLV	Multilamellar Vesicles
NADH	Nicotinamide Adenine Dinucleotide
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PdI	Polydispersity Index
PL1	1% placebo liposomes
PL2	2% placebo liposomes
PL3	3% placebo liposomes
PPL1	1% placebo proliposomes
PPL2	2% placebo proliposomes
PPL3	3% placebo proliposomes
PVDF	Polyvinylidene Difluoride
ROS	Reactive Oxygen Species
RPM	Revolution per Minute
%RSD	Percentage Relative Standard Deviation
SC.E	Stratum Corneum-Epidermis

SD	Standard Deviation
SUV	Small Unilamellar Vesicles
TB	Tuberculosis
TEM	Transmission Electron Microscope
WHO	World Health Organization

CHAPTER ONE

Introduction, aims and objectives

1 Introduction

The skin is classified as a large (2 m^2) complex and integrated diffusion mechanism organ. It consists of a multitude of cells that communicate in a highly efficient manner to control what passes into the body and what is released. The stratum corneum forms part of the basal epidermal layer and constitutes the skin's physical barrier properties, which prevents/resists the entry of toxic substances into the skin layers. This layer then challenges the topical/transdermal delivery of drugs into or through the skin or its layers (Mathes *et al*., 2014:82; Zhang *et al*., 2009:227). The success with which an active pharmaceutical ingredient (API) deposits into the skin is mainly dependent upon a narrow range of specific physiochemical properties, i.e. lipophilicity (log P (octanol-water partition coefficient) between 1 and 4, molecular weight (lower than 500 g/mol) and solubility (> 1 mg/ml) of the API (Chandrashekar & Rani, 2008:94, Naik *et al*., 2000:319).

Clofazimine (CLF) is a highly lipophilic compound, which forms part of an antibiotic subgroup, riminophenazine. This API is used as an antimycobacterial agent and gained the interest of being used as a treatment against the re-emerging multidrug-resistance tuberculosis (MDR-TB). In a small number of TB infected patients, a variety of cutaneous lesions arise, which is referred to as cutaneous tuberculosis (CTB) development. These cutaneous lesions are a secondary effect to that of the primary TB infection and continue to evolve into several different types of skin lesions. The side-effect, associated with the lipophilic properties of CLF, include discolouration of the skin and gastrointestinal disease (Dos Santos *et al*., 2014:219, Mane *et al*., 2012:741, Zhang *et al*., 2012:8410). The highly lipophilic property (log P of 7.66) and the molecular weight of CLF (473.396 g/mol) are indicative of this drug having the potential for accumulating in the stratum corneum, hence making it difficult to permeate to the rest of the skin layers (Zhang *et al*., 2014:4384). However, the main obstacle regarding CLF is its extremely low aqueous solubility of 0.000225 mg/ml, exhibiting a hydrophobic characteristic (PubChem, 2015). Due to this undesired physiochemical property of CLF, it is not seen as an ideal compound for topical delivery, hence giving purpose to investigate. The solubility of CLF may be enhanced by using a vehicle drug carrying system, such as vesicles. According to Çağdaş *et al*. (2014:2) and Wen *et al*. (2006:1187), vesicle systems have the ability to greatly

improve the therapeutic index and bioavailability of drug absorption. The possible improvement of CLF's solubility may magnify the drug capacity for topical delivery of the API.

The research problem for this study entails the investigation into two liposomal vesicle systems, to determine if these systems enhance the topical delivery of the API. The two vesicle systems are the liposomal and the more stable proliposomal vesicle forms containing the API. The proliposomal vesicle system was also investigated due to the stability problems associated with liposomes. Thus, this study involved the formulation of an API into a vesicle and its pro-vesicle form and evaluating the subsequent topical application of these dispersions.

The aim of this study was to evaluate the effectiveness of formulated CLF in liposome and proliposome vesicles, by determining whether such dispersions would enable this API to cross the stratum corneum and penetrate into the skin layers. The permeation results would help determine whether CLF in liposomes and proliposomes would offer a viable route for the topical delivery of this drug and, if it can be applied for the treatment of CTB. Thus, the successful delivery of CLF into the skin layers for the treatment of CTB lesions may only assist in the prescribed oral treatment regimen of drugs for MDR-TB.

The objectives of this research study were:

- The validation and development of a high performance liquid chromatographic (HPLC) method for the determination of the concentration of clofazimine in the liposome and proliposome vesicles.
- The determination of aqueous solubility and octanol-buffer distribution coefficient (log D) of clofazimine.
- The formulation of two vesicular systems (liposomes and proliposomes) containing clofazimine.
- The characterisation (morphology, droplet/particle size, zeta-potential, pH, viscosity and drug entrapment efficiency (EE%)) of the vesicular systems with and without clofazimine.
- The determination of the release of clofazimine from the liposomes and proliposomes through membrane release studies.
- The determination of the transdermal and topical delivery of clofazimine from the liposomes and proliposomes by performing a diffusion study followed by tape stripping, respectively.

- The *in vitro* examination of the cytotoxic effects (cell death) clofazimine has on an immortal human keratinocyte cell line (HaCaT) cells through lactate dehydrogenase (LDH)-release and cell culture studies.

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CHAPTER TWO

Topical penetration of clofazimine using liposomes and proliposomes

2.1 Introduction

The human skin is a viable target regarding the cutaneous delivery of drugs. This is indicated by the worldwide billion dollar industries of transdermal delivery, accounting for an amount of $21.5 billion in 2003 and reaching $32.0 billion by the end of 2015 (Reddy *et al.,* 2014:1094, Larrañeta *et al*., 2016:62). The skin is an important part of the human body, as it plays the role of being a defensive barrier, i.e. in separating and protecting the internal from the external surroundings. The substantial surface area of the skin makes it an appealing route for topical drug delivery, but the barrier function however is the main obstacle for formulation scientists. Even though this barrier can limit the transport of drugs across the skin, it also provides an added advantage by using the large surface of the skin as an easy accessibility point for drug administration (Andrews *et al.,* 2013:1099; Menon, 2002:S4). The skin's barrier function has to be overcome in order to optimise the effective delivery of topical drugs into the skin (Pouillot *et al.,* 2008:143).

It has currently surfaced among scientists (WHO, 2015) that the *Mycobacterium tuberculosis,* known as TB, has begun to develop resistance against the anti-TB first line drugs (isoniazid and rifampicin) used during treatment. The second line anti-TB drug, CLF, emerged into the spotlight when it showed potential against MDR-TB. *M. tuberculosis* isolates have illustrated inefficiency to generate resistance against CLF (Yano *et al.,* 2013:10276). In a small population of TB infected individuals, they start to develop cutaneous lesions, i.e. CTB. These CTB lesions are secondary to the main infection with TB and lead to the development of a variety of different types of skin lesions (Frankel *et al*., 2009:19-21, Sosnik *et al.*, 2010:548)

Due to the limited and inconclusive information regarding CLFs' mechanism of action in the human body, investigating this API for topical drug delivery is essential. This investigation could help in uncovering unknown information considering its possibility to be delivered into the skin and treatment of CTB. The main obstacle in using this highly lipophilic API (CLF) topically is its undesired physicochemical properties, making it nearly impossible to cross the stratum corneum. A possible solution to overcome these limitations is the utilisation of a vehicle system, i.e. liposomes. Liposomes can possibly transport CLF past the stratum corneum barrier and into the layers of the skin to enhance skin penetration. The problem with using liposomes is their instability and it is anticipated that the formulation of CLF into proliposomes would possibly

eliminate the instability of this vehicle. Consequently, the topical delivery of CLF was evaluated during this study to determine whether this drug could be applied topically by using liposome and proliposome vesicles to cross the stratum corneum.

2.2 Clofazimine

In 1954, the API, CLF, was synthesised for the first time from lichen-derived compounds by Dr Vincent Barry and his colleagues. It was proposed that this new wonder could be used as an anti-TB agent. Initially, this newly derived compound was thought to be futile against the mycobacterial disease TB due to the clinical development being withdrawn and the shortage of evidence being a drawback. However, in 1955 Chang identified its effective treatment for two other mycobacterial species which are subject to leprosy, i.e. *M. leprae and M. lepromatosis.* Since the discovery of CLF, the World Health Organization (WHO) has endorsed it as a triple drug regime for the treatment of leprosy (Brennan & Young, 2008:96; Cholo *et al*., 2012:290; Gopal *et al*.; 2013:1001; Wong *et al*., 2013:499).

CLF is also affiliated with the riminophenazine antibiotic family and elicits a variety of responses due to its structural integrity. The characteristic properties of riminophenazines are their alkylimino group (position 2) and phenyl substituents (positions 3 and 10) on the phenazine nucleus. Figure 2.1 provides an illustration of the chemical structure of CLF. CLF has an isopropylimino group on its phenazine nucleus which is important for the antimicrobial action of this riminophenazine family (Cholo *et al*., 2012:291).

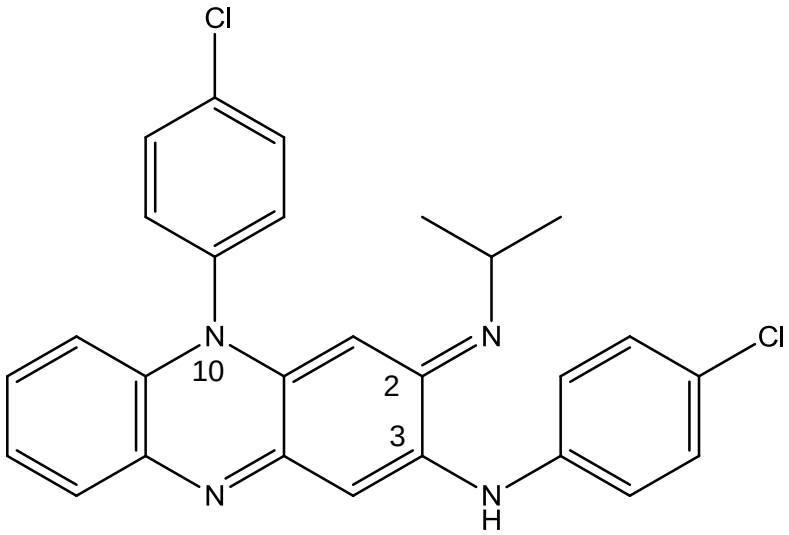

Figure 2.1: A molecular representation of the riminophenazine; CLF

Physicochemically, CLF has a very distinct and visible colour associated with it, as it comes in a spectrum of red colours depending on the pH levels it is exposed to. This brings to light the most important characteristic of CLF, namely its high levels of hydrophobicity as it is distinctively identified by the intense violet colour change when associated with an acidic pH (Reddy *et al.,* 1999:616). Table 2.1 provides general information regarding the physical and chemical properties of CLF (PubChem, 2015).

Table 2.1: Physicochemical and general properties of CLF

Physicochemical and general properties of CLF	
Chemical names	CLF; B-663
IUPAC name	N,5-bis(4-chlorophenyl)-3,5-dihydro-3-[(1-methylethyl)imino]-2-phenazinamine
Molecular weight	473.4 g/mol
Empirical formula	$C_{27}H_{22}Cl_2N_4$
Melting point	210 - 212 °C
Solubility	0.000225 mg/ml
pKa	8.51
Log P (Octanol-water partition coefficient)	7.66

The absorption and distribution properties of CLF are mainly seen in the skin (subcutaneous fat layer), but have also been found throughout the body, i.e. the liver, kidneys, and gastrointestinal tract (GIT). This accumulation of crystallised CLF in different parts of the human body is correlated to the physicochemical properties it possesses. CLF is a highly lipophilic, fat soluble compound (Kar & Gupta, 2015:560; Wong *et al*., 2013:499), with a lengthy half-life of approximately 70 days (Gopal *et al*.; 2013:1004; Wong *et al*., 2013:499). Correlating to the extreme accumulation of CLF, it is evident from Feng *et al*. (1989) that even though CLF can be metabolised, the quantity of metabolic products (metabolites I, II, and III) formed are of minimal value. The metabolic products account for only 1% of the total recovery demonstrating the low metabolite concentration during elimination from the body. It is also not proven, or not yet studied sufficiently, to determine if these metabolites promote the antimycobacterial function of CLF (Kapoor, 2013:820).

2.3 Mechanism of action

The mechanism of action for CLF is unpredictable and not yet clearly determined according to a variety of studies. These studies concluded there are two proposed mechanisms associated with CLF. Firstly, the originally proposed mechanism, which is the antimycobacterial action of

CLF and secondly, CLFs' anti-inflammatory response and immunosuppressive effects (Hwang *et al.,* 2014:2; Kar & Gupta, 2015:56; Reddy *et al.,* 1999:617).

2.3.1 Antimycobacterial effects

Reddy *et al.* (1999) proposed that the hydrogen peroxide found in the intercellular domain supported the antimycobacterial action of CLF. However, Kar and Gupta (2015:560) proposed that CLF prevents the deoxyribonucleic acid (DNA) template function of mycobacterial DNA; this was done by binding to the large guanine regions on the DNA and enhancing the phagocytic competence of macrophages. Through this enhancement, the lysosomal enzyme production was stimulated and in effect inhibited the proliferation of mycobacteria (Gopal *et al.*; 2013:1004, Morrison & Marley, 1976:475). Also according to Mane *et al.* (2012:741), the presence of CLF can activate the stimulation of bacterial phospholipase A2. In doing this, it promotes the release of lysophospholipids, which exhibit toxicity towards mycobacteria and suppresses proliferation.

The most widely accepted mode of action for CLF is its ability to produce reactive oxygen species (ROS) to eliminate mycobacterial organisms. This action was recognised when CLF indicated a redox potential as it oxidised. This is followed by its reduction in concurrence with the production of oxygen from the respiratory chain of the mycobacteria, generating ROS and leading to the death of mycobacterial organisms (Yano *et al.,* 2010:10276).

2.3.2 Anti-inflammatory and immunosuppressive effects

Despite the antimycobacterial properties of CLF it also demonstrates an anti-inflammatory activity. The anti-inflammatory activity operates through the inhibition of lymphocyte and neutrophil action in the body (Yano *et al.,* 2010:10276). This action is associated with the response of macrophages to inflammation leading to the release of prostaglandin to counteract and exhibit immunosuppressive effects in the body (Reddy *et al.,* 1999:621).

2.4 Pharmacology of clofazimine

2.4.1 Tuberculosis

The causative agent in the development of TB is mainly routed in the *M. tuberculosis* bacterial organism. This disease spreads from person to person through the air by coughing, sneezing or spitting, thereby discharging the active TB parasite to individuals in the vicinity, where it is inhaled consequently infecting the respiratory system. Associated with the infection of the lungs, a TB patient usually exhibits symptoms which include, rapid weight loss, high fever, loss of appetite and chills. TB is particularly coherent with patients exhibiting weakened immune

systems, e.g. HIV (human immunodeficiency virus) infected persons (Saravan *et al.,* 2015:2). HIV is a human disease, which undermines the body's immune system. This makes HIV infected patients susceptible to secondary infections, i.e. TB, by deteriorating the defensive effectiveness, which might end in death (Ingraham & Ingraham, 2004:676). The macrophages in the alveolar and epithelial cells of the HIV infected patients attempt to destroy the mycobacterium through phagocytosis, but fall short due to the TB bacteria's unique defence mechanism (Saravan *et al.,* 2015:2).

TB is one of the most deadly diseases worldwide, as its airborne transmission and contagious features make it an easily procurable disease (NIH, 2012). According to Sosnik *et al.* (2010:548), nearly 2 billion people have been infected with the *M. tuberculosis* bacterium, indicating that approximately 30% of the population globally is possibly infected. In approximately 1 % to 2 % of TB cases, dermatological manifestations of TB, i.e. skin lesions, occur indicating a cutaneous development of TB, i.e. CTB. The cutaneous lesion development of TB can be acquired either exogenously or endogenously, where the former is less frequently encountered. The exogenous inoculation of CTB develops directly from the inoculation of the *M. tuberculosis* bacterium into the skin of a susceptible individual through damage or trauma to the skin. Endogenous infections generally transpire from individuals already affected by the bacterium by either contiguous extension, and lymphatic- or hematogenous metastasis leading to the development of a variety of different skin lesions (Dos Santos *et al*., 2014:219, Frankel *et al*., 2009:19-21).

The challenge regarding TB is the recurring resistance of the *M. tuberculosis* bacteria against the first-line anti-TB drugs, i.e. isoniazid and rifampicin. This resistance led to the propagation of a MDR-TB generation (Dooley *et al.,* 2013:1352). According to the WHO (2015), the estimation of individuals infected by the resistance against the first-line anti-TB drugs was projected at 480 000 individuals worldwide in 2014 alone. An estimate of 190 000 people died as a result of MDR-TB (WHO, 2015). The large increase of MDR-TB is most commonly ascribed to the control programmes not being enforced appropriately, which will eventually lead to insufficient therapeutic practices and consequently lowering the effect of anti-TB medication (Lemos & Matos, 2013:239).

The suggested solution to this resistance is to use second-line drugs, i.e. CLF and other drugs, to assist the current treatment regimens of MDR-TB. However, the main obstacle is the inadequate potency and unwanted toxicity profiles of most second-line drugs (Dooley *et al.,* 2013:1352).

2.4.2 Clofazimine as treatment for MDR-TB

The second-line drug CLF was rejected as a treatment option for TB in its initial discovery. This was ascribed to the evidence not being sufficient and the *in vivo* experiments being inconclusive. The emergence of MDR-TB gave new perspective regarding CLFs' rejuvenation as most mycobacteria, including *M. tuberculosis* isolates, showed inefficiency to generate resistance against CLF (Yano *et al.,* 2013:10276). Evidence suggests that CLF, in both animal and *in vitro* studies, retained accepted activity against MDR-TB strains. This indicates that CLF has an equivalent potential to that of the first-line drugs, such as isoniazid and rifampicin, in animal models (Xu *et al.,* 2012:1104).

The current dosage of CLF used for the treatment of MDR-TB is 50 - 100 mg daily (Dooley *et al.,* 2013:1353). Xu *et al.* (2012:1109) made the statement, "CLF contributed to favourable treatment outcomes," indicating its effectiveness with no major side effects or toxicity restrictions. The minimum inhibitory concentration (MIC) fluctuates between 0.06 and 2.00 µg/mL for CLF concerning the *M. tuberculosis* bacteria (Dooley *et al.,* 2013:1353).

The topical delivery of CLF to the lesion areas generated from the CTB may aid during the general treatment of MDR-TB. This option is viable to investigate, in light of the evidence provided, to reduce the treatment time due to the direct application of the drug to the lesions on the skin caused by the CTB, i.e. topical delivery of CLF. Thus, this approach only aims to assist the oral regimen of drugs in the treatment of MDR-TB and not replace it.

2.4.3 Adverse effects of CLF treatment

The well-documented side effects associated with the higher dosages of CLF during treatment are, GIT irritation (40 - 50% of patients) and an orange to brown skin discoloration (75 - 80% of patients). There are reports stating that 8 to 28% of patients develop skin dehydration and ichthyosis, also, approximately 1 to 5% of patients develop rash and pruritus due to the treatment with CLF (Gopal *et al.,* 2013:1005; Mane *et al.,* 2012:742).

The most undesired side effect is the skin discoloration, as this develops only a few weeks after treatment. This discoloration is due to the pharmacokinetic properties of CLF accumulating in the fat tissues creating a brown-orange pigmentation (Zhang *et al.,* 2012:8410). This side effect can be reversed upon the termination of drug treatment, but will need months to years to completely dissipate (Wong *et al.,* 2013:499). Secondly, the GIT irritation is the more serious side effect of the two, as the late onset of this adverse effect can be potentially fatal. The GIT irritation provokes nausea, abdominal pain and may possibly lead to anorexia (Garrels,

1991:529). Evidently these adverse effects caused by using CLF in treatment may affect patient compliance, causing a limited use of this drug in the near future (Wong *et al.,* 2013:499).

2.5 Architecture and design of the human skin

The multilamellar structured skin is construed as an organic and integrate diffusion mechanism organ. This organ contains a multitude of cells that communicate in a highly efficient manner to ensure homeostasis. The skin is of great importance due to it being the main interface between the internal and external environment, thus it controls what passes into the body and what is released (Mathes *et al*., 2014:82; Zhang *et al*., 2009:227).

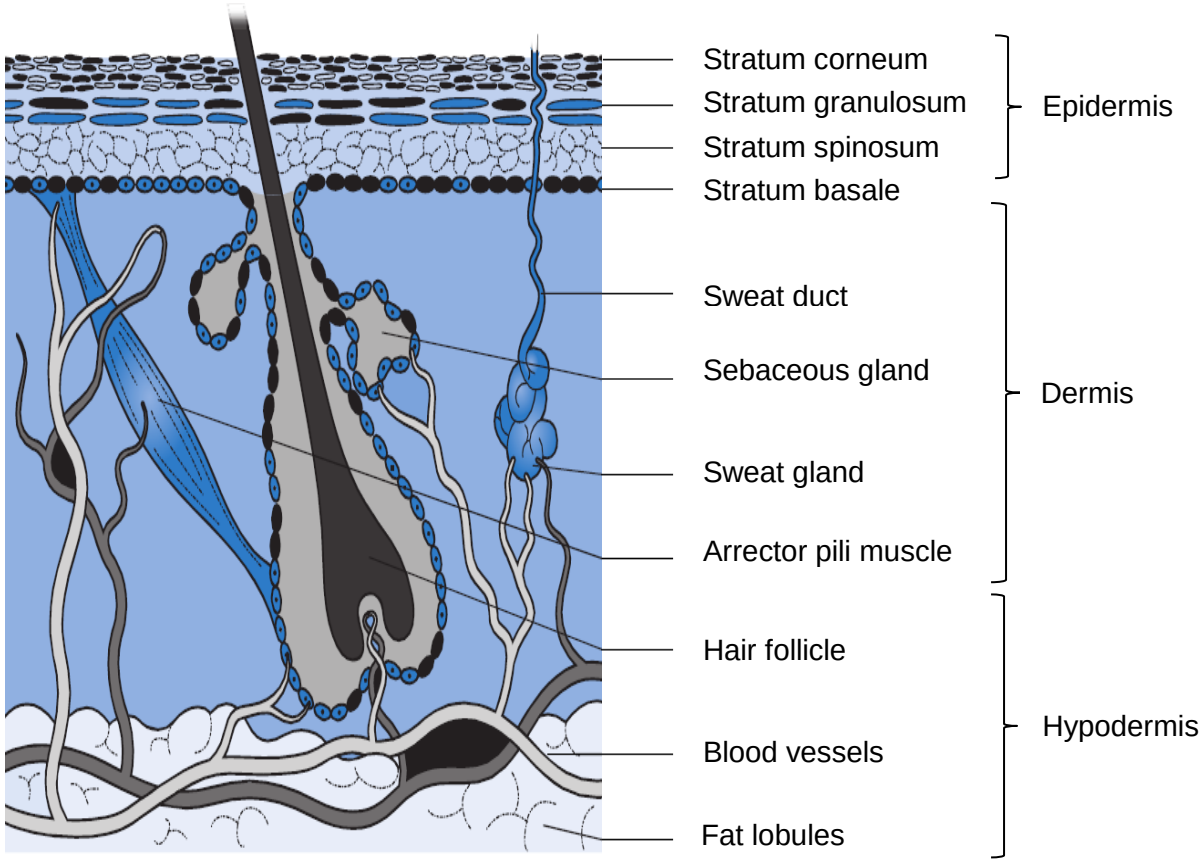

Figure 2.2: Schematic cross-section representation of the three distinct human skin layers: epidermis, dermis and hypodermis, adapted from Aulton (2013:677).

Physiologically the skin has a surface area of approximately $2m^2$ and accounts for a third of the human body's circulating blood (Sharma *et al*., 2013:287). The skin's large mass also constitutes approximately 15% of the total body mass (Kanitakas, 2002:390). The skin has a great variety of functions, i.e. protects against endogenous water loss, contributes to innate immunity, provides thermoregulation, gives sensory perception, synthesises vitamin D, provides

insulation and is an important energy reserve. The main function, however, is assigned to its natural barrier effect as it prevents/resists the entry of cytotoxic chemicals (chemical, pathogen, physical and ultra-violet (UV) radiation) into the skin layers, thereby illustrating the barriers' protective function (Mathes *et al*., 2014:82; McLafferty *et al*., 2012:39; Menon *et al.,* 2002:S4).

The three main identifiable layers of the human skin comprise of the epidermis, dermis and the hypodermis (sub-cutaneous layer), as illustrated in Figure 2.2. The stratum corneum forms part of the outermost epidermal layer and constitutes the skin's physical barrier. Due to the heterogeneous character of the skin, it can also accommodate a variety of appendages: hair follicles, sweat- and sebaceous glands (Mathes *et al*., 2014:82; Zhang *et al*., 2009:227).

2.5.1 Epidermis

The epidermis consists of cells that constantly differentiate to create a variety of layers namely, the viable epidermis and the stratum corneum. The viable epidermis is made up of three main embodying layers: the stratum basal (single layer), the stratum spinosum (5 - 15 layers) and the stratum granulosum (1 - 3 layers). Forming the topmost layer of the epidermis closest to the surface of the skin is the rate-limiting layer, the stratum corneum, which consists of 5 to 10 layers (McLafferty *et al.,* 2012:56).

2.5.1.1 Viable epidermis

This skin layer comprises of stratified epithelium cells, having the ability to continually restore and regenerate to create a 50 - 100 µm thick skin layer (Andrews *et al.,* 2013:1099). The differentiating cells of the viable epidermis are represented by the keratinocytes (Svensson, 2009:247). The keratinocyte cells constitute approximately 90% of the total cells in these layers; they are however not the only cells present as they are accompanied by non-keratinocyte cells namely, Merkel cells, melanocytes, and Langerhans cells, which represent the remaining 10% of cells in the epidermis (Kanitakas, 2002:390; McLafferty *et al.,* 2012:36).

The variety of layers formed by the keratinocyte cells are recognised by the cells undergoing differentiation or maturation stages through several phases to evidently produce flattened corneocyte cells (dead cells). The keratinocyte cells develop from the basal layer (stratum basal) of the skin. The further the cells migrate from this layer, the less nutritional components can be provided to each cell. This causes a deficit in nutrients available to the cells which will naturally be followed by cell death (Kanitakas, 2002:390). Eventually these dead cells represent the stratum corneum, which by this time is completely keratinised (McLafferty *et al.,* 2012:36-38; Menon, 2002:S5; Svenson, 2009:247).

2.5.1.2 Stratum corneum

This skin layer serves as the rate-limiting layer and functions as the main physical barrier of the skin. The stratum corneum contributes to 80% of the defensive action against percutaneous penetration. The stratum corneum is a 10 - 20 µm thick layer largely consisting of two components, i.e. protein-enriched cells and lipid-enriched intercellular domains. These structural components contribute to the model that was proposed by Elias *et al.* (1981) as the "brick and mortar model." The flat cells represent the 'bricks' and the surrounding lipid matrix is represents the 'mortar' (Pouillot *et al.,* 2008:144; Proksch *et al.,* 2008:1063).

The protein-enriched corneocytes are the main component in the formation of the stratum corneum layer. It is bound by corneodesmosomes and is structurally fixed into a lamellar lipid layer of embedded non-polar lipids in the intercellular matrix. In the terminal differentiation phase of keratinisation there are major structural changes occurring in the keratinocytes. Firstly, these cells are altered to form anucleated, flat corneocytes containing keratin filaments, which protect tissue and skin from chemicals, heat and micro-organisms. These cells are then enclosed by cornified envelope proteins and form a covalently bound lipid envelope. The corneocytes that are formed are confined in a hydrophobic matrix of extracellular non-polar lipids (McLafferty *et al.,* 2012:36-38; Proksch *et al.,* 2008:1063). The stratum corneum is the conclusion of a complete differentiation of keratinised cells that eventually forms corneocyte cells, i.e. dead cells, which create the structurally built defensive barrier of the outmost layer of skin.

2.5.2 Dermis

The dermis, with a thickness of 0.55 mm, is located between the basal layer of the epidermis and the subcutaneous layer. This layer is constructed out of interstitial and cellular constituents and mainly consists of two major layers, the upper papillary dermis and reticular dermis. These two layers provide loose and dense collagen fibres respectively, as well as blood and lymph vessels. This may be important in providing a possible percutaneous absorption route (Lai-Cheong & McGrath, 2009:224; Mathes *et al*., 2014:82). The foremost cells found in the dermis are the fibroblasts. They are responsible for producing the important interstitial and cellular components. The main function of the dermis is to provide a secondary resistance against mechanical stress to yield a flexible structure (elastin and collagen fibres); it also operates as a water depot organ (Lai-Cheong & McGrath, 2009:224; Svensson, 2009:248).

2.5.3 Hypodermis (subcutaneous fat layer)

This innermost layer forms the lowest part of the skin. It functions as an isolation buffer to the dermis and epidermis against heat and shock and has the ability to supply energy to the body in the form of fat (Svensson, 2009:248). The hypodermis is mainly composed of adipocytes (fat cells), indicating these cells aid in the production and storage of fat (Reddy *et al.,* 2014:1096; Svensson, 2009:248). The hypodermis is also below the vascular system of the dermis, suggesting that no percutaneous absorption would be able to occur in this layer (Reddy *et al.,* 2014:1096).

2.6 Topical penetration routes for drug absorption

Pouillot (2008:145) explained that the stratum corneum functions as a barrier and governs 80% of the barrier function that the skin provides. The stratum corneum's absorption is specific to liposoluble, non-ionised particles as they are able to penetrate with more ease than ionised and hydrophilic molecules (Pouillot *et al.,* 2008:145).

2.6.1 Percutaneous absorption routes

There are two routes regarding the penetration of drug compounds past the stratum corneum: the transappendageal and the transepidermal route (Bhowmick & Sengodan, 2013:638).

2.6.1.1 Transappendageal route

This route uses appendages, i.e. apocrine and eccrine sweat glands and hair follicles, as the functional units to transport compounds into the different skin layers (Bhowmick & Sengodan, 2013:638). These routes are also referred to as the "shunt" route as they bypass the stratum corneum penetration. According to Barry (2001:101), these appendages only cover approximately 0.1% of the entire skin surface, which indicates that this route would only contribute to the transport of drugs at therapeutic level in a minor way (Bhowmick & Sengodan, 2013:638).

2.6.1.2 Transepidermal route

The transepidermal route can be subdivided into two routes: the transcellular (intracellular) and the paracellular (intercellular) route (Bhowmick & Sengodan, 2013:638; El Maghraby *et al*., 2008:205).

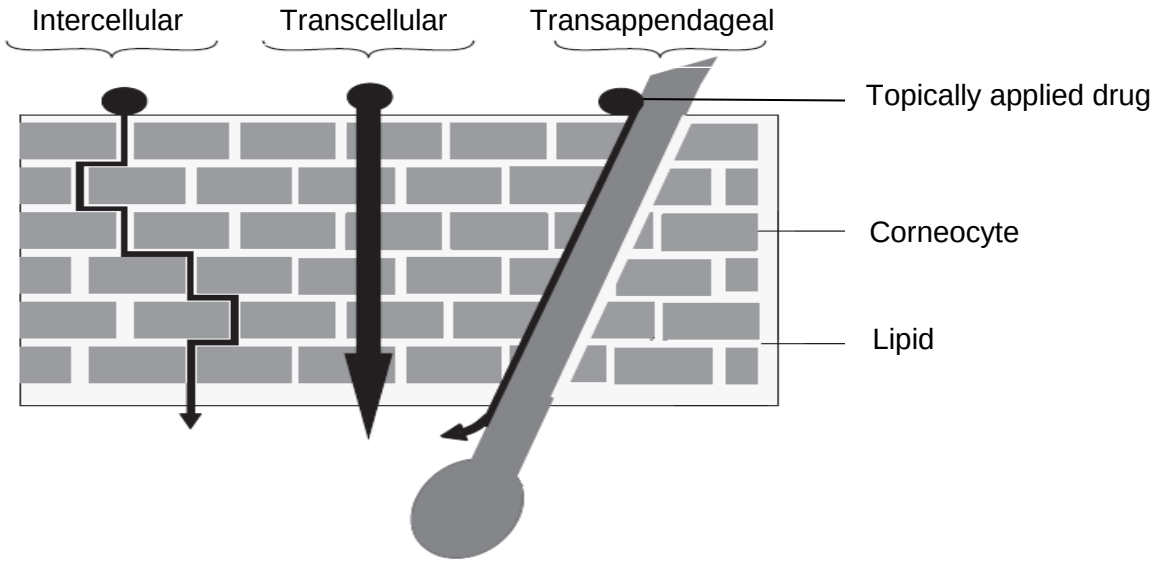

Figure 2.3: Illustration of the percutaneous absorption routes through the stratum corneum 'brick and mortar' model adapted from Chilcott (2008:11). The intercellular route is indicated by the zigzag pattern showing a longer pathway for topically applied drugs, while the transcellular and transappendageal route indicate a more direct pathway through the stratum corneum.

The transcellular route indicates that compounds will be moved across the epithelial cellular membrane, i.e. through the keratinocytes and across the intercellular lipids. Under normal provision, this pathway is not the preferred route, mainly due to the low permeability properties of corneocytes and the increased level of partitioning required between keratin bricks and across intercellular lipids (Bhowmick & Sengodan, 2013:638; El Maghraby *et al*., 2008:205).

The paracellular (intercellular) route is the main penetration route of drugs through the skin. This route uses the lipid rich extracellular space between corneocytes to move compounds from the skin to the designated layer. According to Bhowmick & Sengodan (2013:636), compounds of hydrophilic nature are preferably partitioned into the intercellular domain compared to lipophilic drugs which will use the intracellular route.

2.7 Model transport through the skin

Diffusion into the skin is of the utmost importance for topically applied drugs. This is defined by the movement of molecules from a region of high concentration to a region of low concentration. The transport of drug molecules past the stratum corneum is seen as a process of diffusion and can be explained by means of Fick's laws (Chilcott & Price, 2008:97).

The usage of Fick's laws to describe a model for diffusion through the stratum corneum can only be applied when a drug molecule acts in the 'ideal' fashion. Fick's first and second laws are mathematical models used to determine and explain the transport of drug molecules through the skin (Chilcott & Price, 2008:97).

2.7.1 Fick's first law

This principal aids in the understanding of permeation through the skin by embodying several considerations that can affect topical drug delivery (Aulton, 2013:680; Chilcott & Price, 2008:97; Dragicevic-Curic & Maibach, 2015:112). Fick's first law is a partially differential equation, which construes the diffusional flux relationship of a solute across the stratum corneum through passive diffusion (Naik *et al.,* 2000:320).

$$J = - D \frac{\partial C}{\partial x}$$ **Equation 2.1**

Where:

- J (mass/cm^2 per second) state of compound flux at a predetermined time;
- ∂C states that the flux of a compound is proportional to the differential concentration change;
- ∂x states that the flux of a compound is inversely proportional to the differential distance; and
- –D indicates diffusion coefficient of the drug in the stratum corneum.

The diffusion coefficient is combined with a negative sign to indicate that the net flux is in the direction of descending concentration (Aulton, 2013:681; Benson, 2005:24; WHO, 2006:23).

2.7.2 Fick's second law

Topical delivery is seen as unidirectional due to the assumption that the diffusion gradient is formed from the exterior surface of the skin into the tissue (Aulton, 2013:681). Fick's second law is derived from Fick's first law and is an indication of how much flux will be expected from a limited dose. This law also provides information regarding the rate of changes occurring in the concentration at any given time and space (expressed as 't' in Equation 2.2) (Aulton, 2013:681; Chilcott & Price, 2008:97).

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial^2 x}$$ **Equation 2.2**

Fick's second law is used as a model to express the concentration of a drug within a membrane, but is guided by specific assumptions. The assumptions include that the drug molecules does not bind to the skin, it is not metabolised and the diffusion coefficient remains constant throughout (WHO, 2006:24).

2.8 Physicochemical properties determining skin diffusion

The topical delivery of drugs into the skin is defined by Aulton (2013) as the application of a drug to the skin with the intention of conserving the drug within the skin layers and not to deposit it into the systemic circulation. The physicochemical properties of the API control whether or not a topically applied drug can pass into the skin layers. These physicochemical properties refer to the following factors: the diffusion coefficient, partition coefficient, molecular size and structure, drug concentration, pH, pKa and ionisation, hydrogen bonding, drug solubility and melting point. The ideal physicochemical properties of a drug used for topical and transdermal drug delivery is expressed by Naik *et al.* (2000:319) and Chandrashekar and Rani (2008:94), as seen in Table 2.2.

Table 2.2: Physicochemical considerations regarding topical drug delivery

Physicochemical properties	Ranges
Aqueous solubility	> 1 $mg.mL^{-1}$
Log P	1 - 4
Molecular weight	< 500 g/mol
Melting point	< 200 °C
pH of saturated aqueous solution	pH 5 - 9

2.8.1 Diffusion coefficient

The ease of diffusion with which a permeant crosses through tissue is determined by the diffusion coefficient, which gives light to the property of a permeant in the membrane. The measuring units for the diffusivity of a permeant are conveyed as a measurement of an area over time, i.e. cm^2/h or cm^2/s (Aulton, 2013).

2.8.2 Partition coefficient

Aulton (2013) describes the partition coefficient as the partitioning of molecules between two domains. For topical delivery, it would be between lipid and aqueous phases, which represents the stratum corneum lipids and water present in the skin, respectively. This is usually expressed as the *n*-octanol water partition coefficient (log P). Octanol is closely associated with

the lipid properties of cells and can thus be used as a model for the distribution of molecules in the *n*-octanol phase in comparison with the distribution within the water phase, completing the required two phase system. The ideal log P values for topical drug delivery is between 1 and 4 (Naik *et al.*, 2000:319).

It is evident that due to the lipophilic properties of the stratum corneum, it is deemed more suitable for lipophilic drugs. In accordance with this, CLF (log P of 7.66) would be received with more ease by the stratum corneum, but the high lipophilic properties of this API may negatively influence the topical delivery of CLF (Naik *et al.*, 2000:319). Naik *et al*. (2000:319) states a drug exhibiting a too high lipophilicity may only continue in the stratum corneum layers and not further. On the other hand the hydrophilic nature of the dermis layer would prove more unlikely to receive CLF and would provide even more limitations regarding the APIs topical delivery (Yamaguchi *et al*., 2007:4391).

2.8.3 Molecular size and structure

The size as well as the shape of drug molecules has a great influence on the absorption and transport of drugs into the stratum corneum. The prevention of foreign substances to enter the skin is the main function of the stratum corneum as it is exposed to the external environment on a constant basis. Due to this design, the molecular size of drug molecules will greatly influence the drug transport into the skin (Akhlaq, 2014:178; Aulton, 2013:680). According to Naik *et al.* (2000), it is necessary for drug molecules to be less than 500 g/mol as this is an ideal molecular weight to permeate into the skin layers. The diffusion of drug molecules and molecular size has an inverse relationship, as a decrease in diffusion is seen with an increase in molecular size (Hadgraft, 2004:292).

CLF is a large molecule (molecular weight of 473.4 g/mol) and may experience some difficulty crossing the stratum corneum, which indicates there might be a delay in the rate of diffusion of CLF across the stratum corneum into the underlying skin layers.

2.8.4 Drug concentration

Drug concentration is defined by Reddy *et al.* (2014: 1098) as a flux that is proportional to the concentration gradient across the stratum corneum. This gradient will increase if the concentration of the drug dissolved across the membrane is elevated. Thus, the flux is proportional to the concentration gradient of the drug (Reddy *et al.,* 2014:1098).

2.8.5 pH, pKa and ionisation

At an appropriate pH, the degree of ionisation concerning topical drugs is of considerable value. Compounds in a unionised form largely move through the stratum corneum's lipophilic intercellular domains, but at a more reduced rate. While ionised compounds may diffuse through these regions more rapidly. The skin is seen as cation-preselective, which is due to the negative charge the skin possesses at a physiological pH (Nair *et al.,* 2013:425). Thus, the unionised compounds have a higher permeability coefficient than that of the ionised compounds, due the latter compound possessing a lower log P value (Vitorino *et al*., 2015:2703).

2.8.6 Hydrogen bonding

The stratum corneum constituents, i.e. lipids and proteins, provide the stratum corneum layer with an abundance of hydrogen bonding groups. In the presence of a suitable penetrant, hydrogen-bonding groups, integral to those of the stratum corneum, will form reversible bonds as the drug molecules diffuse through this layer. It is evident that if these hydrogen bonds in the stratum corneum briefly bind to the drug molecules, it will negatively affect the diffusion of the drug molecules through the stratum corneum and retard the diffusion of drug molecules (Chilcott & Price 2008:88).

2.8.7 Drug solubility and solubility parameter

A drug molecule will not be able to cross the stratum corneum if it is highly hydrophilic. If the drug is too lipophilic (log P larger than 6) the drug will remain in the stratum corneum layers (Naik *et al.*, 2000:319). According to Akhlaq *et al*. (2014), the solubility of a drug compound is directly equivalent to its permeation through the skin. Thus, solubility below 1 mg/ml is deemed as a very low solubility and for an ideal aqueous solubility a value between 1 - 10 mg/ml is required (Akhlaq *et al*., 2014:178). This indicates that a beneficial solubility will increase the chance of good permeation into the skin (Hadgraft, 2004:292).

As stated previously, CLF is a highly lipophilic drug and may not be able to pass the stratum corneum layers, but rather accumulate. Since CLF has an exceptionally low solubility (0.000225 mg/ml) it is not an ideal drug for topical drug delivery and would prove remarkably difficult to cross the stratum corneum layer.

2.8.8 Melting point

The desired solubility of a drug in the intercellular lipid region of the stratum corneum is correlated to a low melting point (< 200 °C) (Vitorino *et al*., 2015:2703). This indicates there is a direct link between solubility and melting point, as a low melting point will indicate an increase in the solubility of the permeant and vice versa (Barry; 2001:102). CLF has a high melting point of between 210 to 212 °C. The high melting point of CLF may contribute to its low levels of solubility, which may increase the difficulty for CLF to cross the stratum corneum.

2.9 Particulate drug carrier system

Since 1970, liposomes have been extensively studied as a drug carrying system for the use of delivering drugs, with the aim to eventually treat diseases (Prashar *et al*., 2013:130). Currently, in the pharmaceutical industry the use of drug carrier systems, especially liposomes, has become of great and significant interest to improve the bioavailability of drug absorption (Drulis-Kawa & Dorotkiewicz-Jach, 2010:197; Prashar *et al.*, 2013:130). It is evident from research that the delivery of drug molecules through the utilisation of vesicles to the site of action has advanced immensely, since it is extensively used cosmetically and pharmaceutically (Akbarzadeh *et al.,* 2013:1).

Originally liposomes were only used for biological studies, given they are very similar to biological cells, making them ideal model organelles. As the investigation into liposomes advanced, this biological model cell became of great interest to scientists as the possibility of transporting drug molecules into the human body seemed promising. Liposomes have gained increased importance as potential carrier models for targeted drug delivery among other carriers systems in the pharmaceutical world, since they improve the therapeutic index of drugs (Çağdaş *et al*., 2014:2; Wen *et al.,* 2006:1187).

2.9.1 Structural congregation and components

Liposomes are small artificial colloidal vesicles with a distinct spherical structure. They are constructed out of lipids that form a double layer (bilayer of phospholipids), which encapsulates an aqueous volume in its inner domain. This then assembles an amphipathic liposomal vesicle of hydrophobic (water-hating) and hydrophilic (water-loving) nature, which becomes of importance regarding drug facilitation and drug delivery (Drulis-Kawa & Dorotkiewicz-Jach, 2010:197).

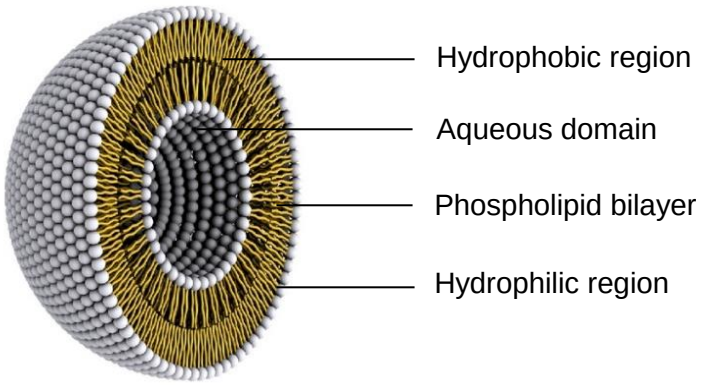

Figure 2.4: Schematically representation of a liposomes fundamental organisation, adapted from Bitounis *et al*. (2012:2).

The rudimentary components of liposomes are phospholipids and cholesterol (serves as a fluidity buffer). The formation of the phospholipid bilayer vesicle design was first discovered by Alec D. Bangham and colleagues in 1964, as they noted the spontaneous aggregation of phospholipids. The polar heads incline to the inner and outer domain to form an enclosed bilayer structure upon hydration in an aqueous medium (Akbarzadeh *et al.,* 2013:1). The liposome then consists of a lipid bilayer, dividing the internal aqueous region from the outside region, creating macroscopic vesicles as illustrated in Figure 2.4.

2.9.2 Liposomal morphology

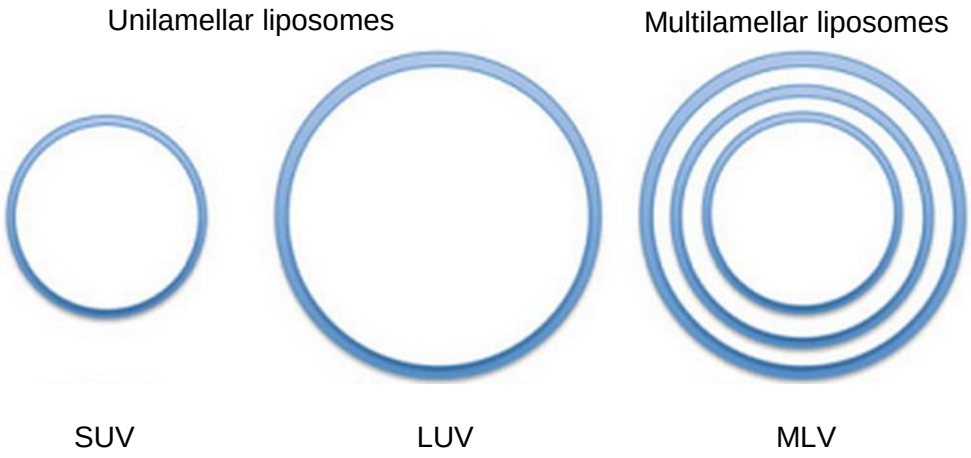

Figure 2.5: Illustration of the three basic liposomal lipid vesicles: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and multilamellar (MLV)

The classification of liposomes is established according to their composition, the number of lamellae and range of sizes (Deepthi & Kavitha, 2014:47). There are two principal classes of

liposomes, i.e. multilamellar vesicles (MLV) and unilamellar vesicles. The latter vesicle class can also be subdivided into two other subclasses, namely small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) (Elzainy *et al*., 2005:282). The MLV has a size range of between 20 -100 µm and resides with five or more layers. The SUV and LUV only embody a single bilayer and are smaller than 100 nm and between 100 -1000 nm, respectively (Elzainy *et al*., 2005:282). Figure 2.5 is adapted from Uchegbu *et al.* (2013:43) and illustrates the difference in number of lamellae and range of sizes between the two classes of liposomes.

2.9.3 Liposomal preparation

The preparation of liposomes is dependent on their usage and functionality, since the method will be used to modify and control the size, shape, structure and other characteristics of liposomes (Çağdaş *et al*., 2014:4). There are three general methods used in the preparation of liposomes, namely: the handshaking method, a sonication method and a reverse evaporation method to create and control the liposomal formation (Rani, 2013:9). However, although the reverse phase evaporation method is the main focus of this study the other methods will also be discussed shortly.

2.9.3.1 Reverse phase evaporation method

The reverse phase evaporation method is a method based on the foundation of creating inverted micelles with a larger aqueous capacity to lipid ratio. The liposomes are formed by sonicating two combined phases, i.e. an aqueous and an organic phase, each containing water soluble molecules and amphiphilic molecules, respectively. The aqueous molecules are encapsulated within the liposome and the organic phase is solubilised. After the combination of these two phases, the organic solvent is evaporated at a slow rate, reconstructing these micelles into a viscous gel nature. At a specific point in the method, the micelles will decay due to the transformation of the gel-like nature adding extra phospholipids into the mixture, which then contribute to the bilayer arrangement of liposomes. This is how bilayer designed liposomes are created through the reverse phase evaporation method (Rani, 2013:9).

2.9.3.2 Handshaking method

The handshaking method is based on the preparation of a myelin figured lamellae that will be agitated mechanically by shaking, vortexing or pipetting to form liposomes. The myelin figures are formed after the hydration of a dry lipid layer film. Following the mechanical agitation, the myelin figures become weakened in order for the hydrophobic edges to become uncovered, ending with the formation of liposomes (Rani, 2013:9).

2.9.3.3 Sonication method

The sonication method is subdivided into two different approach methods to create SUVs from MLVs. The first approach involves the use of a probe sonicator, where the tip is placed straight into the liposomal dispersion applying a high energy input to create SUV liposomes. The second approach is more temperature controlled. Due to the high-energy input in the first approach an ice bath is usually required but not in the second approach, making it an easier method to follow (Akbarzadeh *et al.,* 2013:3).

2.10 Pharmacological potential: Role as drug carriers

The noteworthy structural feature of liposomes makes them perfect drug facilitating vehicles. Hydrophilic drugs can be entrapped within the aqueous core of liposomes and hydrophobic/lipophilic drugs can position themselves into the bilayer membrane of the vesicle system. These properties make liposomes ideal for transporting a variety of drugs across the skin (Madni, 2014:401).

An important feature concerning industries and the liposomal drug carrying systems are the natural components of liposomes. Since liposomes are biodegradable and biocompatible, the use of liposomes becomes more enticing as drug delivery systems (Madni, 2014:401; Wen *et al.,* 2006:1187). Liposomes have also surfaced as carriers for transporting larger molecules and more sensitive drugs, which only benefit their value as drug carrying systems and improving the bioavailability of drugs to the body (Madni, 2014:402).

These fitting features of liposomes are essential in regards to poorly water soluble drugs, i.e. CLF, as liposomes can encapsulate CLF into their micelle structure. The effective advantage of encapsulating a water insoluble drug is that no dissolution of the drug is required prior to absorption as the liposomal vesicle, in conjunction with this drug, is in a solubilised form. Therefore, increasing the solubility of water insoluble drug by using liposomes are key features of these drug delivery systems, contributing to their versatility and usefulness (Harikumar & Aggarwal, 2012:919).

2.10.1 Employing liposomes for topical drug delivery

The role of liposomal drug delivery is to enhance the delivery into the skin by implementing a deposit site for sustained release of topical compounds and improving the bioavailability in the skin. A great capacity for liposomes as effective drug carriers into the skin has been recognised by numerous scientists. The drug carrying liposomes have the ability to fuse/absorb into the cellular membranes when applied topically, followed by the transfer of active material into cells.

The topical delivery of actives in liposomes not only provides a means for targeted delivery to the site of action, but also yields as a slow releasing system for continued exposure over longer periods (Pierre & Costa, 2011:609).

2.10.2 Liposomal-skin interaction

Pierre and Costa (2011) suggested a variety of mechanisms to illustrate the workings of the interaction between liposomes and human skin. It was observed among scientist that liposomes are able to permeate into the skin only when the skins' defensive barrier is weakened, for example through the origin of a skin disease.

A variety of factors affect the capability of liposomes to deliver drugs into the stratum corneum, i.e. size, composition, structure, fluidity and charge. It is also evident that the human stratum corneum lacks any phospholipids, but in the intercellular domain, skin lipids are found in an organised bilayer structure. This bilayer sheet is believed to be in the vicinity where most transported water and drugs will reside. In the case of liposomes it is hypothesised that they can act as tiny reservoirs allowing a slow and controlled release of drug compounds when they cross the stratum corneum (Pierre & Costa, 2011:615).

It is suggested that the mechanism of liposomal drug transfer into the skin is due to the skins' lipid composition being of equal nature to that of the liposomes bilayer, which can promote fusion of liposomes into the intercellular domain (Pierre & Costa, 2011:615). It was also suggested by Du Plessis *et al.* (1994:278) that the mixture of the liposomes' bilayer with the skin lipids of the stratum corneum could result in an intra-cutaneous depository of drugs. The key mechanism of liposomes is their entrapment and drug transfer into the skin due to the near identical correlation between liposomes and the bilayer of the stratum corneum. Upon dehydration, it will strongly connect to the skin surface and act as a reservoir for a continuous drug release across the stratum corneum (Pierre & Costa, 2011:615).

2.10.3 Advantages and disadvantages of liposomes as drug delivery systems

Table 2.3: Summary of the advantages and disadvantages of using liposomes as drug delivery systems (Deepthi & Kavitha, 2014:48; Jain *et al*., 2014:2)

	Advantages	Disadvantages
Liposomes	➢ Suitable for hydrophilic and hydrophobic drug delivery ➢ Protects drug from external environment ➢ Low toxicity ➢ Reduces exposure of toxic drugs and their metabolites to sensitive tissues ➢ Suited to deliver large and small molecular weight drugs ➢ Target specific drug delivery ➢ Biocompatible and biodegradable	➢ Premature drug release/leakage due to porous nature ➢ Expensive ➢ Hydrophilic drugs have poor encapsulation efficiency ➢ Short half-life ➢ Low stability

2.11 Proliposomes

The main disadvantage of liposomes is their low stability, as the maintenance of their physical properties is troublesome. This instability is predominantly caused by the oxidative and hydrolytic degradation of formulations (Çağdaş *et al*., 2014:10). A solution to the instability issues of liposomes is to create dry, free flowing granular particles, i.e. proliposomes (Kalepu & Nekkanti, 2015:449).

These proliposomal-drug encapsulating vesicles are created by using a soluble carrier and coating the lipid and drug to form these granular products. Subsequently, upon hydration with water the granular particles will form a suspension of isotonic multi-lamellar liposomes. The drug and lipids are transferred into carrier materials due to their porous complex, allowing the carrier materials to retain their free flowing surface properties and create granular particles, which eventually form proliposomes. The proliposomes can then be stored and hydrated when needed with water, thus alleviating the stability problem associated with liposomes (Rani, 2013:8; Sirisha *et al*., 2012:31). The major advantages related to proliposomes are their reduced level of toxicity, enhancing bioavailability, catering for targeted drug delivery, safeguards drugs from possible degradation and possessing the capability of controlled drug release (Kalepu & Nekkanti, 2015:449).

The pro-vesicular system is an ideal carrier for drugs with a poor bioavailability character without altering its intrinsic characteristics (Xu *et al*., 2009:61) and provides a solution to product

instability due to problems associated with storage of the aqueous dispersion of liposomes. This is done by bearing a dry product, as explained, to increase the storage capabilities of liposomes, which can then be hydrated directly before it is needed, making proliposomes a fitting solution to the stability obstacle related to liposomes (Kalepu & Nekkanti, 2015:449).

2.12 Conclusion

The resistance of TB against its general treatment regime has become of great interest in recent years and gave light to the investigation of second-line drugs. In a small population of TB patients cutaneous lesions develop as a result of exogenous inoculation and endogenous infection. The second-line drug, clofazimine, has shown promising results against resistance and is the main drug candidate for this study, as it will possibly aid in the treatment of skin lesions caused by TB in conjunction with the general TB regimen. A marked interest regarding the topical and transdermal delivery of drugs has amounted in recent times. CLF does not pose as the ideal drug for topical delivery as its physiochemical properties does not fall within the desired ranges. The high log P, melting point and molecular weight, and low aqueous solubility of this API indicate the possible difficulty in the delivery of this API onto/into the skin. Due to the undesirable physiochemical properties that CLF possesses, the use of a vesicle system was implemented to enhance and improve these characteristics. Vesicle systems have been used and implemented extensively to improve the bioavailability and therapeutic index of drug absorption and seen as potential carrier models (Çağdaş *et al*., 2014:2; Wen *et al*., 2006:1187). As a result of the poor physiochemical properties of CLF, liposomes were chosen as the model carrier of this API to determine if these vesicle systems could improve topical drug delivery. Liposomes are subject to oxidation and are seen as unstable in aqueous dispersion. Thus, the additional formulation of proliposomes creating free flowing granular particles was also subject to investigation to improve the stability of the liposomes. This study chiefly focused on the topical delivery of CLF by encapsulation in liposomes and proliposomes. The topical delivery of CLF will not replace the normal oral treatment regimen for TB but it could possibly be used as a direct applicant for CTB to aid in the treatment of the lesion caused by TB.

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CHAPTER THREE

Article for publishing in Drug Delivery

Chapter 3 is written in article format for publication purposes in the Drug Delivery journal. The contribution to the writing of the article and experimental work was performed and completed by the Master's student. The article was written using a template provided by the Journal using US English and TF-X Harvard referencing (EndNote). In Appendix F, the full author guidelines for publishing are found. The template provided was adapted to make this reader friendly, as the text was changed to a justified style.

Formulation and Topical Delivery of Liposomes and Proliposomes Containing Clofazimine

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Formulation and Topical Delivery of Liposomes and Proliposomes Containing Clofazimine

Even though only a small population (1-2%) of tuberculosis patients develop cutaneous tuberculosis it is still considered important, as approximately 2 billion people have been infected with this bacterium worldwide. The purpose of this study was to improve the solubility of the lipophilic API, clofazimine, for topical delivery using liposomes and proliposomes. Initial studies were performed to characterize the clofazimine encapsulated in liposomes (**(CL2)**) and proliposomes (**(CPL2)**). The release of clofazimine from the vesicle systems was evaluated by membrane release studies employing Franz cells. Skin diffusion studies were performed on excised human skin using Franz cells to investigate the clofazimine dispersions transdermal delivery and a tape stripping method to investigate the topical delivery thereof. An *in vitro* toxicity test using an LDH assay was performed on a HaCaT cell line to evaluate the possible cell damage caused by the free drug and vesicle systems. The results from the membrane release study showed a successful release from both dispersions. It was also found that clofazimine concentrated in the stratum corneum-epidermis and epidermis-dermis, illustrating topical delivery. The liposome vesicle system showed a protective effect *in vitro*, as the vesicle dispersions containing clofazimine were non-cytotoxic, while the free drug samples experienced toxicity.

Keywords: Cutaneous Tuberculosis, Topical delivery, Skin diffusion, Clofazimine, Stratum corneum, Epidermis, Dermis, Liposomes, Proliposomes

1 Introduction

Tuberculosis (TB) is one of the most deadly diseases worldwide as nearly 2 billion people have been infected with the *M. tuberculosis* bacterium, indicating that approximately 30% of the population globally has possibly been infected (Sosnik *et al*., 2010). In approximately 1% to 2 % of TB cases, dermatological manifestations of TB, i.e. skin lesions develop resulting in cutaneous tuberculosis (CTB). CTB development can be acquired either exogenously or endogenously, where the former is less frequently

encountered (dos Santos *et al*., 2014) (Frankel *et al*., 2009). The challenge regarding TB is the recurring resistance of the *M. tuberculosis* bacteria against the first-line anti-TB drugs: isoniazid and rifampicin (Dooley *et al*., 2013). The suggested solution to this resistance is to use second-line drugs, i.e. clofazimine (CLF) and other drugs to assist the current treatment regimens against multiple-drug resistant tuberculosis (MDR-TB). However, the main obstacle is the inadequate potency and unwanted toxicity profiles of most second-line drugs (Dooley *et al*., 2013).

CLF is a highly lipophilic compound that forms part of an antibiotic subgroup, riminophenazine. This active pharmaceutical ingredient (API) is used as an anti-mycobacterial agent and gained interest by being used as a treatment option against the remerging MDR-TB (dos Santos *et al*., 2014) (Zhang *et al*., 2012) (Mane *et al*., 2012). The skin is classified as a large ($2m^2$) diffusion organ, where the stratum corneum constitutes the skin's physical barrier properties controlling the import and export of substances into the skin layers. This layer challenges the topical/transdermal delivery of drugs onto or into the skin (Zhang *et al*., 2009, Mathes *et al*., 2014). The success with which an API deposits into the skin is mainly dependent upon a narrow range of specific physiochemical properties, i.e. lipophilicity (log P between 1 and 4), molecular weight (lower than 500 g/mol) and solubility (> 1 mg/ml) of the API (Chandrashekar and Shobha Rani, 2008) (Naik *et al*., 2000). The lipophilic property of CLF (log P of 7.66) is indicative that this drug has the potential to accumulate in the lipophilic skin barrier, making it difficult for the drug to diffuse into the rest of the skin layers (Zhang *et al*., 2009). However, the main obstacle regarding CLF is its hydrophobic characteristic, giving it an extremely low aqueous solubility (0.000225 mg/ml) (PubChem, 2015) and posing possible future complications; hence, giving purpose to investigate. According to (Çağdaş *et al*., 2014) and (Wen *et al*., 2006), vesicle systems have the ability to greatly improve the therapeutic index and bioavailability of drug

absorption. The possible improvement of CLF solubility may magnify the drug capacity for topical delivery of the API. The particulate systems, i.e. vehicles such as liposomes vesicles, form the focus of this study and aim to possibly enhance topical drug delivery (Sharma *et al*., 2013).

Liposomes are water filled, colloidal, spherical particles, with capsule walls that contain amphiphilic molecules on the inside of a bilayer design (Drulis-Kawa and Dorotkiewicz-Jach, 2010). Liposomes are capable of accompanying both hydrophilic and lipophilic compounds and since they closely resemble biological membranes, its experimental usefulness is ideal. Accordingly, the lipophilic CLF is expected to dock into the liposomes' lipid bilayers. Liposomes furthermore, have the capabilities of acting as drug carrier by transporting the encapsulated drug molecules across the skin barrier to be released further down the line (Pierre and Costa, 2011). The solubility of liposomes is an important consideration in transdermal and topical delivery, since it directly affects the active drugs' penetration and permeation into and/or across the skin (Harikumar and Aggarwal, 2012). An alternative to liposomes, namely proliposomes, was also investigated during this study. Despite the many advantages, liposomes provide a significant disadvantage, which is their instability due to being subject to degradation (oxidation) (Çağdaş *et al*., 2014). It is anticipated that the instability of liposomes would be relieved by reformulating them into a provesicle form (e.g. proliposomes) (Rani, 2013), hence the comparative investigation between liposomes and proliposomes.

The aim of this study was to evaluate the effectiveness of CLF encapsulated in liposomes and proliposomes by determining whether such vesicle systems would enable this API to cross the stratum corneum and penetrate into the designated dermis where CTB resides. The permeation results would help to determine whether the CLF

dispersions could offer a supplementary route for the topical delivery of this API to the normal MDR-TB treatment regime.

2 Materials and Methods

2.1 Materials Used in this Study

Sangrose Laboratories Pvt Ltd (India) donated the raw (powder form) CLF used during this study. To create the liposome vesicles, the following were required for preparation: L-α-phosphatidylcholine, cholesterol and α-tocopherol were all procured from Sigma-Aldrich® (Germany), the D-sorbitol and analytical grade chloroform were purchased from Merck Millipore® (Darmstadt, Germany) and the AR grade methanol was purchased from (ACE Chemicals, South Africa). The acetate buffer used as a hydration medium was prepared from sodium-acetate (tri-hydrate) and purchased from Sigma-Aldrich® (Germany). The ultra-pure Milli-Q® used throughout the study, had a resistivity of 18 $M\Omega.cm^{-1}$ and was acquired from a Rephile Direct Pure® UP water purification system (USA). The mobile phase used for the high performance liquid chromatography (HPLC) analysis was acetonitrile of chromatographic grade (Merck, South Africa). HPLC-grade ethanol was used for the membrane release and skin diffusion studies, along with a pH 7.4 phosphate buffer solution (PBS) prepared from potassium phosphate monobasic, both of which were procured from Merck Chemicals (Wadeville, RSA).

The cell cultures study used an immortalized keratinocyte cell line (HaCaT), which was received as a gift from the University of the Witwatersrand (WITS). The growth media for cell preparation was obtained from HyClone™ (GE Healthcare Life Sciences, South Logan, Utah) and included: Non-Essential Amino Acids, Dulbecco's Modified Eagle Medium (DMEM), L-Glutamine, Phosphate Buffered Saline (1x) and

Fetal Bovine Serum. The other consumables were purchased from Lonza ™ (Basel, Switzerland) (Pen/Strep and Trypsin-Versene® (EDTA)) and Sigma-Aldrich® (St. Louis, Missouri) (Trypan Blue Solution). For the determining the cytotoxicity, a CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit was purchased from Promega™ (Madison, Wisconsin).

2.2 HPLC Analysis Method

A validated HPLC method for CLF was determined to provide an appropriate and reliable method to ascertain the amount of CLF present in the samples. Analysis in the laboratory was performed at room temperature (25°C), on an Agilent 1100 series HPLC equipped with an Agilent 1100 isocratic pump, auto sampler and ultra violet (UV) detector (Agilent Technologies, Palo Alto, CA). The HPLC system was installed with ChemStation rev. A10.02 data acquisition and analysis software. A Venusil XBP C18 (2) column consisting of ultra-pure silica, (150 x 4.6 mm) with a 5 µm particle size was used as the stationary phase during the HPLC analysis (Agela Technologies, Newark, DE). An injection volume of 50 µl was set with a flow rate of 1 ml/min and a wavelength of 284 nm. An amount of 0.005 M of octane sulphonic acid-Na was needed for the preparation of the mobile phase and orthophosphoric acid was used to adjust the pH to 3.5. A 70:30 ratio of acetonitrile and octane sulphonic acid-Na buffer was mixed to create the mobile phase. This method was validated with a limit of detection (LOD) of 0.01290 µg/ml and lower limit of quantification (LLOQ) of 0.10330 µg/ml.

2.3 Aqueous Solubility of Clofazimine

Excess amounts of CLF were dissolved in 7 ml of PBS (pH 7.4) in preparation of the aqueous solubility analysis. The solution of CLF in PBS (pH 7.4) was mixed

thoroughly in a polytop, placed in a pre-heated water bath (32°C – emulating the temperature of the skin) for 24 h, then filtered through 0.45 µm polyvinylidene difluoride (PVDF) filter. The filtered solutions were transferred into vials and analyzed on the HPLC, in triplicate.

2.4 Log D of Clofazimine

The distribution coefficient (log D) of CLF in octanol (lipophilic phase) and PBS (pH 7.4) (aqueous phase) was determined. A pre-weighed mass of 200 mg CLF was added to 20 ml pre-saturated *n*-octanol in a test tube; 3 ml of each solution was then transferred into a test tube followed by an equal volume of the extracted PBS (pH 7.4) phase. The prepared solution containing the two phases was then placed into a mechanical shaker bath overnight, which caused a disrupting effect in the solution. Following the overnight period, 1 ml of the *n*-octanol-phase and PBS-phase in each polytop was extracted, respectively, placed into different polytops and diluted in 10 ml of methanol. 1 ml of the diluted *n*-octanol solution and PBS (pH 7.4) extracted from the separate polytops was transferred into vials for HPLC analysis to determine the concentration of the API in each separate phase. The experiment was performed in triplicate. To determine the log D, Equation 1 was used.

$$\text{Log D} = \text{concentration in } n\text{-octanol} / \text{concentration in PBS} \qquad (1)$$

2.5 Vesicle Preparation

The preparation of a 2% concentrate dispersion of CLF encapsulated in liposomes (**(CL2)**) samples consisted of the same ratios as proposed by Patel and Misra (1999). Proliposomes (**(CPL2)**) were also prepared using the same ratios used for the liposomes, with the addition of sorbitol (soluble water carrier). By keeping the ratios of

the two vesicle systems components fixed, any possible variation between vesicle formations was removed and simplified the explanation regarding the comparison.

A CLF:phosphatidylcholine (PC):cholesterol ratio of 1.00:7.85:1.00 was used to prepare a 10 ml **(CL2)** dispersion (Patel and Misra, 1999). The cholesterol, PC and CLF were dissolved in a solvent mixture of methanol and chloroform (1:2), where after α-tocopherol was added and the mixture was evaporated on a Buchi® Rotavapor® RII. After obtaining a thin lipid film, an acetate buffer (pH 5) was used as a hydration medium and added to the flask, which was stirred until the lipid film in the flask was completely hydrated. This was followed by sonicating the hydrated mixture with a sonication probe, six times for 2 min intervals and left for 2 h.

A soluble carrier approach was used during the preparation of **(CPL2)**. The same ratio of cholesterol, PC and CLF, as mentioned above, were prepared and dissolved in a solvent mixture of methanol and chloroform (1:2), where after α-tocopherol was added to the mixture. In a round bottomed flask containing the sorbitol, the lipid mixture was added drop wise and evaporated until the entire solution was added. The coated sorbitol granules were then placed in a desiccator for overnight drying. Upon use, an acetate buffer (pH 5) was utilized as a hydration medium and added to the flask containing the granules. The flask was stirred until a liposomal suspension had formed, followed by sonicating the suspension with a sonication probe, six times for 2 min intervals and left for 2 h.

2.6 Physical characterization of the final vesicle and provesicle dispersion

2.6.1 Morphology

Visualization was carried out at approximately 200 kV on a FEI Tecnai G2 high resolution TEM (FEI, Holland). API, α-tocopherol and acetate buffer (pH 5) were

absent during the preparation of the dispersion, as they may cause damage to the TEM microscope. The dispersion was prepared for the TEM by diluting (x10) the sample in Milli-Q® water and stirring thoroughly. A single drop of the sample was taken and added to a copper carbon-coated 300-mesh grid, followed by a drying stage; a drop of osmium was added to the completely absorbed solution to stain the lipid films. After the stained dispersion dried, it was treated with uranyl acetate and lead citrate and left to dry. The vesicles were viewed at magnifications between 5000 and 20000x.

2.6.2 Droplet Size and Distribution

The average droplet size and size distribution (polydispersity index-PdI) of the vesicles in the dispersions was established by a Malvern Zetasizer Nano ZS (Zen 3600) (Malvern® Instruments Ltd, Worcester, United Kingdom). Three low concentration samples were prepared per **(CL2)** (n=3) and **(CPL2)** (n=3) sample. Each low concentration sample was administered to three disposable cells (2 ml), respectively. The analysis was performed in triplicate to establish a good average size and distribution.

2.6.3 Zeta-potential

A Malvern Zetasizer Nano ZS (Zen 3600) (Malvern® Instruments Ltd, Worcester, United Kingdom) was employed to determine the zeta-potential to evaluate the stability of the dispersions. An identical procedure was followed to the one used in droplet size and distribution during sample preparation. The samples were measured in triplicate a day after sample preparation (n=3) to calculate a good average.

2.6.4 pH

The pH of the vesicle lipid bilayers was measured using a calibrated Mettler Toledo pH

meter FE20/FG2 (Greifensee, Switzerland). The measurements were taken by placing the probe of the pH meter into the freshly prepared sample (10 ml); two measurements per sample were taken in triplicate.

2.6.5 Viscosity of the vesicle systems

The viscosity of the samples was determined using a Brookfield Viscometer DV2T-LV (Stoughton, USA. The **(CL2)** (n=3) and **(CPL2)** (n=3) samples were prepared, respectively, the day of the viscosity measurements. 2 h prior to measuring, the samples were placed in a pre-heated water bath (25.5°C), where after the viscosity readings were taken. The T-spindle (Stoughton, MA) of the Brookfield Viscometer was inserted into each sample (6.5 ml), respectively, and set at a fixed rotating rate. In total, 13 measurements (10 sec intervals over a 2 min) per sample were procured.

2.6.6 Encapsulation efficiency

The validated HPLC method was used to determine the EE%. Using Eppendorf® tubes, the prepared dispersions (10 ml) were transferred and centrifuged (25 000g for 30 min at room temperature) in an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa). The supernatant that formed was then diluted (x50) with the aforementioned solvent used in the HPLC section. After diluting the supernatant, 1 ml was extracted and added to HPLC vials for analysis. The analysis was performed in triplicate. A standard solution was also prepared and injected to acquire a linear standard curve. The EE% of each vesicle system was calculated accordingly, as adapted from Xiang *et al*., (2009).

$$EE\% = Drug\ (total) - Drug\ (supernatant) / Drug\ (total) \times 100 \qquad (2)$$

2.7 Membrane Release Study

A membrane release study was performed for the **(CL2)** and **(CPL2)** dispersions, with the aim of evaluating the API's release from the vesicle and provesicle systems. Twelve Franz cells where used per experiment, consisting of 10 cells containing the prepared dispersion and two placebo dispersions stemming as the control samples. The donor phase consisted of the prepared dispersions, and the receptor phase contained HPLC-grade ethanol. The donor and receptor phases where placed into two water baths respectively; one was used to emulate temperature of the skin (32°C) and the other to emulate the temperature of blood flow (37°C).

The Franz cells were assembled by applying vacuum grease to both the receptor (containing a magnetic stirrer bar) and donor phase components. A cut PVDF hydrophilic membrane filter (pore sizes of 0.45 μm) was placed between the two components, followed by the adjoining of the donor and receptor unit with a horseshoe clamp to ensure no leakage occurred during the studies. Into each Franz cells receptor compartment, 2 ml of HPLC-grade ethanol was injected and into each donor compartment, 1 ml dispersion was injected and finally sealed with Parafilm®. The fully assembled Franz cells were then placed onto a magnetic stirrer plate (Variomag®) in a pre-heated water bath set at 37°C. The receptor phase contents were extracted every hour for a 6 h period and transferred to HPLC vials for analysis. The receptor component was then immediately replaced with an equal volume of the pre-heated HPLC-grade ethanol. The HPLC was used to determine the concentration of CLF released through the membrane.

2.8 Human Skin Preparation for Diffusion Studies

Full-thickness skin was obtained from the abdomen of Caucasian woman after plastic

surgery by qualified surgeons. The skin was obtained and immediately stored in a fridge (-20°C) at the Bio-safety Laboratory of the North-West University, for a maximum of 24 h (ethical approval reference number: NWU-00114-11-A5). A Zimmer™ electric dermatome was used to cut the full-thickness skin at appropriate and identical thicknesses of 400 µm, after which it was set on Whatman® filter paper and wrapped in aluminum foil, then stored in a freezer at -20°C; the left over subcutaneous fat was safely discarded. Skin is only valid for a period of six months, after which it is dispensed of correctly.

2.9 Skin Diffusion

The passive diffusion of CLF was determined and evaluated by performing topical diffusion studies using Franz cells. The method and general setup used for the membrane release studies was implemented during these experiments with the only difference being the use of dermatomed skin instead of a PVDF membrane filter and a different receptor phase (HPLC-grade ethanol:PBS (pH 7.4)). The skin was prepared as mentioned above and placed on the receptor compartment (stratum corneum facing upward) after being cut into 15 mm circles, followed by the complete assembly of the Franz cell as previously mentioned. Every 2 h for a 12 h period the receptor phase was extracted and injected into vials for analysis by HPLC. A pre-heated buffer (HPLC-grade ethanol:PBS (pH 7.4)), in a ratio of 1:9, was immediately used to replace the extracted receptor phase. The concentration calculated from the HPLC data was used to determine if any transdermal diffusion occurred.

2.10 Tape Stripping

After completion of the diffusion studies, the localization of the API in the stratum

corneum-epidermis, or epidermis-dermis, was determined to establish the extent to which CLF had diffused into each skin layer using a tape stripping approach. This provides evidence regarding the topical delivery of CLF (12 h).

Firstly, the Franz cells were disassembled and the skin samples carefully removed and pinned onto a solid surface covered with Parafilm®. The only viable section of the skin samples that can be used for tape stripping is where diffusion occurred (marked). These sections were cleaned with tissue paper, followed by the placement of the 3M Scotch® Magic™ tape onto the marked diffusion area of each skin sample, which represented the stratum corneum-epidermis layer. After removal, the first piece was discarded into a biosafety bin, then 15 consecutive 3M Scotch® Magic™ tape strips were removed until the samples appeared lucent. The strips were placed into a polytop, consisting of 5 ml HPLC-grade ethanol and stored in a fridge (4°C) overnight. After tape stripping, every skin sample (where diffusion occurred) was cut into small pieces, i.e. the epidermis-dermis, and placed into a polytop consisting of 5 ml HPLC-grade ethanol and stored in a fridge (4°C) overnight. The HPLC-grade ethanol was extracted after 8 h and filtered (PVDF filters) into vials for HPLC analysis (Pellett *et al.*, 1997). This approach was implemented for the **(CL2)** and **(CPL2)** dispersions.

2.11 Data and statistical analysis of release and diffusion studies

Data analysis was performed on the **(CL2)** and **(CPL2)** results received after HPLC analysis, which included membrane release data and tape stripping (stratum corneum-epidermis and epidermis-dermis) data. The membrane release between the **(CL2)** and **(CPL2)** was compared to determine the statistical difference between the release of the API from the liposomes and proliposomes. The API concentrated in the stratum corneum-epidermis and epidermis-dermis were statistically analyzed to determine the

statistical difference between the **(CL2)** and **(CPL2)** dispersions in each skin layer, respectively. Parametric and non-parametric t-tests were performed and yielded similar results. A two-way analysis of variance (ANOVA) test was performed for the tape stripping data to determine the statistical difference between the skin layers and the dispersions. A p-value smaller than 0.05 ($p<0.05$) illustrated an acceptable significance level for the ANOVA, t-tests and non-parametric tests.

2.12 In vitro Cytotoxicity

2.12.1 Preparation of stock and dispersions

The stock solution (free drug) and dispersion (**(PL2)** and **(CL2)**) samples were prepared in 0.1, 0.2 and 0.4 mg/ml concentrations by diluting the samples with the growth medium. The samples were prepared in triplicate and this process was repeated twice for each sample.

2.12.2 Cell Culture Cultivation

An epidermal cell line, i.e. HaCaT, was used to determine the cytotoxicity of the API. A standard seeding of mammalian cells, SOP, (Gouws, 2014) was adapted for the cultivation of the HaCaT cell line. The selected cell line was cultivated in a 75cm^2 flask with a growth medium of DMEM in a humidified incubator at 37°C and 5% CO_2. The cells were enriched with 10% fetal bovine serum, 1% Pen/Strep, 4mM L-glutamine and 1% non-essential amino acids supplements. Every day the cells were fed with fresh growth media and when a confluence of 80% was reached, the cells were split.

2.12.3 Seeding of cells for toxicity assay

A hemocytometer was used to determine and visualize the cell viability by following a

Trypan Blue dye exclusion approach to ensure that in each well of the 96-well plate, 20000 cells were present. Trypan Blue 0.4% (w/v) was used to stain a uniform cell suspension of 10 µl. After the cells were stained (3 min), they were transferred to an etched counting chamber (9 mm^2) for evaluation. The live (viable) cells do not uptake the dye while the dead (non-viable) cells do.

2.12.4 Determining cell death using LDH assay

A commercially available CytoTox 96® Non-Radioactive Cytotoxicity Assay Detection Kit was purchased to determine the cell death, i.e. LDH release from the cells. The measurements were conducted with (1) a negative control (medium containing no API, nor vesicle system), (2) a positive control (100% LDH release, i.e. total cell damage), (3) a **(PL2)** sample, (4) a **(CL2)** sample and (5) a medium containing the free drug CLF (Ahn *et al*., 2012).

In the 96-well assay plate, the growth medium and cells were placed and made up to volume (100 µl) with the prepared samples at the predetermined concentrations. The CytoTox 96® Non-Radioactive Cytotoxicity Assay Detection Kit's instructions were followed to determine the LDH-release for the cells.

3 Results and Discussion

3.1 Aqueous Solubility and Log D

The results regarding CLF solubility in PBS (pH 7.4) at 32°C was inconclusive. According to Lu *et al*., (2011) CLF is deemed as practically insoluble at pH 7.0. The almost complete insolubility of CLF in similar mediums is supported by Narang and Srivastava (2002), Ganesh *et al*. (2013), Bevan and Lloyd (2000) and Bolla and Nangia (2012), as they also expressed the extremely low solubility of the API due to its highly

hydrophobic nature.

As stated before, CLF is a highly lipophilic compound (Ganesh *et al*., 2013) and in this study, it was determined that CLF had a log D of 4.60 corresponding closely to what literature proposes (Baik *et al*., 2013). The small difference in the experimental and literature log D values may be due to differences in the experimental conditions.

The experimental log D value indicates the lipophilic nature of CLF and this would evidently illustrate the inverse relationship with aqueous solubility, i.e. high lipophilicity and low aqueous solubility of CLF in an aqueous medium.

3.2 Characterization of Vesicle System

The TEM photomicrographs show the morphology of the liposomes and proliposomes, and are illustrated in Figure 1. The captured images illustrated the successful formation of vesicles from the liposome and proliposome dispersions (Figure 1). All the vesicles had successfully formed and possessed spherical shapes, which fell within the range as proposed by literature (Elzainy *et al*., 2005). The liposomes in Figures 1 a) and b) presented a more rigid and irregular membrane compared to the proliposomes' more smooth appearance in Figures 1 c) and d).

The average values of the zeta-potential, PdI, pH, viscosity and EE% of the dispersions are summarized in Table 1. Figure 2 provides an illustration regarding the droplet size distribution of the **(CL2)** and **(CPL2)** dispersions.

The PdI of both vesicle systems are relatively high and the dispersions are seen as heterogeneous according to literature, but are still within an acceptable range (Shah *et al*., 2014). The largest curves in each graph were relatively narrow and predominantly fell within the droplet sizes that were measured compared to the two other smaller peaks.

The peaks indicate the particles have a close range to each other, even though a large distribution of particles can be found in each dispersion (Figure 2).

The zeta-potential of the liposomes and proliposomes were all predominantly positive and were large enough to ensure stable membranes and that no flocculation or agitation of the vesicles would occur.

The pH of both dispersions was almost equivalent to the pH of the hydration buffer (pH 5), illustrating that none of the ingredients used influenced the membrane charge on the formed vesicles. Therefore this pH is in the ideal range (pH 5-9) for the topical drug (Naik *et al*., 2000).

The viscosity of the liposomes was higher than the proliposomes, but both exhibited a relatively larger viscosity, which is advantageous for topical delivery as the dispersion would not disperse as easily and increase skin exposure.

The EE% of both dispersions was relatively close to what is proposed by literature (Patel and Misra, 1999). This illustrates the successful encapsulation of CLF in the vesicles.

[Figure 1 near here]

[Table 1 near here]

[Figure 2 near here]

3.3 Membrane Release Studies

The results from the membrane release studies, illustrated by the box-plot in Figure 3, show the comparison between the releases of the API from both dispersions through the membranes, respectively. Clearly, the CLF diffused through the synthetic membrane and into the receptor phase from both vesicle dispersions over a 6h period, indicating

the API was successfully released from both dispersions and helped to eliminate the potential problems during topical diffusion studies, e.g. absence of the API in the skin.

The average flux and median flux in the box-plot (Figure 3) of the liposomes and proliposomes illustrates a slight difference in their flux values. Although the median flux values are higher than the average flux, these results are almost equivalent illustrating the closely relatable release from the vesicle systems. The median value is usually used to represent the flux value as the in the presence of outlier, but due to the values being closely relatable, the average was used to represent the flux value for the membrane release study (Schmidlkofer, 2013). This is confirmed by the box-plots, as the distribution is similar and almost identical regardless of the outlier in the **(CPL2)** data. Hence, the average flux data is a valid representation of the APIs release from a vesicle and provesicle system through a membrane over 6 h.

[Figure 3 near here]

3.4 Skin Diffusion Study

By performing skin diffusion studies, the transdermal delivery of CLF was determined; this was also followed by a tape stripping approach to evaluate the topical delivery of CLF. Consequently, the skin diffusion study illustrates the presence (concentration) of the API in either the extracted receptor phase (represent blood plasma), i.e. transdermal, or the presence of the API in the skin layers (stratum corneum-epidermis or epidermis-dermis), i.e. topical. No traces of the API was found in the receptor phase for both dispersions, ruling out any transdermal diffusion.

Figures 4 and Figure 5 illustrates the box-plots comparing the **(CL2)** and **(CPL2)** dispersions in the stratum corneum-epidermis and epidermis-dermis, respectively. The results show there were detectable traces of the API found in stratum

corneum-epidermis and epidermis-dermis from both dispersions after HPLC analysis, therefore supporting the aim of topical delivery. The stratum corneum-epidermis results of **(CL2)** and **(CPL2)** indicated the successful penetration of the lipophilic API into this lipophilic skin layer, verified by the box-plots in Figure 4. This was expected, as the lipophilic character of the API and stratum corneum-epidermis have a high affinity towards each other. The proliposomes exhibited a larger average concentration, but this difference may be attributed to the inhomogeneous composition of this continually differentiating layer (Bolzinger *et al.*, 2012). The epidermis-dermis results show that the **(CPL2)** had a more than 7x higher concentration than the **(CL2)**, verified by the average concentrations in the box-plots in Figure 5. The detection of the API in this hydrophilic layer illustrated a successful permeation of the API using both vesicle systems, which corresponds well with aim of the study by reaching the target layer despite the API being lipophilic and almost insoluble in a hydrophilic environment. The permeation can be linked to the brick and mortar model, where CLF permeated into the epidermis-dermis layer either through the corneocytes or the intercellular spaces along the lipid matrix of the stratum corneum-epidermis (Schneider *et al.*, 2009). The difference between the liposomes and proliposomes API concentration in both layers may also be linked to the fact that two different skin donors were used due to shortage of skin and the stratification difference (age, temperature and hydration) found between two different skin donors may have influenced the data.

[Figure 4 near here]

[Figure 5 near here]

3.5 Statistical Analysis of Release and Diffusion Studies

The parametric and non-parametric t-tests illustrated similar results for both the membrane release and tape stripping data, therefore only the parametric t-test was used. There was no statistical difference found on a 5% level between the average flux ($\mu g/cm^2.h$) values of liposomes and proliposomes ($p=0.08$) from the membrane release data.

The results of the two-way analysis of variance (ANOVA) test found a statistical significance between the data of the dispersions ($p=0.000002$), but no statistical significance was found between the two skin layers ($p=0.4$) data. A statistical significance was also found in the interaction effect of the dispersions and the skin layers ($p=0.04$). The t-test of the liposomes ($p=0.02$) and proliposomes ($p=0.00006$) showed a statistical significance between their concentrations in both the stratum corneum-epidermis and the epidermis-dermis layers; the liposomes ($p=0.006$) represented a statistical significance in both layers, whereas the proliposomes ($p=0.46$) were statistically the same and represented no difference.

3.6 In-vitro Cytotoxicity

The LDH-release from cells is a reliable indicator of the amount of cell death that has occurred when exposed to substances, in this case the API clofazimine (Perche *et al*., 2009). The API was encapsulated in a liposome vesicle system and compared to placebo and free drug (stock) samples. A control sample was used as a non-cytotoxic reference and the samples percentage cell death was evaluated according to the following ranges: below 40% - non-cytotoxic, between 60% and 40% - weak cytotoxicity, between 80% and 60% - moderate cytotoxicity and between 100% and 80% - strong cytotoxicity (Lopez-Garcia *et al*., 2014).

Figure 6 illustrated the difference between the **(PL2)** and **(CL2)** dispersions and the free drug at increasing concentrations (0.1, 0.2 and 0.4 mg/ml), respectively. Compared to the control samples, the **(PL2)** and **(CL2)** dispersions both yielded non-cytotoxic levels on the HaCaT cells regardless of the increase in the sample concentrations. The free drugs' percentage cell death increased proportionally from weakly cytotoxic to strongly cytotoxic as the samples concentration increased. Liposomes are generally seen as pharmacologically inactive and non-toxic due to their composition (natural phospholipids) and correlate with the non-cytotoxic result obtained from the **(PL2)** dispersions data in Figure 6 (Sercombe *et al*., 2015). The **(CL2)** dispersions non-cytotoxic result may be atoned to the protective property liposomes have on a drug by safeguarding the drug against natural biodegradation processes (Bozzuto and Molinari, 2015) (Sercombe *et al*., 2015). This protective effect is also noticed through the free drug samples results, since there is an increased level of cytotoxicity in the absence of a vesicle system. These results also correspond to a study performed by Metha (1996), as similar results were obtained from two different cell lines, i.e. red blood cells and macrophages. Liposomes also possess a hydrophobic characteristic, which may have an influence on the release of the API in an aqueous (hydrophilic) growth medium, as it evidently creates an inhomogeneous mixture which may have led to the dispersions non-cytotoxic results (Sercombe *et al*., 2015).

[Figure 6 near here]

4 Conclusion

This study shows that CLF was successfully entrapped within the liposome and proliposome vesicle systems. The vesicle systems were capable of releasing the API effectively to ensure possible diffusion from the vesicle systems to the targeted skin

layer, i.e. epidermis-dermis, where CLF can possibly elicit a therapeutic effect against CTB. The proliposomes delivered the highest amount of CLF to the epidermis-dermis between the two vesicle systems. This illustrated that the topical delivery of CLF to the dermis layer of the skin is indeed possible. The *in vitro* cytotoxicity showed a significant decrease in the API cytotoxicity *in vitro* when encapsulated in the liposome vesicle system and may possibly be ascribed to the protective effect liposomes have on a drug, or due to the liposomes (hydrophobic) being inhomogeneous in a hydrophilic medium.

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Disclosure of Interest

Any opinions, findings, conclusions or recommendations expressed in this material are those of the authors, and therefore the NRF does not accept any liability with regard thereto.

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Table 1: Summary of the **(CL2)** and the **(CPL2)** physical characterization results

Vesicle System	**(CL2)**	**(CPL2)**
Average zeta-potential	36.80±2.640 mV	27.97±4.000 mV
Average droplet size	330.83±4.400 nm	203.97±18.250 nm
Average PdI	0.52±0.032	0.41±0.006
Average pH	4.87±0.010	5.24±0.013
Average viscosity	43.79±0.133 cP	31.90±0.118 cP
Average EE%	84.15±4.200%	64.99±0.024%

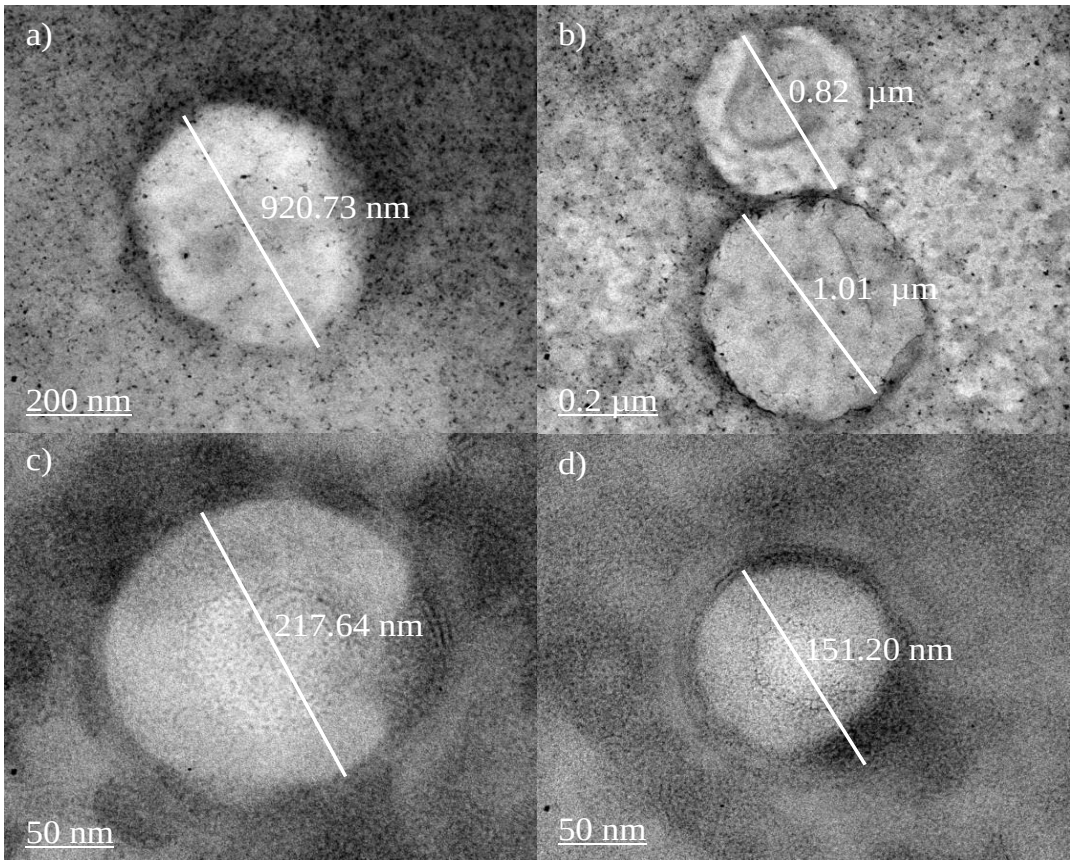

Figure 1: Micrographs of **(CL2)** and **(CPL2)** captured on the TEM at 200 kV: a) a single liposome and b) two liposome vesicles that formed, c) and d) are both liposomes that formed from proliposomes.

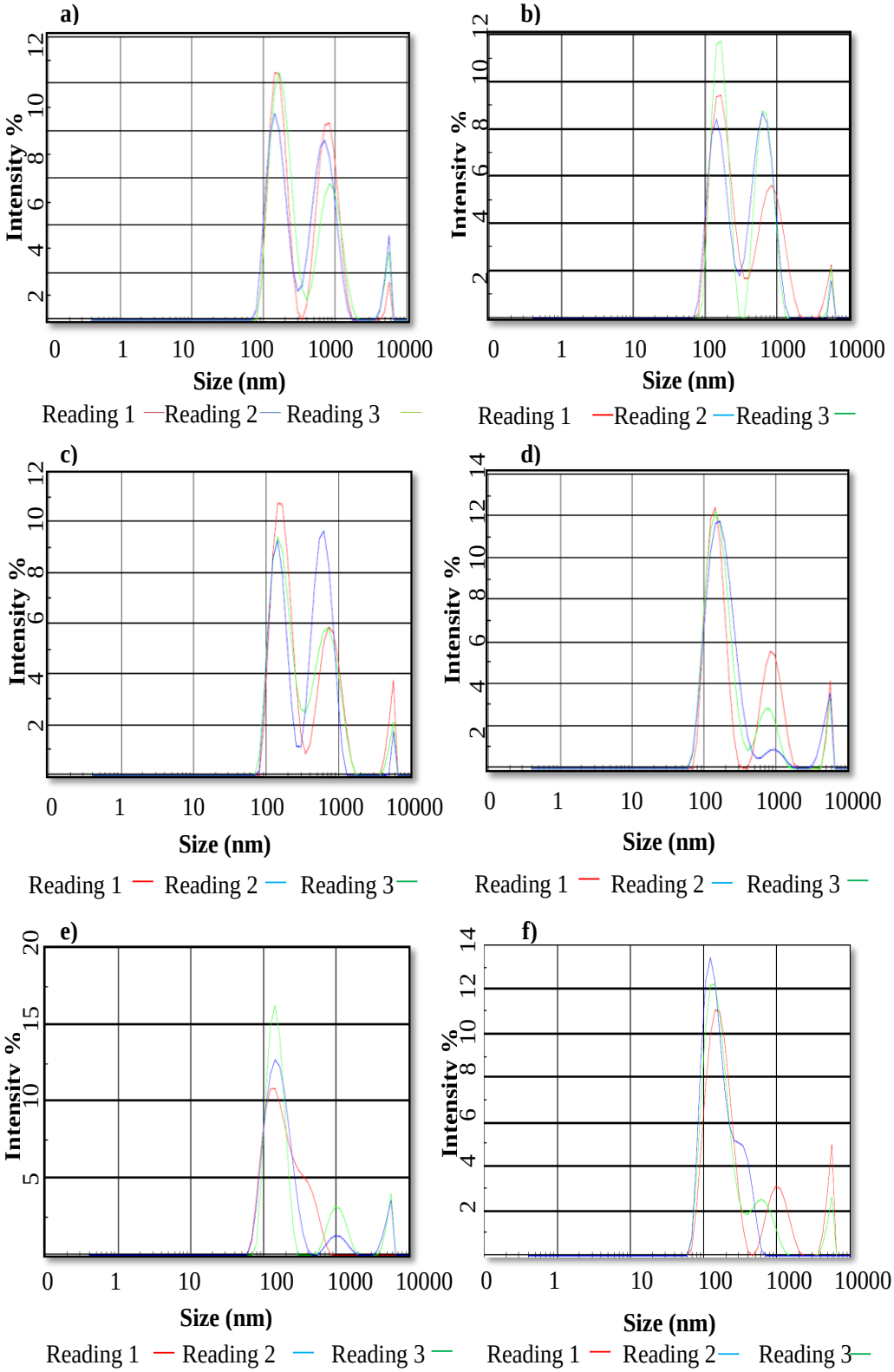

Figure 2: Average particle size of the final dispersions: a), b), c) illustrate the **(CL2)** (n=3) measurements and d), e), f) illustrate the **(CPL2)** (n=3) measurements.

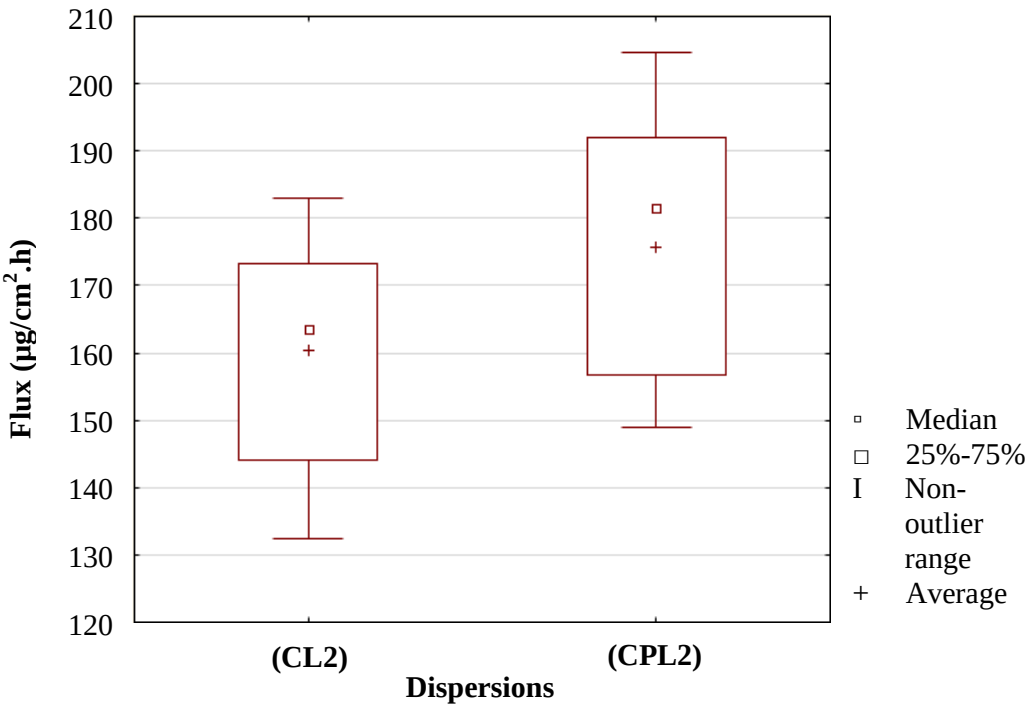

Figure 3: Box-plot representing the flux (µg/cm^2.h) of **(CL2)** and **(CPL2)** present in the receptor phase during the 6 hr membrane release studies. The median and average concentrations are respectively shown by the small square and plus symbols.

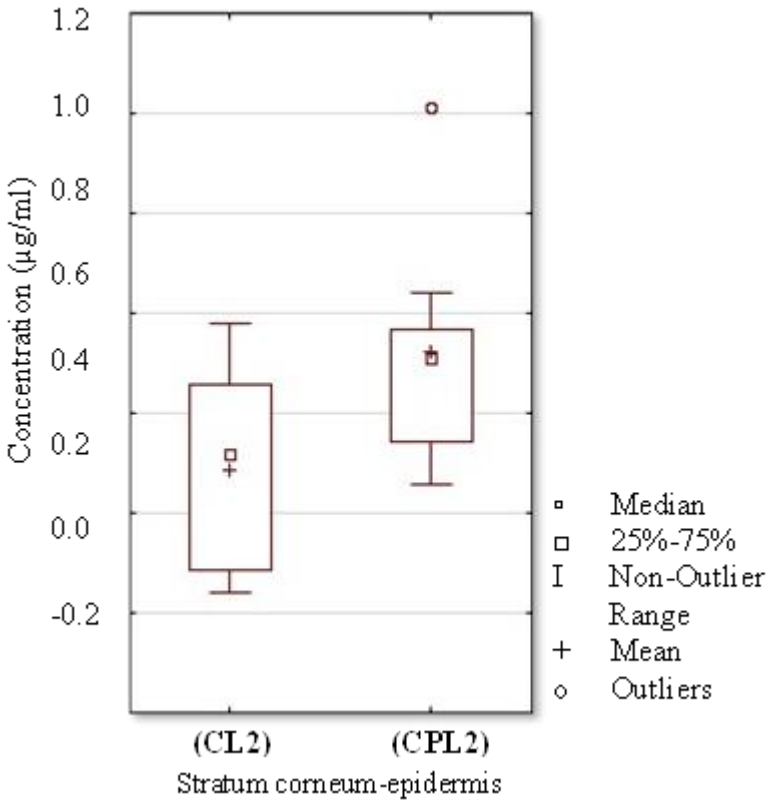

Figure 4: Box-plot of the **(CL2)** and **(CPL2)** concentrations (µg/ml) found in the stratum corneum-epidermis during tape stripping. The small box illustrates the median value and the plus symbol, the mean calculated from the data.

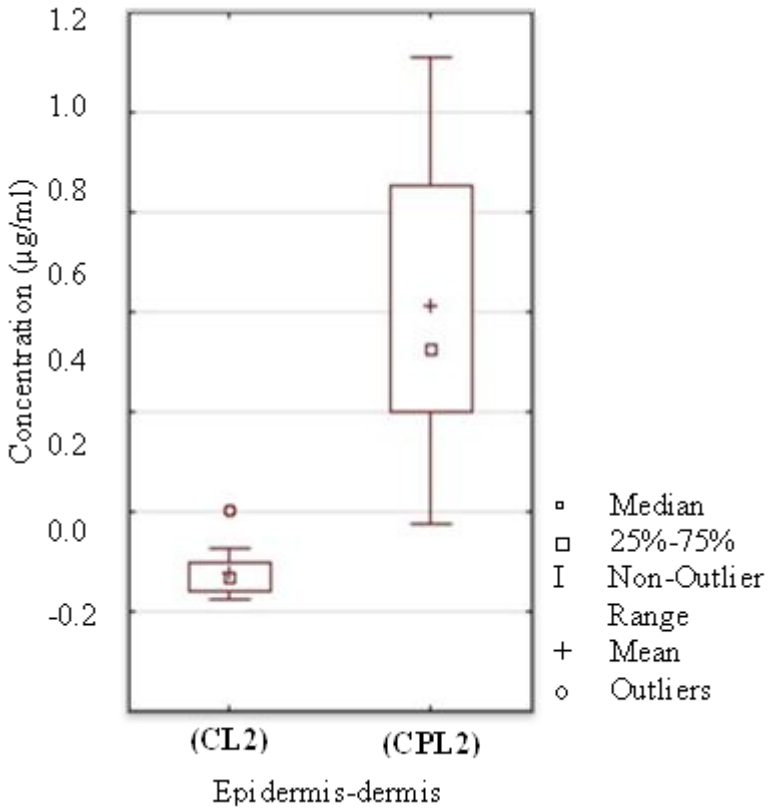

Figure 5: Box-plot of the **(CL2)** and **(CPL2)** concentrations (µg/ml) found in the epidermis-dermis after tape stripping. The small box illustrates the median value and the plus symbol the mean calculated from the data.

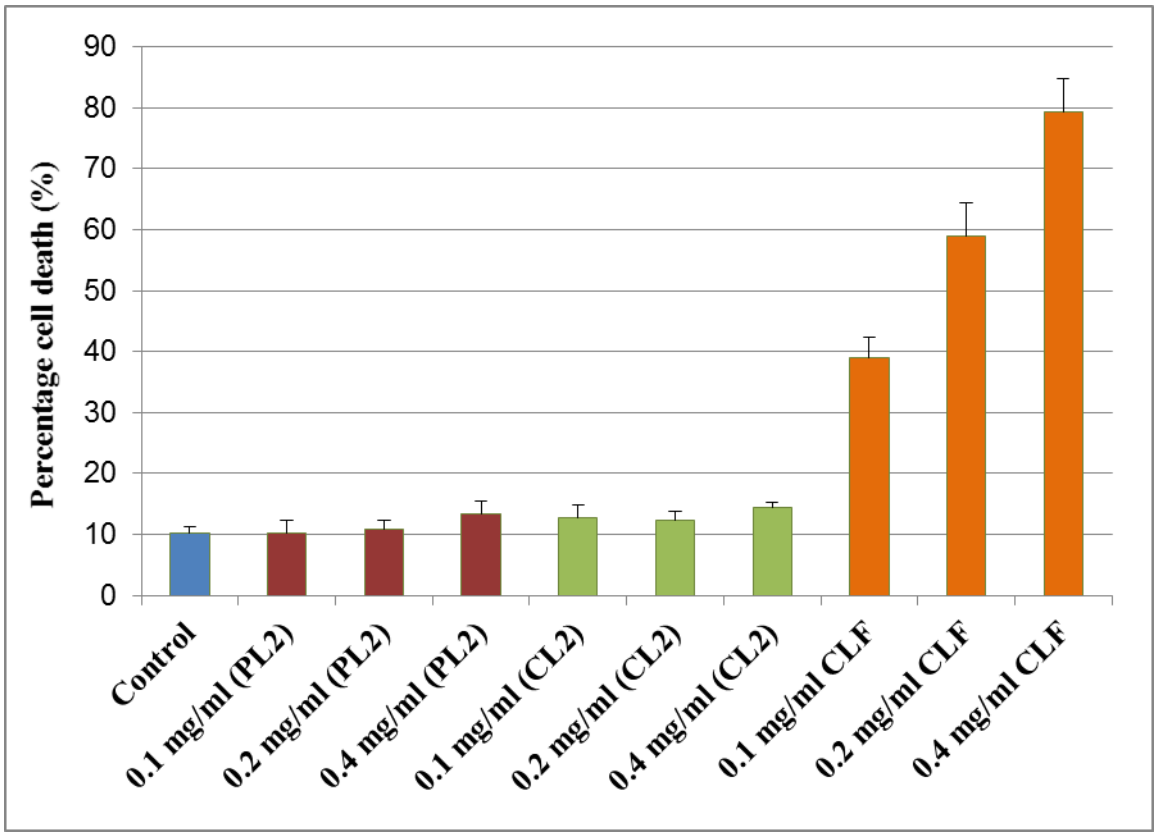

Figure 6: Percentage cell death of the HaCaT cells after exposure to the **(PL2)** and **(CL2)** dispersions and the stock solution (free drug) at different concentrations (0.1, 0.2 and 0.4 mg/ml).

CHAPTER FOUR

Conclusion and future prospects

The defensive barrier of the skin, i.e. stratum corneum, plays an intricate role in preventing/resisting the entrance of exogenous substances into the skin due to its structural composition, and evidently poses as an obstacle for dermal formulation scientists (Mathes *et al*., 2014:82; Zhang *et al*., 2009:227). This creates opportunity to challenge this layer by using enhanced methods for topical and transdermal drug delivery. CTB skin lesions emerge in only a small population of TB patients through the exogenous or endogenous manifestation of the *M. tuberculosis* bacterium (Dos Santos *et al*., 2014:219, Frankel *et al*., 2009:19-21). Although rifampicin and isoniazid are part of the first-line drug treatment regime for TB, a generation of MDR-TB population has developed (Dooley *et al.,* 2013:1352). This resistance led to the investigation of the second-line drug, clofazimine, as it experienced no resistance from the *M. tuberculosis* bacterium. Hence, the investigation into the topical delivery of clofazimine, however its extremely poor solubility posed an obstacle for topical delivery (Pubchem, 2015). The solubility problem associated with clofazimine gave light to investigating the use of vesicle systems (liposomes) to enhance the solubility of the API by entrapping it within its hydrophobic lipid bilayer. By increasing the solubility, using liposomes, would positively attribute to specific limits regarding successful topical diffusion (e.g. aqueous solubility of > 1 mg/ml). In order to evaluate the effect of the vesicle systems designed, experiments were performed to determine the ideal dispersion for possible topical and/or transdermal diffusion by characterising the liposome and proliposome dispersions containing the API. *In vitro* cytotoxicity studies were also performed to evaluate the API's (clofazimine) cytotoxicity profile.

The objectives of this research study were:

- The validation and development of an HPLC method for the determination of the concentration of clofazimine in the liposome and proliposome vesicles.
- The determination of aqueous solubility and log D of clofazimine.
- The formulation of two vesicular systems (liposomes and proliposomes) containing clofazimine.
- The characterisation (morphology, droplet/particle size, zeta-potential, pH, viscosity and drug EE%) of the vesicular systems with and without clofazimine.

- The determination of the release of clofazimine from the liposomes and proliposomes through membrane release studies.
- The determination of the transdermal and topical delivery of clofazimine from the liposomes and proliposomes, by performing a diffusion study followed by tape stripping, respectively.
- The *in vitro* examination of the cytotoxic effects (cell death) clofazimine has on HaCaT cells through LDH-release and cell culture studies.

In collaboration with Prof Jan Du Preez, an HPLC method was validated for the successful determination of the APIs concentration throughout this study, i.e. encapsulation efficiency, release and diffusion studies. The validation proved that the method was reliable, repeatable, accurate and precise for the API, which illustrates reputable HPLC results. The limit of detection (LOD) was determined to be 0.01290 µg/ml and the limit of quantification (LOQ) was 0.10330 µg/ml.

To determine the aqueous solubility of clofazimine, it was examined in a phosphate buffer solution (PBS) (pH 7.4) at 32° C (pH and temperature mimicked blood). Lu *et al*. (2011:5189) stated clofazimine is virtually insoluble in a medium at pH 7.0, which is also supported by numerous other authors and is linked to its highly hydrophobic nature. The result regarding the aqueous solubility of clofazimine in PBS (pH 7.4) was inconclusive, but according to recent literature from Pubchem (2015), the API has a solubility of 0.000225 mg/ml. Due to the hydrophobic (low aqueous solubility) nature of clofazimine; it was expected to have a high log D value. A log D value of 4.60 was determined and correlated to the expected outcome, but does not appear to be ideal API for topical delivery as a range between 1 and 4 is required for optimal penetration (Naik *et al*., 2000:319).

The final dispersion was chosen by characterising a range of clofazimine liposome and proliposome concentrations according to the optimal EE% ratio determined by Patel and Misra (1999:359). The characterisation included transmission electron microscopy (TEM), droplet size and distribution, zeta-potential, pH, viscosity and EE%. The concentration that represented the best suited characterisation results and highest EE% was selected for further studies. The ideal concentration was a 2% concentration of clofazimine encapsulated in liposomes ((**CL2**)) prepared in a ratio of 1.00:7.85:1.00 (phosphatidylcholine:cholesterol:clofazimine) (Patel & Misra, 1999:359). The same concentration and ratio implemented for the liposomes was used in the preparation of the proliposomes ((**CPL2**)) to eliminate any possible variation when

comparing the two dispersions. Thus, two vesicle systems were prepared with the encapsulated API for further investigation.

Characterisation of the final dispersions illustrated the best results for suitable dispersions. The TEM images gave a good representation of successfully formed vesicles for both systems, also ranging within the size parameters proposed by literature (Elzainy *et al*., 2005:282). The dispersion samples illustrated a broad range of droplet sizes; hence a more heterogeneous distribution was seen. The liposomes had larger droplet sizes than the proliposomes, which was accounted for in the heterogeneity between the dispersions, and corresponded acceptably to literature regarding size and distribution (Elzainy *et al*., 2005:282, Shah *et al*., 2014:65). A highly positive zeta-potential was found for both dispersions, illustrating a good stability for the dispersions as no flocculation or aggregation would occur (Sutradhar *et al*., 2013:1). The pH of the dispersions corresponded with the hydration buffer (pH 5), indicating none of the constituents used during the preparation influenced the surface charge of the vesicles. Both dispersions' viscosity was rather increased and ideal for longer exposure time to the skin. Even though the liposomes exhibited a higher viscosity, both would be advantageous for topical delivery. The EE% of the liposomes correlated closely to what was proposed by Patel and Misra (1999:359) and was higher than the proliposomes encapsulation of the API. These ideal characteristic properties were proven for the use of the 2% dispersions for further studies.

The membrane release studies showed a similar linear release of clofazimine from the liposomes and proliposomes, illustrating a successful release of the API from both vesicle systems. The successful release excluded any release problems, thus if no topical delivery occurred during the skin diffusion studies the release would not be the problem. Liposomes had the best release between the two systems but the difference was insignificant, as stated by statistical analysis.

Transdermal diffusion of the API from the dispersions was ruled out due to the absence of any API in the receptor phase. Topical diffusion was confirmed with the presence of the API in the stratum corneum-epidermis and epidermis-dermis from both dispersions. The penetration of the API into the stratum corneum-epidermis was the expected result due to the lipophilic nature of this skin layer and clofazimine. The permeation of the API to the targeted epidermis-dermis illustrates how the vesicle systems theoretically improved the solubility of the API by concentrating the lipophilic clofazimine in a hydrophilic skin layer. The proliposomes experienced the highest concentration to the intended layer and consequently, had the best topical delivery between the two dispersions.

The cytotoxicity of the vesicle systems *in vitro* showed a big difference compared to the free drug samples. Compared to the control sample, the vesicle system experienced an almost non-cytotoxic effect on the HaCaT cells regardless of the increase in sample concentration; a noticeable difference was seen from the free drug, as the sample concentrations increased. The free drugs' cytotoxicity increased proportionately to the increase in sample concentration and the cytotoxicity advanced from a level of weakly cytotoxic to strongly cytotoxic. These results also correlated to literature (Metha, 1996:1896), as the employed liposomes vesicle system improved the level of cytotoxicity of clofazimine by exerting its protective effect on the drug. Hence, the implementation of a vesicle system decreases the degree of cytotoxicity of clofazimine on the HaCaT cell line.

Future prospects include:

- Investigate and formulate the vesicle systems into a gel, cream or ointment and compare by membrane release, topical and transdermal diffusion studies.
- Stability testing of dispersions, gel, cream and ointment to ensure its validity over an extended period.
- Metabolic investigation of the API to evaluate its biotransformation in the skin.
- *In vivo* studies to determine the cytotoxicity effect the API has on human skin.
- Investigate the *in vitro* efficacy and minimum inhibitory concentration of the dispersions against a TB strain infected in a cell line.

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APPENDIX A

Analytical method for the determination of clofazimine

A.1 Validation

The HPLC analytical system was used throughout this research study for the quantification of CLF. In order to acquire reliable results, a validation process of the analytical procedure was needed to establish a successful method that will match or better the minimum requirements in accordance to the Food and Drug Administration (FDA) guidelines. The HPLC is a dependable analytical apparatus and in order to validate a method for CLF, the following HPLC parameters have to operate within acceptable criteria, i.e. linearity, accuracy, limit of detection (LOD), lower limit of quantification (LLOQ), precision, ruggedness, system repeatability, specificity and robustness, to establish viable and publishable results. The analysis and detection of CLF encapsulated in liposomal vesicle systems for topical delivery was quantified by HPLC.

A.2 Chromatographic conditions

The development of a suitable analytical method for CLF was performed in collaboration with Prof J L du Preez from the Analytical Technology Laboratory (ATL) at the North-West University, Potchefstroom. Miss Helene Joubert used the same API and thus the validation results are the same. The laboratory conveyed a controlled environment, at a room temperature of 25 °C, when the analysis was performed. The following conditions were established for the method:

Analytical instrument: The analysis of CLF was performed on an Agilent 1100 series HPLC. This instrument was equipped with an Agilent 1100 isocratic pump, auto sampler and ultra violet (UV) detector (Agilent Technologies, Palo Alto, CA). The HPLC system was installed with ChemStation rev. A10.02 data acquisition and analysis software.

Column: A Venusil XBP C18 (2) column consisting of ultra-pure silica, (150 x 4.6 mm) with a 5 µm particle size was used during the HPLC analysis (Agela Technologies, Newark, DE).

Mobile phase: HPLC water with 0.005 M of octane sulphonic acid-Na was needed for the preparation of the mobile phase. Orthophosphoric

	acid was used to adjust the pH to 3.5. A 70:30 ratio of acetonitrile:buffer was mixed to create the mobile phase.
Stop time:	10.0 min
Flow rate:	1.0 ml/min
Injection volume:	25 µl
Detection:	UV detector at wavelength 284 nm
Retention time:	± 5.2 min
Solvent:	A 50:50 mixture of methanol and ultra-pure water with 1% glacial acetic acid was used throughout the validation process during the preparation of all standards and samples and will hereafter be referred to as solvent throughout this chapter.

A.3 Standard preparation

The preparation of a standard was required to have a known concentration of CLF in a specific volume, which can then be used to determine unknown concentrations of the API. The preparation of standard solutions was as follows: accurately weigh a mass of approximately 5 mg of CLF and transfer it to a volumetric flask (100 ml). The volumetric flask with the CLF content was dissolved and made up to volume with the solvent. To ensure the contents were properly dispersed, the solution was placed in an ultra-sonic bath. Thereafter two consecutive ten-fold dilutions were made to extend the concentration range for the determination of linearity; where after 1 ml of each standard solution was transferred to HPLC vials for analysis according to the desired chromatographic conditions. Different standard concentrations were used at certain parameters and will be indicated at each instance.

A.4 Sample preparation

The samples were prepared in accordance to the criteria for each validation parameter. The weighed CLF was dissolved in the solvent and diluted according to each parameters specification.

A.5 Parameters for HPLC validation

An HPLC method for the API was validated according to the following parameters: linearity, accuracy, LOD and LLOQ, precision, ruggedness, system repeatability, specificity and robustness.

A.5.1 Linearity

An analytical method's linearity is its capability to acquire experimental results, within a working range, that are corresponding to the concentration of a compound in a sample. The linearity of a method is determined by injecting a succession of concentration samples, ranging from 70 – 130% of the target's concentration, into the HPLC system for analysis to acquire results that can be plotted on a graph (concentration (µg/µl) versus response relationship) (ICH, 2005:5). The ideal linearity is portrayed by the relationship between the concentrations (µg/µl) and responses, i.e. the correlation coefficient (r^2). A high r^2 of larger than ≥ 0.97 for drugs is indicative of good linearity for a method (ICH, 2005:8; UNODC, 2009:11).

A.5.1.1 Sample solution preparation

The linearity of the analytical method was performed using a range of 12 different concentrations, as indicated in Table A.1, covering 70 – 130% of the target concentration of CLF to establish a standard curve (Figure A.1). The samples were transferred into HPLC vials for analysis. The analysis was performed in duplicate.

Table A.1: Peak area ratio and concentration of CLF

Standard (µg/ml)	Average peak area
0.075	18.3
0.188	32.6
0.377	58.4
0.753	113.7
1.130	171.2
1.506	228.6
3.765	554.7
7.530	1101.7
11.295	1662.0
15.060	2201.8
37.650	5587.1
75.300	11062.2
R^2	**1**
y-intercept	**-4.01×10^{-13}**
Slope	**146.04**

In Table A.1 and Figure A.1 it is observed that the concentration range for CLF is linear for the analytical method over a range of 0.075 – 75.300 µg/ml. The high correlation coefficient (r^2) of 1 indicates proficient stability from the analytical system and that the method is acceptable within the desired specifications.

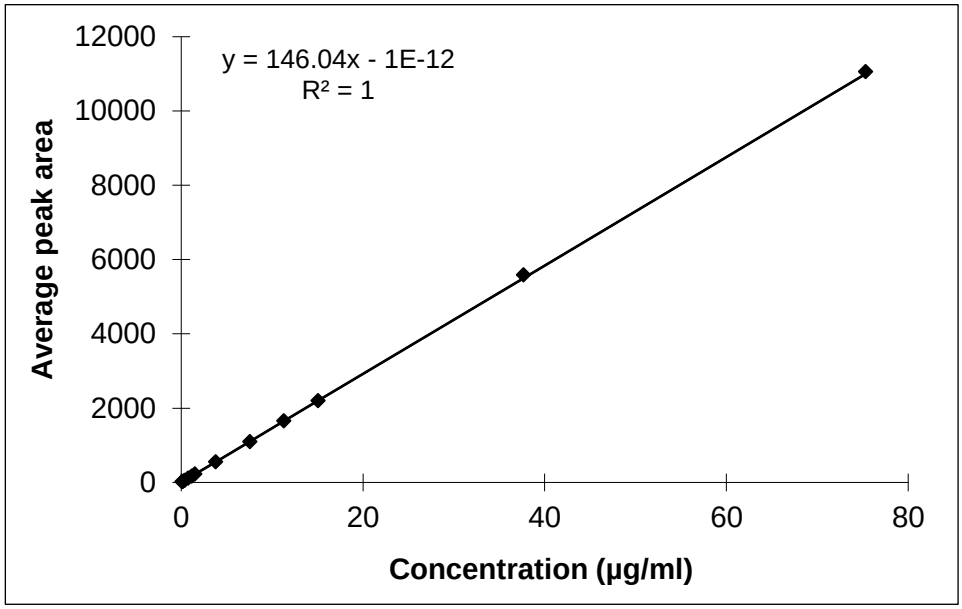

Figure A.1: Average peak area versus concentration to portray the linearity of the analytical method for CLF. The graph also indicates the correlation coefficient (r^2) used to evaluate linearity.

A.5.2 Accuracy

Accuracy is defined by the degree of closeness of the detected concentration to that of the true or accepted reference concentration. The accuracy is determined by means of evaluating the %recovery of an assay of known concentration in a sample (ICH, 2005:4). The criteria for acceptable recovery ranges within 90 – 110%, according to Shabir (2005:322), for active ingredients over a target concentration range of 80 – 120%, and that the bias for drug substance has to be smaller than 1%. The standard deviation (SD) and %relative standard deviation (%RSD) is used to determine the precision of the results obtained.

A.5.2.1 Preparation of standard solution

A standard solution of CLF was prepared by adding CLF (Table A.2) into a 100 ml volumetric flask, which was then made up to volume with the prepared solvent. The standard was transferred into HPLC vials and injected at five different volumes (1, 2, 5, 10, 15, and 20 µl) on the HPLC.

A.5.2.2 Preparation of sample solution

The samples were prepared according to the ICH criteria of using nine determinants over three concentration levels. Table A.2 indicates the weighed masses of CLF which was dissolved in the solvent and used for each concentrations level. Table A.3 is a representation of the %recovery of CLF over the three different concentration ranges. Analysis was performed in duplicate.

Table A.2: The mass of CLF weighed for the standard and different concentration levels

Preparation	Mass CLF weighed (mg)
Standard	17.66
Sample A	12.41
Sample B	10.33
Sample C	14.19

Table A.3: The accuracy results for CLF

Concentration spiked (µg/ml)	Peak area A	Peak area B	Mean peak area	Recovery	
				(µg/ml)	%
18.6 (1)	2681.5	2675.0	2678.3	18.7	100.4
15.5 (1)	2248.2	2241.8	2245.0	15.6	100.5
21.3 (1)	3023.9	3052.5	3038.2	21.3	99.9
43.4(2)	6221.8	6224.4	6223.1	44.0	101.4
36.2 (2)	5232.3	5225.4	5228.9	36.9	102.1
49.7 (2)	7081.2	7065.7	7073.5	50.1	100.9
93.1 (3)	13282.7	13291.9	13287.3	94.6	101.6
77.5 (3)	11162.1	11166.1	11164.1	79.4	102.5
106.4 (3)	15039.1	15030.0	15034.6	107.1	100.6
				Mean	**101.1**
				SD	**0.8**
				%RSD	**0.8**

From Table A.3, the mean recovery percentage of CLF from the system can be seen as 101.1%, which is within the acceptable criteria range. Thus, the accuracy is acceptable for the analytic system.

A.5.3 Limit of detection and lower limit of quantification

The LOD and LLOQ express the lowest concentration of an analyte that can be truly measured using an established analytical method. The LOD is thus important to detect the absence or presence of an analyte with no quantification by the analytical method. The LLOQ is important to accurately measure low levels of an analyte in sample matrices within acceptable accuracy and precision. The ICH (2005:11) and Shabir (2009:322) uses a signal-to-noise ratio to determine the accepted criteria. According to these sources the accepted criteria for LOD are a 3:1 signal-noise-ratio and a %RSD of less than 20%. For LLOQ the best assessment for low concentrations is a %RSD below 10% and not above 15%.

A.5.3.1 Preparation of sample solution

The LOD and LLOQ sample preparation requires only a single concentration of CLF. The sample was transferred into a HPLC vial where it was injected seven times per injection volume (2.5, 5.0, 10.0, 15.0, and 20.0 µl), as indicated in Table A.4.

Table A.4: Limits of detection (LOD) and quantitation (LLOQ) data

Injection volume (µl)	**2.5**	**5.0**	**10.0**	**15.0**	**20.0**
Concentration (µg/ml)	0.01290	0.02580	0.05165	0.07750	0.10330
Peak area	3.250	6.358	11.597	16.350	20.686
	3.403	6.460	11.478	16.168	20.795
	3.489	6.277	11.507	15.759	20.696
	3.288	6.443	11.605	16.046	20.642
	3.362	6.177	11.321	16.078	20.673
	3.341	6.294	11.138	16.187	20.573
	3.114	6.049	11.186	16.206	20.666
Mean peak area	3.321	6.294	11.405	16.113	20.676
SD	0.111	0.135	0.177	0.171	0.062
%RSD	3.338	2.147	1.551	0.131	0.024

Table A.4 indicates the LOD of CLF for the analytical system as 0.01290 µg/ml with a %RSD of 3.338% and the LLOQ for CLF was 0.10330 µg/ml with a %RSD of 0.024%. The %RSD is within the required range for the detection of CLF and indicates the system provides the minimum amount of variation between samples.

A.5.4 Precision

The precision of an analytical method is a measurement of the degree of agreement between separate test results obtained from repeat samplings of a uniform sample. This then expresses the random errors occurring in the method. The precision of an analytical procedure is subject to two measurements, i.e. reproducibility (inter-day repeatability) and repeatability (intra-day repeatability). Reproducibility is used to assure the same results are acquired in different laboratories, while repeatability is similar, but refers to using the same analyst with the same instrument over a short time frame in a laboratory (ICH, 2005:4; UNODC, 2009:11).

A.5.4.1 Repeatability (intra-day precision)

According to the USP, precision is suggested as the RSD of an array of measurements. The RSD is expressed in terms of percentage and the accepted %RSD criterion for intra-day repeatability is ± 2% for drug products (Shabir, 2009:322).

A.5.4.1.1 Preparation of standard solution

A standard solution of CLF was prepared by adding CLF (Day 1: 17.66 mg) into a 100 ml volumetric flask that was made up to volume with the prepared solvent. The standard was transferred into HPLC vials and injected at six different volumes (1, 2, 5, 10, 15 and 20 µl) on the HPLC.

A.5.4.1.2 Sample solution preparation

Table A.5: Intra-day repeatability data for CLF

Concentration spiked (µg/ml)	Peak area A	Peak area B	Mean peak area	Recovery	
				(µg/ml)	%
18.6 (1)	2681.5	2675.0	2678.3	18.7	100.4
15.5 (1)	2248.2	2241.8	2245.0	15.6	100.5
21.3 (1)	3023.9	3052.5	3038.2	21.3	99.9
43.4 (2)	6221.8	6224.4	6223.1	44.0	101.4
36.2 (2)	5232.3	5225.4	5228.9	36.9	102.1
49.7 (2)	7081.2	7065.7	7073.5	50.1	100.9
93.1 (3)	13282.7	13291.9	13287.3	94.6	101.6
77.5 (3)	11162.1	11166.1	11164.1	79.4	102.5
106.4 (3)	15039.1	15030.0	15034.6	107.1	100.6
				Mean	101.1
				SD	0.8
				%RSD	0.8

The sample solution consisted of nine different concentrations covering a range of three different concentration levels (3 x 80%, 3 x 100 % and 3 x 120%) of CLF, respectively (Table A.5). The samples were transferred into HPLC vials for analysis on the same day. Analysis was performed in duplicate.

The %RSD (Table A.5) for the intra-day repeatability is 0.8%, which is within the accepted criteria recommended by the ICH, indicating a precise method.

A.5.4.2 Reproducibility (inter-day precision)

The acceptable %RSD for inter-day repeatability, according to Shabir (2009:322), is ± 2% for drug products and according to Du Preez (2010a:7), a %RSD of ≤ 5% is acceptable.

A.5.4.2.1 Preparation of standard solution

A standard solution of CLF for Days 2 and 3 were prepared by adding CLF (Day 2: 5.31 mg; Day 3: 5.22 mg) into a 100 ml volumetric flask which was then made up to volume with the prepared solvent, respectively. The standard was transferred into HPLC vials and injected at five different volumes (5, 10, 15, 20 and 25 µl) on the HPLC.

A.5.4.2.2 Sample solution preparation

The sample solution for Days 2 and 3 were prepared by weighing three different masses of CLF per day and adding it to three separate 100 ml volumetric flasks, which was then made up to volume with the prepared solvent. The samples were transferred into HPLC vials for analysis on Days 2 and 3, respectively. Analysis was performed in duplicate.

Table A.6: Inter-day repeatability data for CLF

CLF	Day 1	Day 2	Day 3	Mean between days
%	101.40	102.30	98.10	
	102.13	99.92	98.09	
	100.93	97.08	98.38	
Mean	101.49	99.76	98.20	99.82
SD	0.49	2.12	0.13	1.84
%RSD	0.49	2.13	0.13	1.85

A reproducibility %RSD value of 1.85%, as shown in Table A.6, indicates CLF was acceptable within the required criteria for inter-day repeatability.

A.5.5 Ruggedness

The USP regards ruggedness within laboratory variations. This suggests that ruggedness is a measurement of the capacity to which an analytical system can withstand any uncontrollable variation, such as different analysts, or different days, or different equipment being used within the same laboratory (UNODC, 2009:63).

A.5.5.1 Sample stability

The resistance of compounds against chemical changes or physical disintegration and decomposition is referred to as the stability of a sample (UNODC, 2009:63). The stability is measured as the amount of degradation which is indicated by the %RSD and is usually performed under predetermined conditions and analysed at specific time intervals. The acceptable amount of degradation is indicated by a %RSD of ± 2%.

A.5.5.2 Preparation of sample solution

The sample solution contained 5.20 mg of CLF, which was made up to volume in a 100 ml volumetric flask with the solvent; 1 ml of the solution was transferred into a HPLC vial for analysis. Over a period of 24 h, 10 µl per hour of sample was injected for analysis on the HPLC (Table A.7). Analysis was performed in duplicate.

Table A.7: Ruggedness results of CLF during hourly injections for 24 h

Time(h)	Peak area	Recovery (%)	Time(h)	Peak Area	Recovery(%)
0	5300.8	100.0	13	5297.4	99.9
1	5359.3	101.1	14	5311.7	100.2
2	5338.3	100.7	15	5312.6	100.2
3	5320.3	100.4	16	5293.2	99.9
4	5321.2	100.4	17	5296.8	99.9
5	5290.7	99.8	18	5290.3	99.8
6	5283.9	99.7	19	5288.8	99.8
7	5299.4	100.0	20	5291.1	99.8
8	5287.8	99.8	21	5286.8	99.7
9	5288.7	99.8	22	5292.4	99.8
10	5295.9	99.9	23	5278.4	99.6
11	5299.4	100.0	24	5305.0	100.1
12	5305.8	100.1			
			Mean	5301.4	100.0
			SD	17.72	0.33
			%RSD	0.33	0.33

Table A.7 indicates the results after a 24-hour period. CLF had a peak %RSD of 0.33% and verified as stable over a 24 h period. This is ideal for release and diffusion studies that would range over 6 h and 12 h periods, respectively, and would not degrade.

A.5.6 System repeatability

The USP defines the repeatability as using an analytical procedure over a short time frame in a laboratory where the analyst and equipment stays unchanged and uniform to determine the closeness of agreement of consecutive sample measurements. The acceptable %RSD for the peak area and retention time of the system is less than 2%.

A.5.6.1 Preparation of sample solution

A single CLF sample was prepared at 100% of the target analyte concentration. Six replicates of the sample solution were injected for analysis on the same day under the same conditions. The analysis was performed in duplicate.

Table A.8: System repeatability data of CLF

Injection number	Peak area	Retention times (min)
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
Mean	5279.0	3.879
SD	13.69	0.009
RSD%	0.26	0.224

The %RSD of 0.224% for the systems repeatability is extremely good and within the acceptable criteria range.

A.5.7 Specificity

According to the ICH (2005:4), the specificity of an analytical method is its ability to accurately detect an analyte in the presence of e.g. the matrix, impurities, or degradants. Thus, the specificity would be acceptable with the pure identification of the target analyte between compounds with similar structures (ICH 2005:4). A 100% standard solution was prepared for the evaluation of specificity.

A.5.7.1 Preparation of sample solution

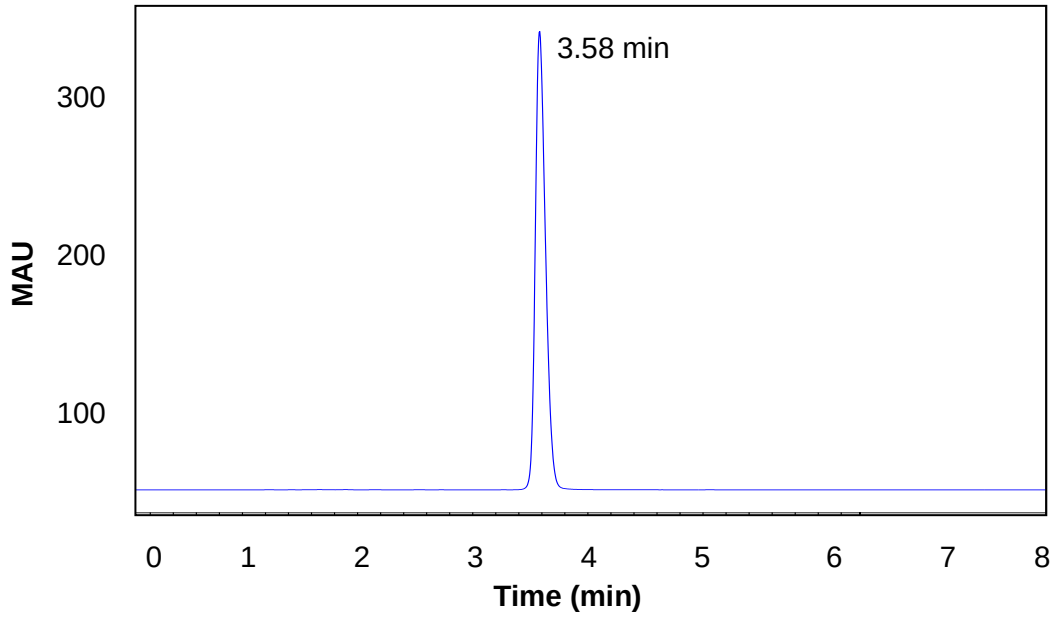

Figure A.2: CLF standard for specificity analysis

The standard solution (1 ml; Figure A.2) was placed in four different test-tubes and to each test tube, 1 ml of the following reagents was added respectively: distilled water (Figure A.3), 0.1 M hydrochloric acid (Figure A.4), 0.1 M sodium hydroxide (Figure A.5, and 10% peroxide (Figure A.6). The prepared test-tube samples were analysed immediately on the HPLC and then analysed again the following day.

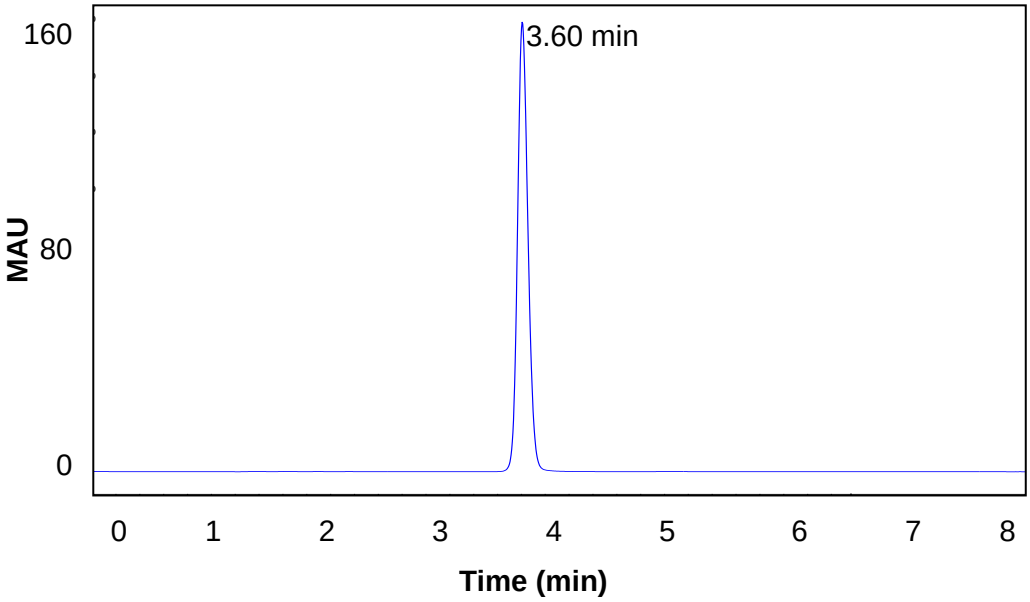

Figure A.3: Specificity analysis results using distilled water as a reagent

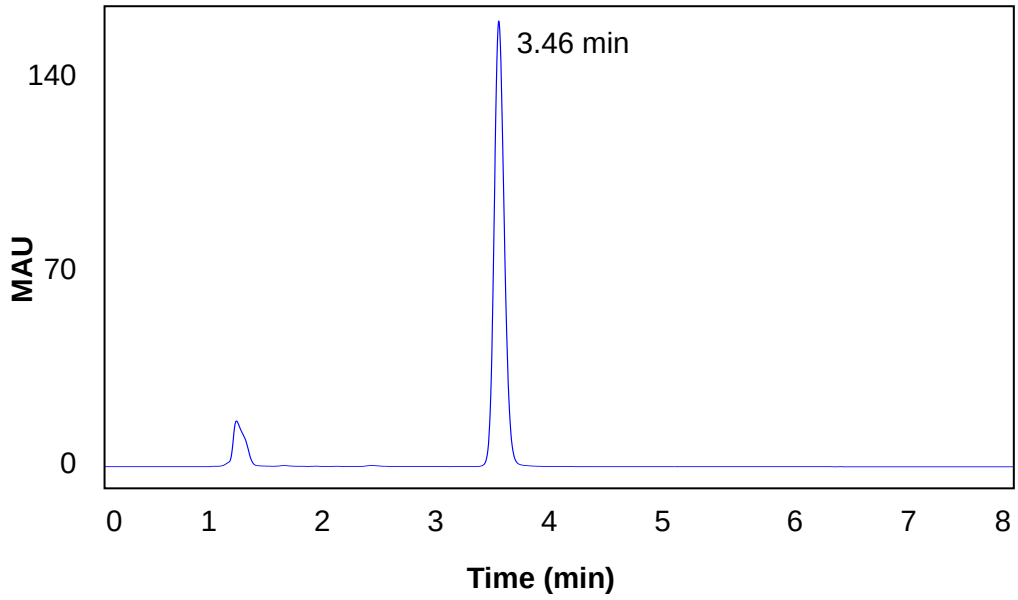

Figure A.4: Specificity analysis results using 0.1 M hydrochloric acid as a reagent

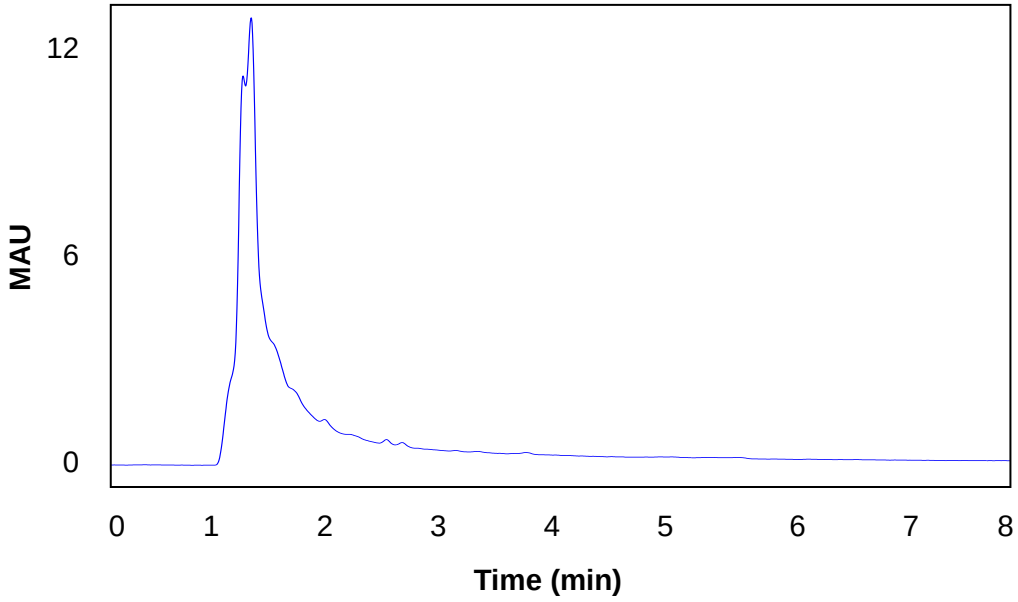

Figure A.5: Specificity analysis results using 0.1 M sodium hydroxide as a reagent

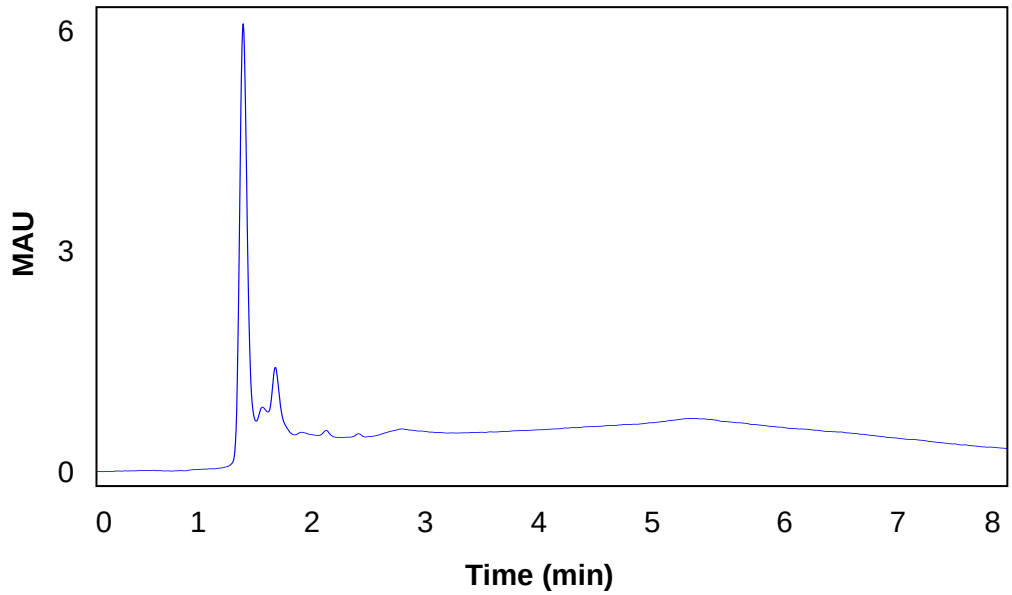

Figure A.6: Specificity analysis results using 10% peroxide as a reagent

It is important the degraded samples do not interfere with the determination of CLF. Figure A.2 is the standard to which Figures A.3, A.4, A.5 and A.6 was compared to illustrate the specificity of CLF in the four different reagents. From the chromatograms in Figures A.3 and A.4, no additive interfered with the detection of the CLF peak. Figures A.5 and A.6 showed the reagent completely degraded the API, yielding no result. Despite this the results prove the analytical methods specificity to be acceptable.

A.5.8 Robustness

The robustness of an analytical method is when meagre, but premeditated variations are deliberately applied to the primary parameters to test the procedures capacity to remain unaltered. This will provide an indication of the systems suitability during normal usage (UNODC, 2009:63).

A.5.8.1 Preparation of standard solution

A standard solution of CLF was prepared by adding 5 mg of CLF into a 100 ml volumetric flask and made up to volume with the prepared solvent. The standard was transferred into HPLC vials and injected at three different volumes (5, 4, and 6 µl) and detected at different wavelengths (284, 280, and 288 nm) on the HPLC, respectively.

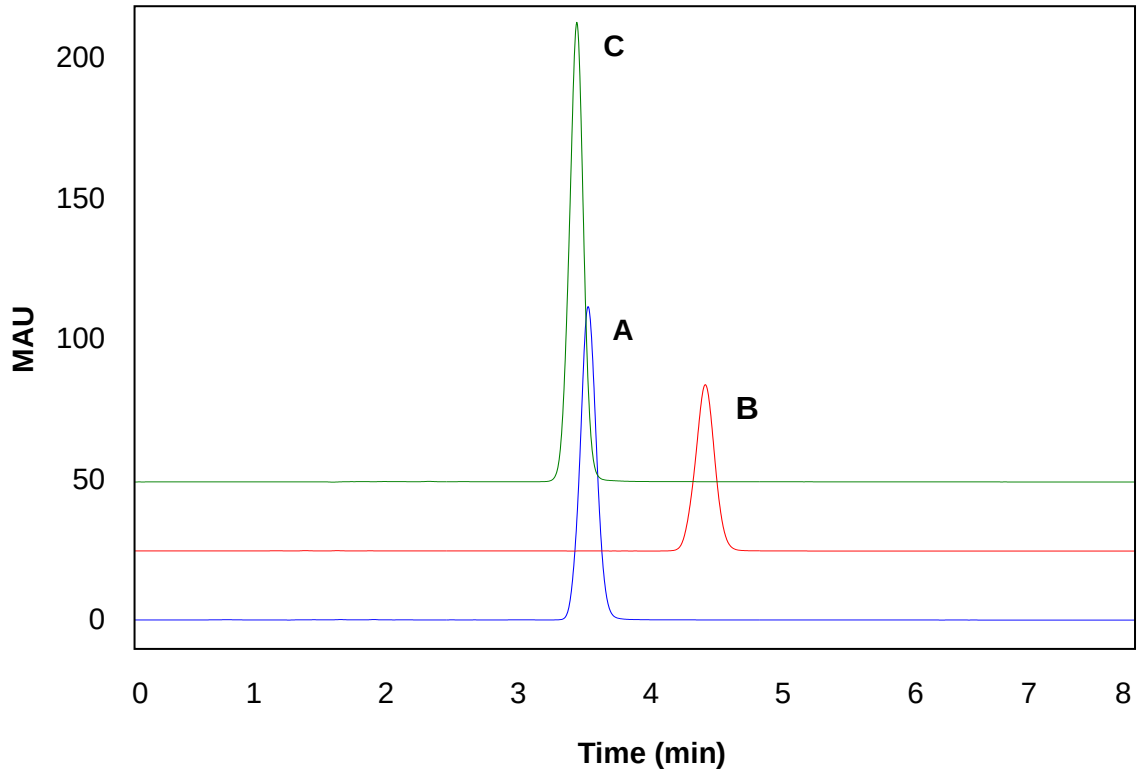

Figure A.7: Chromatogram results after the different injection volumes and wavelengths. Peak **A** had an injection volume of 5 µl and was detected at a wavelength of 284 nm. Peak **B** had an injection volume of 4 µl and was detected at a wavelength of 280 nm. Peak **C** had an injection volume of 6 µl and was detected at a wavelength of 288 nm.

The chromatogram in Figure A.7 illustrates the peaks after specific changes were made to the system to determine the robustness of the system. From Peaks A, B and C it is clear the system provides reliable results and proves to be acceptable for analysis.

A.6 Conclusion

A basic HPLC method for the determination of CLF has been established and found reliable and suitable. The validation of the method is approved in accordance to linearity, accuracy, limits of detection and quantification, precision, ruggedness, system repeatability, specificity and robustness. The validation of the HPLC method has been established as being sufficiently dependable and sensitive for the verification of the concentration for CLF. The developed method responded effectively and should be suitable for the analysis of CLF in the vesicle systems during the encapsulation efficacy, diffusion studies, metabolism and quality control.

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APPENDIX B

Formulation of liposomes and proliposomes containing clofazimine

B.1 Introduction

The importance of formulating is to create a drug administration system which can consequently deliver drug molecules at a therapeutic level to a targeted region (Wang *et al*., 2005:59). One of the main areas of focus for this research study was to formulate CLF encapsulated in liposomes and proliposomes to evaluate their suitability as physical, chemical and biologically stable topical products. It is evident that the properties of the stratum corneum seem limiting for topical drug delivery, but there have been significant advances in understanding vesicle systems regarding their physicochemical properties and their ingredients to possibly bypass the stratum corneum (Walters, 2002:319).

Liposomes have the advantage of controlled drug release and drug protection, but these vesicle systems are also subject to degradation by means of oxidation, leakage of the drug and hydrolysis. Thus, the efficient formulation and lyophilisation is essential for successful liposomal formulation and drug encapsulation (Chang *et al*., 2012:2). In order to determine the final dispersion to be used, two vesicle systems (i.e. liposomes and proliposomes) were preformulated and evaluated according to their morphology, droplet size distribution, zeta-potential, pH, viscosity and drug encapsulation efficacy (EE%). The formulation procedure by Patel and Misra (1999:359) was followed and adapted accordingly. The preformulations spanned three concentration ranges, i.e. 1%, 2% and 3%, for both vesicle systems in order to identify the suitable dispersion for further studies. The characteristic properties of the preformulations were evaluated to determine a final dispersion, which was also characterised in Appendix C.

B.2 Materials and methods

B.2.1 Ingredients used during formulation

The ingredients in Table B.1 were used for the preformulation of the vesicle and provesicle systems.

Table B.1: Ingredients in preparing vesicle systems

Ingredients	Purpose	Supplier	Batch number
CLF	API	Sangrose Laboratories Pvt. Ltd	Flagship Programme
Egg lecithin (L-α-Phosphatidylcholine)	Penetration enhancer	Sigma-Aldrich	61755
Cholesterol	Membrane stabiliser	Sigma-Aldrich	421393
α-Tocopherol	Lipophilic antioxidant	Sigma-Aldrich	T3376
Chloroform	Solvent	Merck Millipore	102245
Methanol analytical reagent (AR) grade	Solvent	ACE Chemicals	V800258
Sodium acetate (tri-hydrate)	Acetate buffer salt	Sigma-Aldrich	S7670
Glacial acetic acid	Adjust pH of buffer	Sigma-Aldrich	695092
D-Sorbitol	Water-soluble carrier	Merck Millipore	107758

B.2.1.1 Clofazimine

CLF is a riminophenazine and possesses a highly lipophilic character (log P of 7.6) and due to this characterisation, it will be entrapped in the lipid bilayer of the liposomes system (Mane *et al*., 2012:741, PubChem, 2015). The lipid bilayer is more suited for the hydrophobic nature of CLF. The concentration of the API for the preformulations ranged between 1%, 2% and 3% for both vesicle systems.

B.2.1.2 Egg lecithin (PC)

Liposomes are formed by an adverse reaction when phospholipids are exposed to water, which is due to phospholipids having an amphiphilic character, i.e. their hydrophilic head and hydrophobic tail sections aggregate towards each other. This character leads to the formation of a phospholipid bilayer with an encapsulated water domain (Gómez-Henz & Fernández-Romero, 2005:10).

In this study, egg phosphatidylcholine (PC) was used as the phospholipid of choice. Phosphatidylcholine is part of the glycerophospholipid family and is most prevalent in mammalian cells. The fundamental role of PC is to protect the integrity of the membrane and cell structure (Wiedmer *et al*., 2002:427); it also possesses a neutral charge and is not especially expensive (Gómez-Henz & Fernández-Romero, 2005:10).

B.2.1.3 Cholesterol

One of the main components of a biological cell membrane is cholesterol. Cholesterol has the ability to affect the mechanical character of cells, i.e. enhancing mechanical strength, changing the elastic property of cells and strengthening the packing density of lipids. As a result, the cholesterol creates a less permeable and stronger membrane. Cholesterol has been used for liposomal formulations in the past and was also used in the preformulation and the final vesicle- and provesicle system for this study (Magarkar *et al.,* 2014:1).

B.2.1.4 α-Tocopherol

The vitamin α-tocopherol, more commonly known as a natural vitamin E, plays the role of acting as a fundamental anti-oxidant. The chemical structure of this vitamin gives α-tocopherol its distinct anti-oxidant property, i.e. possesses a free hydroxyl group on its aromatic ring. This anti-oxidant vitamin is found in the low density lipoproteins (LDL) of cell membranes. Due to its anti-oxidant effects in the cell membranes of humans, α-tocopherol protects the membranes from oxidation by capturing the free radicals (Engin, 2009:855, Num *et al*., 2016:4). This is important during the formulation of liposomes as they are prone to oxidation, hence, adding an anti-oxidant will increase the stability of the liposomes.

B.2.1.5 Chloroform

Chloroform is also known as trichloromethane and is a volatile, colourless substance with an ether-like odour. It was formally used as an anaesthetic when inhaled before it was banned, but now it is more commonly used as a solvent in laboratories (Pubchem, 2016). In this study, chloroform was mixed with methanol and used as a solvent when mixed with the PC to produce a lipid solvent mixture, which was then evaporated during preformulation and the final vesicle- and provesicle system of this study.

B.2.1.6 Methanol

Methanol is a flammable and colourless liquid with a wide variety of uses. It can be used for chemical synthesis, e.g. constructing acetic acid or formaldehyde and can be used in laboratories as a solvent (Pubchem, 2016). During this study methanol was mixed with chloroform in a 1:2 ratio and used as a solvent during preformulation and the final vesicle and provesicle system.

B.2.1.7 Sodium acetate (tri-hydrate)

Sodium acetate (tri-hydrate) is a tri-hydrate sodium salt of acetic acid. This salt form of acetic acid can alter the pH of a solution by generating a sodium bicarbonate, which will cause the pH to increase (Pubchem, 2016). In this study, the sodium acetate (tri-hydrate) was one of the components used to prepare an acetate buffer. The prepared buffer was used as a hydration medium after the solvent mixture was evaporated.

B.2.1.8 Glacial acetic acid

Glacial acetic acid is one of the most basic carboxylic acids and is used as a chemical reagent and industrial chemical (HMBD, 2016). It possesses an extremely potent vinegar odour and is a clean colourless liquid (Pubchem, 2016). During this study, it was used to adjust the acetate buffer to a pH of 5 after the sodium acetate (tri-hydrate) was added to water. The acetate buffer was then used to hydrate the thin-film that formed on the surface of the round bottomed flask after evaporation.

B.2.1.9 Sorbitol

Sorbitol is used as an aid to improve the stability of the vesicle system. Sorbitol is a type of sugar and was used as a water soluble carrier system to prepare the formulation of the provesicle system in this study (Tee *et al*., 2000:111).

B.2.2 Preformulation of the vesicle- and provesicle system

B.2.2.1 Formulation of the vesicle system

In this study, two different vesicle systems were prepared and evaluated, both with and without the API, i.e. placebo liposomes, liposomes containing CLF, placebo proliposomes and proliposomes containing CLF. These vesicle systems were prepared according to Patel and Misra (1999:359), which uses the thin-film hydration method. The method used by Patel and Misra (1999:359) was thereafter adapted and combined with the carrier method of Madni *et al*. (2014:412) to formulate the provesicle system, which includes a hydration step in order to form a liposomal dispersion.

B.2.2.2 Preformulation and testing of the vesicle system

In this study, the lipid film hydration technique was used to prepare the preformulations and final liposomes. The preformulations were all prepared at different concentrations (1%, 2% and 3%) to determine the ideal liposomes to be used during further studies. The liposomes containing CLF were formulated according to the procedure Patel and Misra (1999:359) described and the

ratio of the constituents for the liposomes was chosen according to their best EE% (approximately 71.0%). The ratio of the constituents were as follows, PC:cholesterol in the ratio of 7.70:1.00 for the placebo liposomes and CLF:PC:cholesterol in the ratio of 1.00:7.85:1.00 for the liposomes containing CLF (Patel & Misra, 1999:359) and these were kept constant throughout the evaluation of the liposomes. Thus, the following vesicle system concentrations were prepared for the characterisation: 1% placebo liposomes (**PL1**), 2% placebo liposomes (**PL2**), 3% placebo liposomes (**PL3**), liposomes containing 1% CLF (**CL1**), liposomes containing 2% CLF (**CL2**), liposomes containing 3% CLF (**CL3**).

B.2.2.3 Preformulation method of placebo liposomes

The placebo preformulations were prepared in a PC:cholesterol ratio of 7.70:1.00. The amount of constituents increased as the concentration ratio of the dispersion increased (Table B.2). The PC and cholesterol were dissolved in 10 ml of a methanol:chloroform (1:2) system in a round bottomed flask, which contained the solution and α-tocopherol, which was added equivalent to 1% of the amount of PC taken. The solvent mixture was then evaporated with a Büchi® Rotavapor® RII at a temperature of 45 °C until a dry lipid film was procured on the flask's surface. To hydrate the film, 10 ml of an acetate buffer (pH 5) was used, where after it was sonicated with a sonication probe. The liposomal dispersion was then placed on an ice bath and sonicated six times for 2 min intervals (Patel & Misra, 1999:359).

Table B.2: Formula for the placebo liposomes

Ingredients	(PL1)	(PL2)	(PL3)
Cholesterol	100 mg	200 mg	300 mg
PC	770 mg	1540 mg	2310 mg
α-Tocopherol	8 µl	16 µl	24 µl
Methanol/chloroform	10 ml	10 ml	10 ml
Acetate buffer (pH 5)	10 ml	10 ml	10 ml

B.2.2.4 Preformulation method of liposomes containing CLF

The liposomes containing CLF were prepared in CLF:PC:cholesterol (1.00:7.85:1.00). The API, PC and cholesterol were all correctly weighed according to the ratios that Patel and Misra (1999:359) used. The amount of constituents increased as the concentration ratio of the dispersion increased. The constituents were then dissolved in 10 ml of a methanol:chloroform (1:2) system in a round bottomed flask. In the flask containing the solution, α-tocopherol was also added equivalent to 1% of the amount of PC taken. The solvent mixture was then evaporated with a Büchi® Rotavapor® RII at a temperature of 37 °C until a dry lipid film was

procured on the flask surface. An acetate buffer (pH 5) was used to hydrate the film; where after it was sonicated. The liposomal dispersion was then placed on an ice bath and sonicated six times for 2 min intervals (Patel & Misra, 1999:359). Table B.3 indicates the amount of constituents used per concentration.

Table B.3: Formula for the liposomes containing CLF

Ingredients	(CL1)	(CL2)	(CL3)
CLF	100 mg	200 mg	300 mg
Cholesterol	100 mg	200 mg	300 mg
PC	785 mg	1570 mg	2355 mg
α-Tocopherol	8 µl	16 µl	24 µl
Methanol/chloroform	10 ml	10 ml	10 ml
Acetate buffer (pH 5)	10 ml	10 ml	10 ml

B.2.2.5 Formulation of provesicle system

Due to the stability problems associated with liposomes, an alternative vesicle system was formulated, i.e. proliposomes. The preformulation of proliposomes (placebo and proliposomes containing CLF was also prepared at concentrations of 1%, 2% and 3% in the same ratios as described by Patel and Misra (1999:359). Thus, the following provesicle system concentrations were prepared for the characterisation: 1% placebo proliposomes (**PPL1**), 2% placebo proliposomes (**PPL2**), 3% placebo proliposomes (**PPL3**), proliposomes containing 1% CLF (**CPL1**), proliposomes containing 2% CLF (**CPL2**), proliposomes containing 3% CLF (**CPL3**).

B.2.2.6 Preformulation method of placebo proliposomes

The placebo preformulations were prepared in PC:cholesterol (7.70:1.00), as described by Patel and Misra (1999:359). Three preformulations of proliposomes per concentration (1%, 2% and 3%) in the same ratios were prepared. The amount of constituents increased as the concentration ratio of the dispersion increased (Table B.4). The PC and cholesterol were dissolved in 10 ml of a methanol/chloroform system (1:2), in a flask, to which was added α-tocopherol equivalent to 1% of the amount of PC used (Table B.4). The solvent lipid mixture was added drop-wise to 4 g of sorbitol in a round bottomed flask and evaporated with a Büchi® Rotavapor® RII at a temperature of 50 °C. This was performed until all the solvent lipid mixture was added to the sorbitol and dried. The granules that formed were placed in a desiccator overnight for further drying. Upon use, the granules were hydrated with 10 ml of an acetate buffer (pH 5) and sonicated six times for 2 min intervals.

Table B.4: Formula for the placebo proliposomes

Ingredients	(PPL1)	(PPL2)	(PPL3)
Cholesterol	100 mg	200 mg	300 mg
PC	770 mg	1540 mg	2310 mg
α-Tocopherol	8 µl	16 µl	24 µl
Methanol/chloroform	10 ml	10 ml	10 ml
Acetate buffer (pH 5)	10 ml	10 ml	10 ml
Sorbitol	4 g	4 g	4 g

B.2.2.7 Preformulation of proliposomes containing CLF

Three preformulations of proliposomes, containing CLF of each concentration (1%, 2%, and 3%) in the same ratios, were prepared as described by Patel and Misra (1999:359). The API, PC and cholesterol were all correctly weighed according to their ratios, i.e. CLF:PC:cholesterol (1.00:7.85:1.00). The amount of constituents increased as the concentration ratio of the dispersion increased as indicated in Table B.5. The constituents were then dissolved in 10 ml of a methanol/chloroform system (1:2) in a flask. In this flask containing the solution, α-tocopherol was also added equivalent to 1% of the amount of PC taken (Patel & Misra, 1999:359). The total solvent mixture was then added drop-wise to 4 g of sorbitol in a round bottomed flask. The solvent mixture was then evaporated with a Büchi® Rotavapor® RII at a temperature of 50 °C. This was performed until the total mixture was added to the sorbitol and dried. The granules that formed were then placed in a desiccator for overnight drying. Upon use the granules was hydrated with 10 ml of the acetate buffer (pH 5) and sonicated six times for 2 min intervals (Patel & Misra, 1999:359).

Table B.5: Formula for the proliposomes containing CLF

Ingredients	(CPL1)	(CPL2)	(CPL3)
CLF	100 mg	200 mg	300 mg
Cholesterol	100 mg	200 mg	300 mg
PC	785 mg	1570 mg	2355 mg
α-Tocopherol	8 µl	16 µl	24 µl
Methanol/chloroform	10 ml	10 ml	10 ml
Acetate buffer (pH 5)	10 ml	10 ml	10 ml
Sorbitol	4 g	4 g	4 g

B.2.3 Physical characterisation of the preformulated liposomes and proliposomes

B.2.3.1 Morphology

A transmission electron microscope (TEM) was used to determine the presence of vesicles and their morphology. The API, α-tocopherol and acetate buffer (pH 5) were absent during the preparation of the liposomes as these constituents may contain components that can crystallise and cause damage to the TEM microscope. Milli-Q® water was used as a hydration medium during the formulation instead of the acetate buffer (pH 5). Visualisation was performed on a FEI Tecnai™ G2 high resolution TEM (FEI, Holland) at approximately 200 kV and operated by Dr A Jordaan (Laboratory for Electron Microscopy, North-West University, Potchefstroom Campus). The preformulations were prepared for the TEM by firstly diluting (x 10) the liposomes in Milli-Q® water and mixing it thoroughly. One drop of the solution was extracted and dropped on a copper carbon-coated 300 mesh grid. The grid was left to dry (10 min) and any excess amount of solution was removed by filter paper. After the solution was completely absorbed, a drop of osmium was used to stain the lipid films and left to evaporate for approximately 25 min. The samples were then treated with uranyl acetate and lead citrate and left to dry completely before the visualisation on the TEM (Ammar *et al.*, 2011:143, Wibroe *et al*., 2016:2)

B.2.3.2 Droplet size distribution

The Malvern Zetasizer Nano ZS (Zen 3600) (Malvern® Instruments Ltd, Worcester, United Kingdom) was used to determine the average droplet size and size distribution of the vesicles in the preformulations. The droplet size distribution indicates the average size of the vesicles in the liposomes and was used to determine whether or not the vesicles are uniformly spread throughout the dispersion; usually referred to as the polydispersity index (PdI). The sizes of the vesicles in the liposomes are indicated by the z-average, which indicates the average diameter (d.nm) of the vesicles. Three samples per system of each concentration were prepared, respectively. Three low concentration samples were prepared from each individually formulated dispersion (1%, 2%, and 3%), and injected into three disposable cells, respectively. The analysis was performed in triplicate.

B.2.3.3 Zeta-potential

A Malvern Zetasizer Nano ZS (Zen 3600) (Malvern® Instruments Ltd, Worcester, United Kingdom) was used to measure the zeta-potential of the prepared vesicle and provesicle systems. Zeta-potential refers to the degree of repulsion between charged particles of a similar nature to establish the potential stability of a colloidal system, i.e. dispersion (Shah *et al*.,

2014:66). The zeta-potential was measured a day after the samples were prepared. Three low concentration samples were prepared from each preformulation (n = 3) and injected into three disposable cells, respectively. A total of three measurements per sample were procured to calculate an average zeta-potential of each preformulation.

B.2.3.4 pH

The pH is an important factor when measuring the zeta-potential, as this may have an influence on the surface charge of the vesicles and alter the stability, agglomeration and dispersion of the vesicle systems. These effects can influence the zeta-potential and in addition to this, the pH was also measured (Berg *et al*., 2009:276). A Mettler Toledo pH meter FE20/FG2 (Greifensee, Switzerland) was used to determine the vesicle and provesicle systems' pH values under the same conditions; the pH meter was calibrated accordingly. The probe of the pH meter was placed into the 10 ml preformulated dispersions for measurements. Two measurements of each dispersion were taken in triplicate.

B.2.3.5 Rheology: viscosity of liposomes

The viscosity of a formulation is the quantitative measurement of the resistance of a fluid to flow. Thus, a level of high resistance to flow would indicate a high level of viscosity (Quiñones & Ghaly, 2008:62). The degree of a formulations viscosity can impact the delivery of a drug through directly influencing a drugs diffusion rate at a microstructural position (Ueda *et al*., 2009:753). A Brookfield Viscometer DV2T-LV (Stoughton, USA) was used to determine the viscosity of the preformulations. Three preformulated samples of each concentration (1%, 2%, and 3%) were prepared and on the day of the experiments (2 h prior) were placed in a pre-heated water bath at 25.5 °C .The samples reached a temperature equivalent to that of the water bath, where after the viscosity readings were taken. The Brookfield Viscometer contains a T-spindle (Stoughton, MA), which was inserted into 6.5 ml of each sample (without disturbing it) and set at a predetermined rotating rate, respectively. The viscosity measurements were measured at 10 sec intervals over a 2 min period, giving a total of 13 measurements per sample.

B.2.3.6 Encapsulation efficacy

The EE% calculation measures the difference between the total amount of drug encapsulated in the vesicles and the free drug in a sample, depicting the yield obtained. The EE% was measured by means of HPLC. Three prepared preformulations (10 ml) were added to individual Eppendorf® tubes and centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa). The conditions of the ultracentrifuge was set at a speed of 25 000 g for 30 min at

room temperature. After centrifugation, a pellet of the vesicles and a supernatant (containing un-encapsulated CLF formed. The supernatant represents the free drug that was not encapsulated in the vesicles. The supernatant was then diluted (x 50) with the solvent and added to HPLC vials for analysis, which was performed in triplicate. A standard solution was also prepared and injected to acquire a linear standard curve. The determination of the drug concentration in these vesicle systems was performed using the equation (y = mx + c) of the linear standard curve of CLF. The EE% of each vesicle system was calculated accordingly, as adapted from Xiang *et al*. (2009:186):

$$EE\% = \frac{\text{Drug (total)} - \text{Drug (supernatant)}}{\text{Drug (total)}} \times 100 \qquad \textbf{Equation B.1}$$

B.3 Results and discussion

B.3.1 Liposomes

B.3.1.1 Morphology

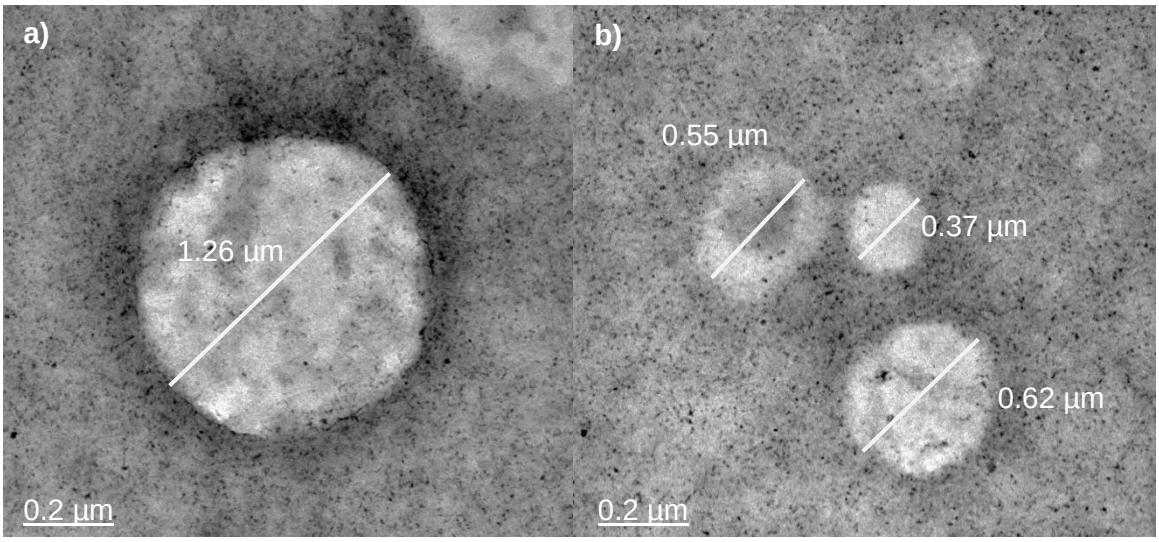


Figure B.1: Micrographs illustrating liposomes captured with the TEM at 200 kV; a) a single liposome and b) cluster of liposomal vesicles

The images of the liposomes were captured with a Gatan bottom mount digital camera on the TEM. In Figure B.1, a clear image is given in light of the formation and presence of vesicles, illustrating a single vesicle with a spherical shape. The dark section illustrates where the osmium rooted in the vesicle's lipid layer. The membrane (Figure B.1.a) is quite pronounced, indicating a strong membrane had formed. Figure B.1.b demonstrates an assembly of liposome vesicles of a similar size. The dark section, illustrating the membrane of the vesicles, is not as prominent as the vesicle in Figure B.1.a, but still formed a vesicle with a sphere shape. There is a clear formation of vesicles in Figure B.1.b, which indicates the success in formulating

liposomal vesicles. The vesicles shape in Figures B.1.a and B.1.b illustrate the accomplishment of formulating liposome vesicles.

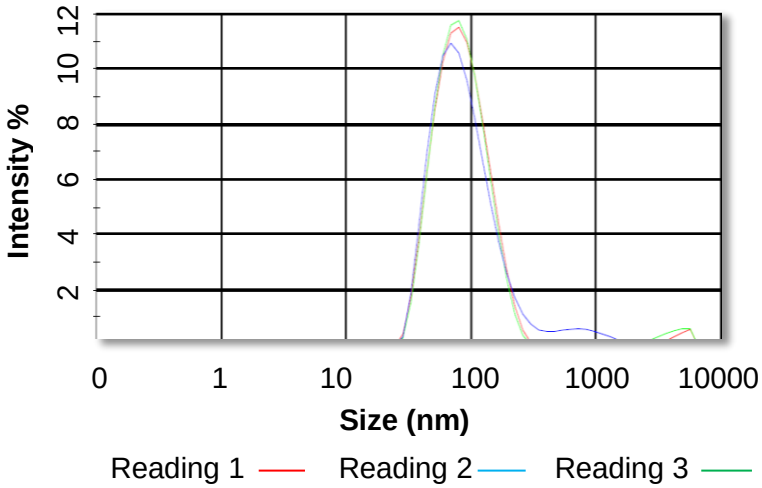
B.3.1.2 Droplet size distribution

Figures B.2 and B.3 illustrate the size distribution and the size of the vesicles in the prepared liposomes. The size distribution of a droplet is quantitatively evaluated according to its PdI value. The PdI measures the quality of the size distribution of particles in formulations and ranges between 0 and 1. A PdI value of less that 0.1 is defined as the highest quality of dispersion, i.e. monodisperse, while a value of less that 0.3 is also deemed as optimum by researchers. A PdI of less than 0.5 is also within acceptable limits, with regards to the dispersion quality, but would indicate a level of heterogeneity in the dispersion (Shah *et al*., 2014:65). According to Elzainy *et al*. (2005:282), the multilamellar vesicles (MLV) sizes range between 100 nm and 20 µm.

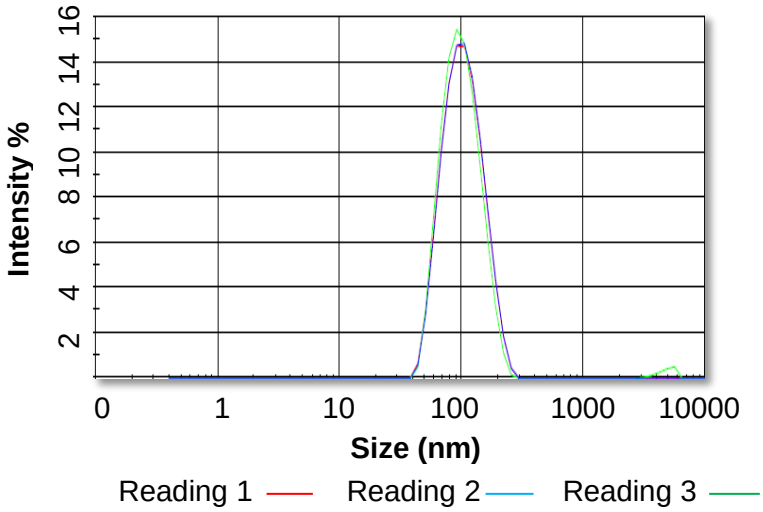
The placebo liposomes showed a good distribution between the (**PL1**), (**PL2**) and (**PL3**). The (**PL1**) had an average size of 74.690 ± 1.205 nm with a PdI of 0.19. The (**PL2**) had an average size of 81.720 ± 5.690 nm with a PdI of 0.13. The (**PL3**) had an average size of 95.930 ± 0.220 nm with a PdI of 0.10. This shows a good homogeneity spanning over the concentration range of the placebo liposomes, due to the low PdI values and the small standard deviation of the three measurements taken from each sample. The size of the placebo liposomes are smaller than expected, but are still close and within an acceptable range proposed by literature. These sizes are also compatible with the size range proposed by Naik *et al*. (2000:319) for topical delivery.

The liposomes containing CLF also showed a good distribution in the (**CL1**) and (**CL2**) dispersions. The (**CL3**) were not successful due to the formation of a paste and could not be sufficiently prepared for analysis. (**CL1**) and (**CL2**) experienced a higher level of heterogeneity than the placebo liposomes as both exhibited PdI values of 0.50 and 0.52, respectively. This indicated a more dispersed dispersion for both (**CL1**) and (**CL2**) dispersions, which represent an increased heterogeneity. The particle size of the (**CL1**) was 293.00 ± 89.26 nm, while the (**CL2**) showed an increase in particle size of 330.83 ± 4.40 nm. These sizes correlate to what is proposed in literature and within the acceptable size limit for topical delivery (Naik *et al*., 2000:319). The large standard deviation of the (**CL1**) is also indicative of the higher level of polydispersity in this dispersion.

a)



b)



c)

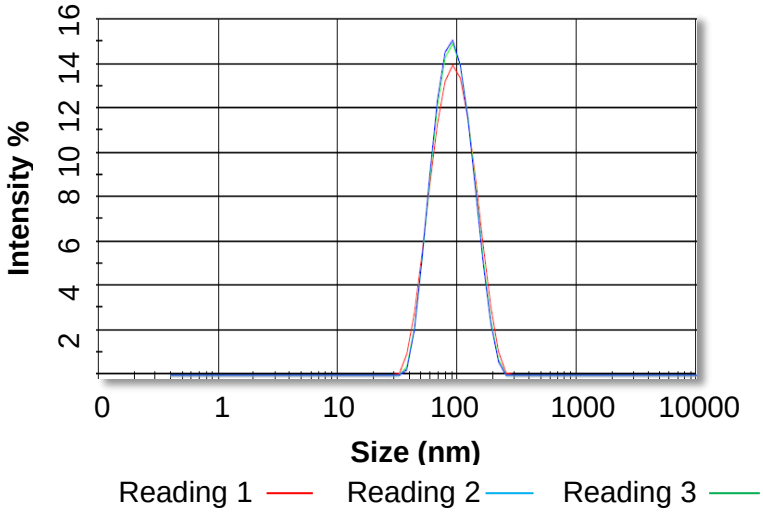
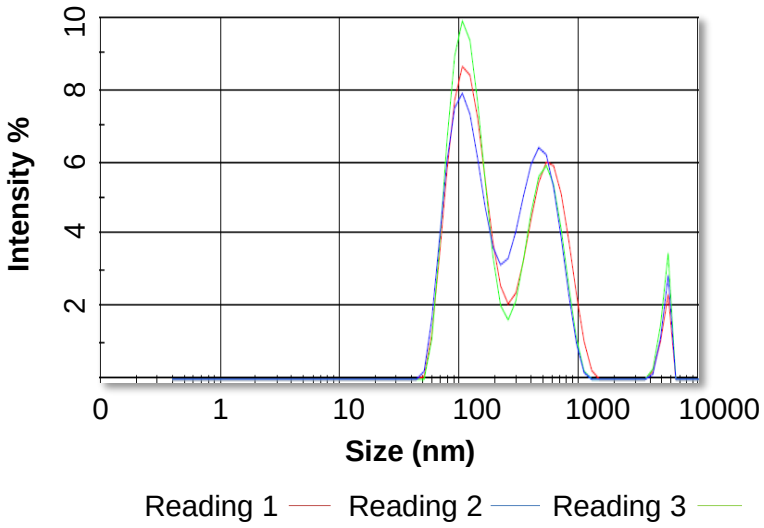


Figure B.2: Average particle size of the placebo liposomes; a) (**PL1**), b) (**PL2**) and c) (**PL3**)

a)



b)

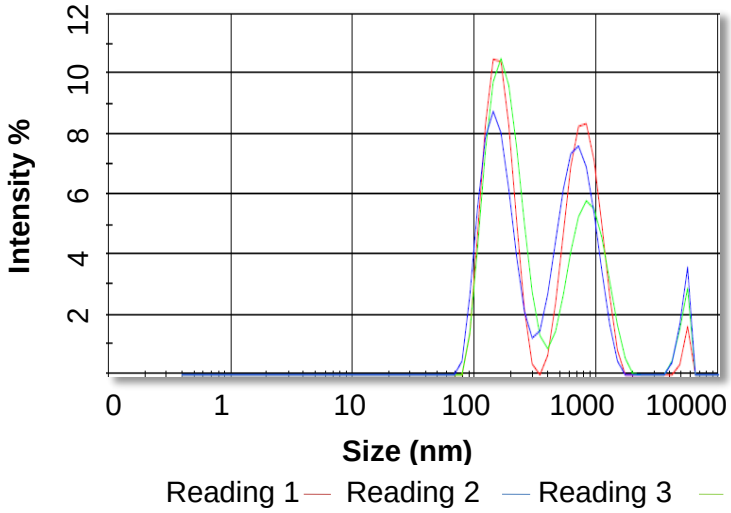


Figure B.3: Average particle size of the liposomes containing CLF; a) (**CL1**) and b) (**CL2**)

From Figures B.2 and B.3, the size and distribution of the droplets of each vesicle system is provided. The graphs of (**PL1**), (**PL2**) and (**PL3**) in Figure B.2 show a single narrow and uniform curve for each placebo preformulation. This then confirms the close relationship between the low PdI and the vesicle sizes. Figure B.3 illustrates a difference compared to the placebo formulations, as more than one particle size is present in (**CL1**) (Figure B.3.a) and (**CL2**) (Figure B.3.b) dispersions. This is shown by the presence of more than one peak in each graph, which in turn confirms the reason for a large PdI value between the (**CL1**) and (**CL2**), which indicated a heterogenic dispersion. Thus the vesicle systems containing CLF were more dispersed in size and distribution compared to the placebo dispersions.

B.3.1.3 Zeta-potential

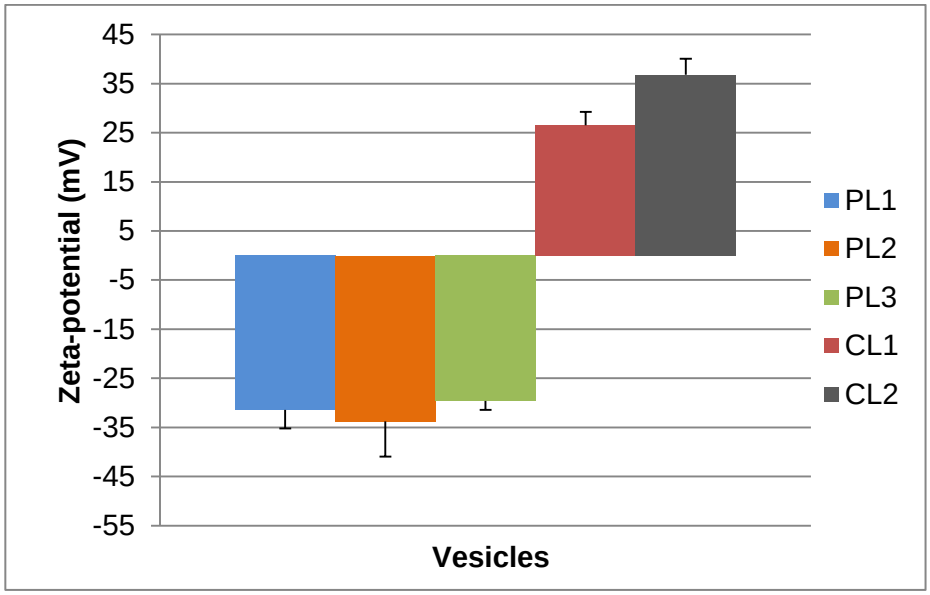


Figure B.4: Zeta-potential of placebo liposomes ((**PL1**), (**PL2**) and (**PL3**)) and liposomes containing CLF ((**CL1**) and (**CL2**))

A zeta-potential value (negative or positive) represents the level at which the particles in a dispersion repel each other. A zeta-potential that is more positive than + 30 mV, or more negative than - 30 mV dictates that the particles repel each other and would not lead to flocculation (Sutradhar *et al*., 2013:1). Figure B.4 illustrates the zeta-potential of the placebo liposomes and liposomes containing CLF. The placebo liposomes were all in the desired range for a stable dispersion as they were all more negative than - 30 mV. This indicates there would be no flocculation due to the vesicles having a largely repelling property. The (**PL2**) had a more negative zeta-potential (-33.78 ± 7.12 mV) than the other liposomes and would therefore prove to be more stable. The liposomes containing CLF exhibit a positive zeta-potential. Although (**CL1**) had a lower positive zeta-potential (26.48 ± 2.73 mV) than desired, it was still close to the limit range and would still be deemed as a possibly stable dispersion. The (**CL2**) had a much more positive zeta-potential (36.80 ± 3.24 mV) than the (**CL1**), 27.97 ± 4.90 mV), and would prove to be the more stable dispersion of the two. Thus, the zeta-potential values of the (**PL2**) and the (**CL2**) proved to be the more stable dispersion as there would be less chance of flocculation in comparison to the other dispersions.

B.3.1.4 pH

The pH of the liposome preformulations is shown in Table B.6. The placebo liposomes had similar pH values of approximately 5 for all three concentrations. This pH is mainly due to the acetate buffer (pH 5) that was used as a hydration medium, which indicates that none of the

constituents used during formulation affected the pH of the liposomes by either increasing or decreasing it in any significant manner. The same is noticed with the liposomes containing CLF, both measured approximately with the same pH values, which can also be linked to the acetate buffer (pH 5) that was used during formulation. It is evident that the pH for both the placebo liposomes and liposomes containing CLF were constant and that none of the constituents used during the formulation affected the pH.

Table B.6: Average pH of liposomes

Liposomes	Average pH
(**PL1**)	5.02
(**PL2**)	4.83
(**PL3**)	4.86
(**CL1**)	4.97
(**CL2**)	4.87

B.3.1.5 Viscosity

Table B.7 shows the speed in revolution per minute (rpm), average torque (%) and the average viscosity values (cP) for the concentration ranges of the preformulations. The placebo liposomes' and liposomes containing CLF's average torque are directly proportional to the viscosity under the same conditions, i.e. speed of 200 rpm. The (**PL3**) exhibited the highest viscosity of the three different concentrations. All the placebo liposomes indicated a low level of viscosity. The (**CL2**) had the highest viscosity average of 43.79 ± 0.133 cP, which was more than fifteen times higher than that of the (**CL1**). This makes it ideal for topical delivery as it would not be extremely free-flowing. This increase in viscosity is due to the proportional increase of cholesterol in the 1%, 2% and 3% preformulations (Archakov *et al*., 1983:89).

Table B.7: Viscosity results of the liposomes

Liposomes	Speed (rpm)	Average torque (%)	Average viscosity (cP)
(**PL1**)	200	16.33	2.45 ± 0.006
(**PL2**)	200	36.98	5.54 ± 0.007
(**PL3**)	200	45.58	6.88 ± 0.011
(**CL1**)	200	19.35	2.90 ± 0.011
(**CL2**)	35	51.09	43.79 ± 0.133

B.3.1.6 Encapsulation efficacy

In Table B.8 the average EE% of (**CL1**) and (**CL2**) are shown. When comparing the two concentrations, the (**CL2**) had the highest average EE% (84.15 ± 4.2%). The two vesicle dispersions were close to the average EE% of 71% proposed by Patel and Misra (1999:359). Thus, an increase in the concentration of the API in the dispersion yielded a higher average EE% of the liposomes containing CLF.

Table B.8: Average encapsulation efficacy of (**CL1**) and (**CL2**)

Liposomes	Average EE%
(**CL1**)	78.16 ± 0.9%
(**CL2**)	84.15 ± 4.2%

B.3.2 Proliposomes

B.3.2.1 Morphology

Figure B.5 shows the presence and successful formation of liposomes after hydration of the proliposomes. In Figures B.5.a and B.5.b the vesicles have distinct spherical shapes. The darker regions (lipid layers reacting with the osmium) around the edges of the vesicles illustrates the formation of the lipid membrane layers. In both images, the dark regions are prominent indicating the formation of a strong lipid membrane and evidently demonstrating the successful formulation of liposomes from a proliposomal preformulation.

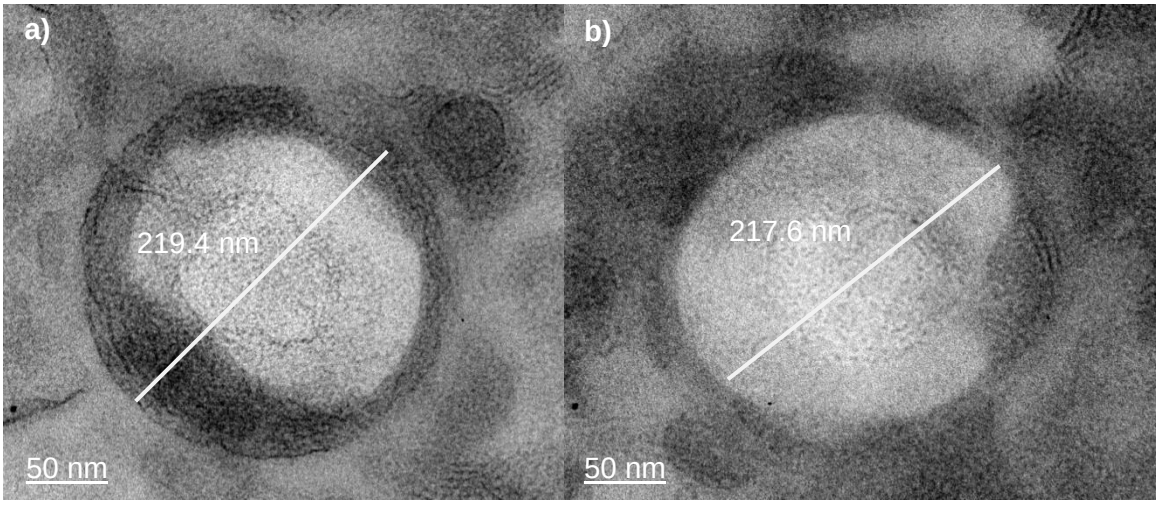


Figure B.5: Micrographs of two different proliposomes (a and b) captured with the TEM at 200 kV

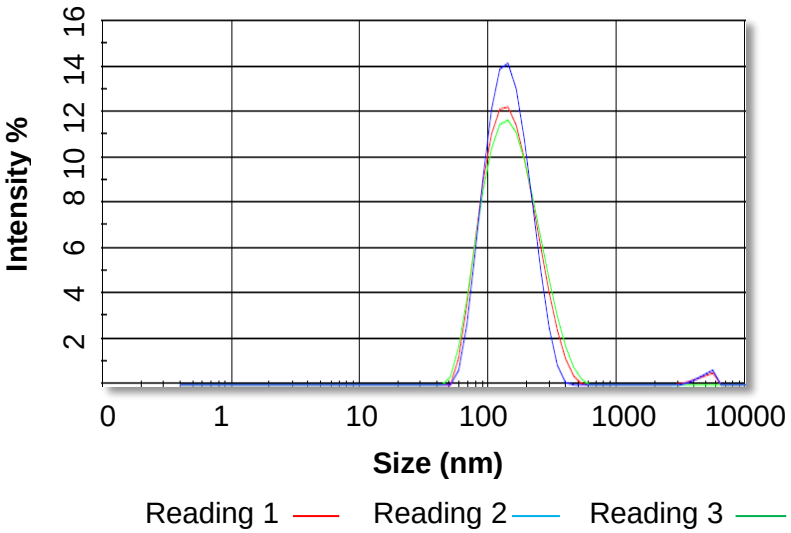
B.3.2.2 Droplet size distribution

The size distribution of the vesicles prepared from proliposomal preformulations are illustrated in Figures B.6 and B.7. (**PPL1**), (**PPL2**) and (**PPL3**) showed 'good' distribution data between the concentrations. The (**PPL1**) indicated a PdI value of 0.22, which indicated a good homogeneity of the vesicles and is seen as an optimum PdI. The (**PPL2**) and (**PPL3**) had closely related PdI values as they both had a PdI of 0.10 and 0.12, respectively. This indicates a monodispersity of vesicles in all of the placebo preformulations. The average particle sizes of (**PPL1**), (**PPL2**) and (**PPL3**) were 165.10 ± 46.05 nm, 114.97 ± 0.65 nm and 105.63 ± 0.74 nm, respectively. Even though the sizes of the liposomes are small, it still correlates to the range proposed by literature. The small standard deviation between the samples of the same concentrations indicates the level of vesicle uniformity in these dispersions.

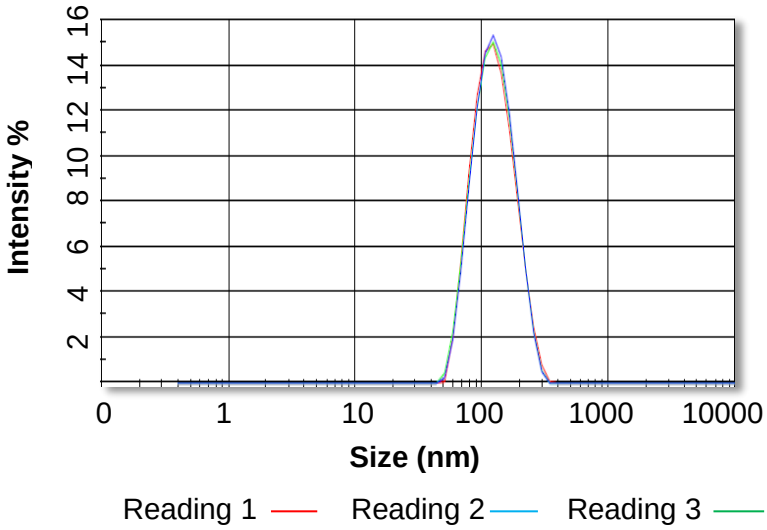
'Good' distribution data was obtained between the concentrations of the (**CPL1**) and the (**CPL2**). The PdI values for the (**CPL1**) and the (**CPL2**) were 0.45 and 0.41, respectively, which indicates an acceptable level of heterogeneity of vesicles in the proliposomes at both concentrations. It is still below a PdI value of 0.5, which indicates these dispersions are still acceptable within the formulations quality. The average size of the vesicles in the (**CPL1**) was 290.75 ± 89.85 nm and with the (**CPL2**), the vesicles had an average size of 203.97 ± 18.25 nm. In comparison to the placebo proliposomes, the size of the vesicles increased. Even though the proliposomes containing CLF vesicles are larger than those of the placebo proliposomes, they are still within the range suggested by literature. The larger standard deviation of the proliposomes containing CLF is due to the higher level of polydispersity in these dispersions.

Figures B.6 and B.7 illustrate the average particle size and distribution for the different provesicle concentrations. The results are almost identical to those of the liposome systems, as the placebo provesicle systems are uniform and had a single narrow curve that illustrates a homogeneous dispersion (Figure B.6). Figure B.7 also shows three peaks for the (**CPL1**) and the (**CPL2**), respectively, which indicates three levels of vesicles sizes. The aforementioned corresponds to the large PdI that presents a heterogenic dispersion. Thus, the placebo provesicle dispersion was more uniform in size and distribution compared to the provesicle dispersions containing CLF.

a)



b)



c)

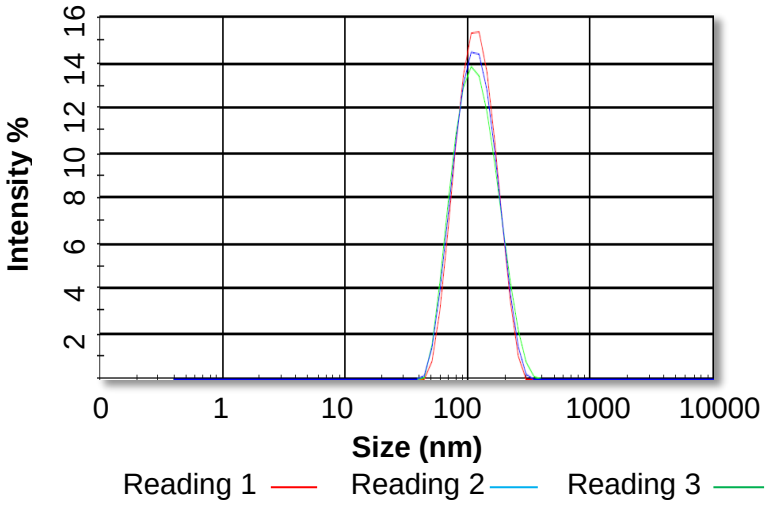
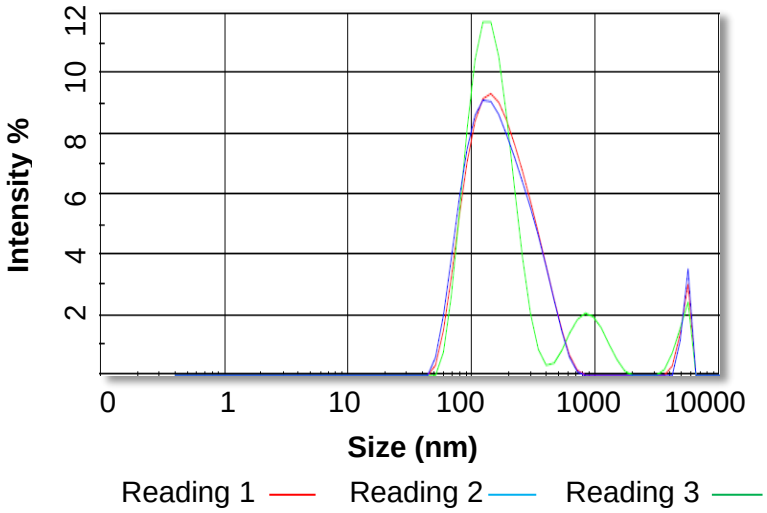


Figure B.6: Average particle size of the placebo proliposomes; a) (**PPL1**), b) (**PPL2**) and c) (**PPL3**)

a)



b)

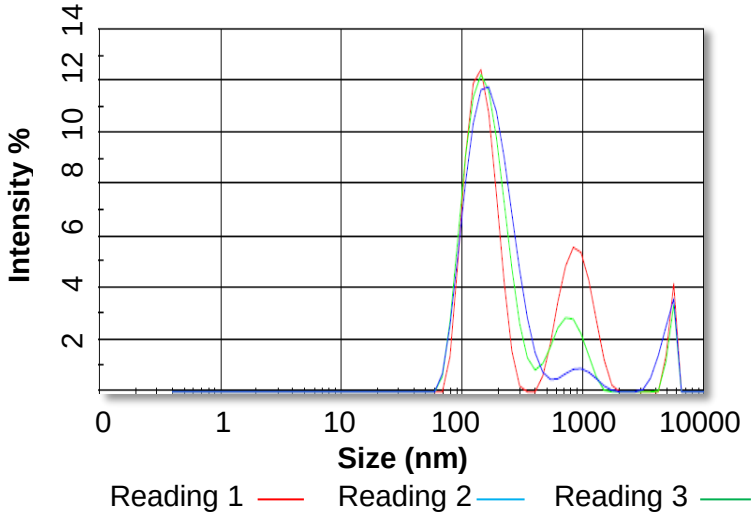


Figure B.7: Average particle size of the proliposomes containing CLF; a) (**CPL1**) and b) (**CPL2**)

B.3.2.3 Zeta-potential

As mentioned in Section B.3.1.3, the ideal zeta-potential is more positive than + 30 mV or more negative than - 30 mV. All the placebo proliposomes (1%, 2%, and 3%) had a zeta-potential that was more negative than - 30 mV, as illustrated in Figure B.8. The (**PPL1**) had the highest zeta-potential of - 44.20 ± 3.46 mV; while the rest of the placebo proliposomes were still within acceptable limits for a stable dispersion. This indicates the placebo proliposomes are all stable and that no agitation would occur. The (**CPL1**) and the (**CPL2**) exhibited a slightly lower and positive zeta-potential (30.60 ± 1.27 mV and 27.97 ± 3.39 mV) than the placebo proliposomes, but are also still within the acceptable range for stable dispersions. Even though the (**CPL2**) had the lower zeta-potential, it should still represent a stable dispersion and have no flocculation due to the repelling action of the particles.

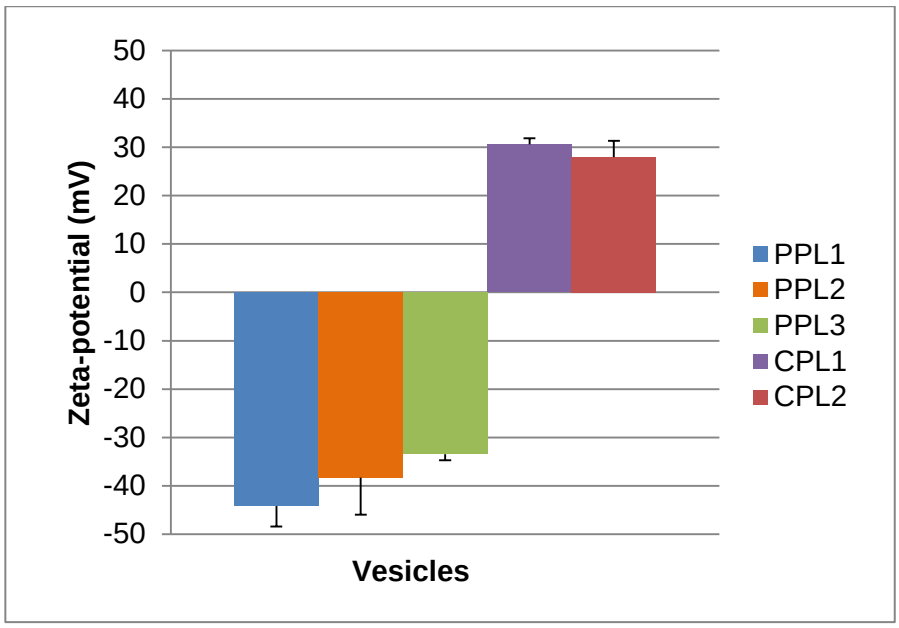


Figure B.8: Zeta-potential results of the placebo proliposomes ((**PL1**), (**PL2**), and (**PL3**)) and the proliposomes containing CLF ((**CPL1**) and (**CPL2**))

B.3.2.4 pH

Table B.9 shows the pH measurements taken from the placebo proliposomes and proliposomes containing CLF. The pH values for the placebo proliposomes were slightly lower than the pH values of the proliposomes containing CLF. The difference is not large enough to indicate any significant change in the charge on the lipid surface. The low pH values of the dispersion are linked to the acetate buffer (pH 5) that was used as a hydration medium.

Table B.9: Average pH of the proliposomes

Proliposomes	Average pH
(**PPL1**)	5.01
(**PPL2**)	5.13
(**PPL3**)	4.87
(**CPL1**)	5.16
(**CPL2**)	5.24

B.3.2.5 Viscosity

The speed (rpm), average torque (%) and average viscosity (cP) values are displayed in Table B.10 for each of the proliposomes. As the concentrations of the proliposomes increased, the average viscosity of the proliposomes increased, which is due to the proportional increase of cholesterol (Archakov *et al.*, 1983:89). The placebo proliposomes, (**PPL3**) had the higher

average viscosity, which correlates with literature. The same conclusion can be made regarding the proliposomes containing CLF, as (**CPL2**) exhibited a higher average viscosity, levels which are ideal for topical delivery as the viscosity is high enough so as not be hugely free-flowing and give opportunity for longer topical exposure.

Table B.10: Average viscosity results of the proliposomes

Proliposomes	Speed (rpm)	Average torque (%)	Average viscosity (cP)
(**PPL1**)	200	42.09	6.31 ± 0.020
(**PPL2**)	100	63.81	19.16 ± 0.030
(**PPL3**)	50	59.62	35.73 ± 0.120
(**CPL1**)	200	40.10	6.01 ± 0.015
(**CPL2**)	35	53.17	31.90 ± 0.118

B.3.2.6 Encapsulation efficacy

The average EE% of the proliposomes containing CLF is shown in Table B.11. (**CPL1**) had a higher average EE% than (**CPL2**), however its standard deviation is larger than that of the (**CPL2**). This indicates a larger difference between the prepared (**CPL1**) samples (n = 3) compared to the prepared (**CPL2**) samples (n = 3). The average EE% for both proliposomes containing CLF still closely correlate to what was proposed by Patel and Misra (1999:359). This indicates the successful encapsulation of CLF in liposomes using the proliposomal procedure to increase the stability of the dispersions. These concentrations are also high enough for successful topical delivery studies.

Table B.11: Average encapsulation efficacy of proliposomes containing CLF

Proliposomes	Average EE%
(**CPL1**)	71.09 ± 7.620%
(**CPL2**)	64.99 ± 0.024%

B.4 Final formulation of vesicle and provesicle system for topical delivery of CLF

The choice of the ideal final dispersion containing the API and vesicle system corresponded to their droplet size distribution, zeta-potential, pH, viscosity and EE%. In order to keep the study constant and eliminate any influence that may occur in further studies, the same ratio had to be used for the final liposome and proliposome dispersions. The two vesicle systems containing the API were prepared as previously discussed, as these represent the affirmed final

dispersions. Therefore for topical delivery of CLF, the following two prepared dispersions were characterised.

B.4.1 Final formulation of liposomes containing CLF

The established final liposomes containing CLF, according to previously stated results were the (**CL2**) dispersion. The ratios of cholesterol, PC and CLF were fixed throughout the characterisation, as only the amount of constituents increased as the concentration increased.

B.4.1.1 Procedure for preparing liposomes containing CLF

Section B.2.2.4 describes the general procedure that was used to prepare the (**CL2**) dispersion. The cholesterol, phosphatidylcholine and CLF were dissolved in a solvent mixture of methanol and chloroform, where after α-tocopherol was added and the mixture was evaporated until a thin-film formed (Table B.12). An acetate buffer (pH 5) was used as a hydration medium and added to the flask containing the lipid film. The flask was stirred until the lipid film in the flask was completely hydrated. This was followed by sonicating the hydrated mixture with a sonication probe, six times for 2 min intervals.

Table B.12: Liposomes containing CLF formula

Ingredients	(CL2)
CLF	200 mg
Cholesterol	200 mg
PC	1570 mg
α-Tocopherol	16 µl
Methanol/chloroform (1:2)	10 ml
Acetate buffer (pH 5)	10 ml

B.4.1.2Outcome

The (**CL2**) formed a dispersion with a dark red colour and had no precipitation of particles in the sample.

B.4.2 Final formulation of proliposomes containing CLF

The stability problems associated with liposomes gave rise to an additional method to increase their stability. Thus, the use of a water-soluble carrier (sorbitol), coated with the lipid mixture to form a dry dispersion, i.e. proliposome granules, is used to relieve the stability problems. This is followed by a hydration step upon use to produce liposomes. The same ratios used in preparing the (**CL2**) were used to prepare the (**CPL2**) dispersion to maintain consistency.

B.4.2.1 Procedure for preparing proliposomes containing CLF

Section B.2.2.7 describes the general procedure that was used to prepare the (**CPL2**) dispersion. The cholesterol, PC and CLF were dissolved in a solvent mixture of methanol and chloroform, where after α-tocopherol was added to the mixture. In a round bottomed flask containing the water soluble carrier (sorbitol), the lipid mixture was added drop-wise and evaporated until the entire solution was completely added (Table B.13). The coated sorbitol granules were then placed in a desiccator overnight to dry. An acetate buffer (pH 5) was used as a hydration medium and added to the flask containing the granules. The flask was stirred until liposomes had formed, followed by sonicating the dispersion with a sonication probe, six times for 2 min intervals.

Table B.13: Proliposome containing CLF formula

Ingredients	(CPL2)
CLF	200 mg
Cholesterol	200 mg
PC	1570 mg
α-Tocopherol	16 µl
Methanol/chloroform (1:2)	10 ml
Acetate buffer (pH 5)	10 ml
Sorbitol	4 g

B.4.2.2 Outcome

The CLF completely stained the sorbitol and after evaporation formed dark red granules. These granules were a lot larger than the pure sorbitol and upon hydration, formed a dark red dispersion similar to the (**CL2**) dispersion.

B.5 Conclusion

In order to choose the ideal dispersion concentration for the use in further studies, the prepared vesicle systems spanned a range of concentrations. These preformulations were tested according to their morphology, droplet size distribution, zeta-potential, pH, viscosity and EE%. The (**CL2**) dispersion resembled the top properties in comparison to the other dispersions. An average droplet size of 330.83 ± 4.40 nm and PdI of 0.52, an average zeta-potential of 36.80 mV, an average pH of 4.87, an average viscosity of 43.79 ± 0.133 cP and an average EE% of 84.15 ± 4.2% deems the (**CL2**) as the most stable dispersion. The provesicle system, (**CPL2**), followed the similar procedural steps to produce the (**CL2**) with the added exception of the carrier method. This was done to eliminate any possible aspect hindering the comparison of the two dispersions, i.e. vesicle versus provesicle and keeping them at a comparable level.

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APPENDIX C

Characterisation of final vesicle- and provesicle systems

C.1 Introduction

Clofazimine forms part of the riminophenazine antibiotic family (Cholo *et al*., 2012:291). Physicochemically, this API does not possess the ideal properties for topical delivery, therefore to bypass some of the physiochemical disadvantages that clofazimine has, a vesicle system is proposed, i.e. liposomes. Liposomes are equipped with a great property of being amphiphilic, making them ideal for improving physiochemical properties of low solubility APIs. This characteristic of liposomes makes it possible for both hydrophilic and hydrophobic drugs to be entrapped within the phospholipid bilayer or the water domain (Drulis-Kawa & Dorotkiewicz-Jach, 2010:197).

The final dispersions ((**CL2**) and (**CPL2**)), as determined in Section B.6, was characterised and evaluated according to their morphology, droplet size distribution, zeta-potential, pH, viscosity and EE%. The characterisation assists in evaluating the physical properties of the vesicle systems in the final dispersions. These properties were used to choose an optimum concentration level useable in support of the following studies, i.e. diffusions studies (Appendix D) and *in vitro* cytotoxicity studies (Appendix E).

An important consideration regarding the preformulation was to research the level of toxicity of the API and the vesicle constituents as this plays a major role in the formulation design and route of administration. Regarding clofazimine, no significant level of toxicity was detected, according to its MSDS, and the liposomes had a non-toxic and biodegradable character. This indicates that using these ingredients in the final dispersion should not be hazardous or harmful (Pierre & Costa, 2011:608).

C.2 Physical characterisation of the final vesicle- and provesicle dispersion

C.2.1 TEM

A FEI Tecnai G2 high resolution TEM (FEI, Holland), at approximately 200 kV, was used to visualise the morphology and presence of the liposomes in the dispersion. The final dispersions were freshly prepared for the TEM. The API, α-tocopherol and acetate buffer (pH 5) were absent during the preparation as these constituents may crystallise and cause damage to the microscope. During the formulation of the final dispersion, Milli-Q® water was used as a hydration medium instead of the acetate buffer (pH 5). Dr A Jordaan (Laboratory for Electron

Microscopy, North-West University: Potchefstroom Campus) operated the TEM in order to capture the images of the vesicles. The dispersions were prepared for the TEM by firstly diluting (x 10) it in Milli-Q® water and mixing it thoroughly. A drop of diluted solution was dropped on a copper carbon-coated 300 mesh grid in order for the vesicles to attach to the carbon substrate. The grid was left to dry (15 min) and any excess amount of solution was removed by filter paper. After the solution was completely absorbed, a drop of osmium was used to stain the lipid films and left to evaporate for approximately 30 min. The dispersions were then rinsed and treated with a 2% aqueous solution of uranyl acetate and left to air dry completely before visualisation on the TEM (Ammar *et al*., 2011:143, Wibroe *et al*., 2016:2).

C.2.2 Droplet size and distribution

The droplet size distribution of vesicles was evaluated by determining the average vesicle size in the dispersions. This determines whether the vesicles were uniform throughout the dispersion, usually referred to as the PdI. This is an important parameter to characterise for the vesicle systems. A Malvern Zetasizer Nano ZS (Zen 3600) (Malvern® Instruments Ltd, Worcester, United Kingdom) was used to determine the average size and size distribution of the vesicles (Badran *et al*., 2012:3). Three samples per system were freshly prepared the day before analysis. Three low concentration samples were prepared from each vesicle sample and injected into three disposable cells (2 ml), respectively. The analysis was performed in triplicate for each sample. A He-Ne laser (633 nm), at a scattering angle of 175 °, was used for the analysis at room temperature.

C.2.3 Zeta-potential

The zeta-potential was measured to indicate the probable stability of the dispersion. Zeta-potential indicates the degree of repulsion between charged particles of a similar nature to establish the physical stability of the formulation (Shah *et al*., 2014:66). The zeta-potential was measured with a Malvern Zetasizer Nano ZS (Zen 3600) (Malvern® Instruments Ltd, Worcester, United Kingdom). Three low concentration samples were prepared from three final vesicle and provesicle samples, respectively. 2 ml of each low concentration sample was injected into three disposable cells, respectively. A day after preparation, the zeta-potential of each dispersion sample was measured in triplicate and the average calculated.

C.2.4 pH

The pH plays a role in the chemistry of the vesicles as a change in pH may influence the stability, agglomeration and dispersion of the vesicle systems. These effects can also influence the zeta-potential therefore the pH was measured (Berg *et al*., 2009:276). A Mettler Toledo

pH meter FE20/FG2 (Greifensee, Switzerland) was used to determine the pH values of three prepared samples ((**CL2**) and (**CPL2**)) per system under the same conditions. The probe of a pH meter was submerged into each sample (10 ml) and used to measure the pH of the dispersions. The measurements of each freshly prepared sample were performed in triplicate.

C.2.5 Viscosity of the vesicle systems

The viscosity of a formulation is the quantitative measurement of the resistance of a fluid to flow. Thus, a level of high resistance to flow would indicate a high level of viscosity (Quiñones & Ghaly, 2008:62). The degree of a formulations' viscosity can affect the delivery of a drug by directly influencing a drugs diffusion rate at a microstructural position (Ueda *et al*., 2009:753). A Brookfield Viscometer DV2T-LV (Stoughton, USA) was used to determine the viscosity of the dispersions. Three final dispersion samples per vesicle system were prepared the day before the viscosity characterisation. The samples were placed in a pre-heated water bath at 25.5 °C the following day to reach a temperature equivalent to that of the water bath (30 min), where after the viscosity readings were taken. The Brookfield Viscometer contains a T-spindle (Stoughton, MA), which was inserted into 6.5 ml of each sample (without disturbing it) and set at a predetermined rotating rate. The viscosity measurements were measured at 10 sec intervals over a 2 min period giving 13 measurements per sample.

C.2.6 Encapsulation efficiency

The EE% calculation measures the difference between the total amount of drug encapsulated in the vesicles and the free drug in a sample, depicting the yield obtained. The EE% was measured by means of HPLC. Dispersion samples of (**CL2**) and (**CPL2**), of 10 ml each, was prepared respectively and added to Eppendorf® tubes and centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa). The conditions of the ultracentrifuge was set at a speed of 25 000 g for 30 min at room temperature. After centrifugation, a pellet of the vesicles formed and a supernatant (containing un-encapsulated clofazimine). The supernatant was then diluted (x 50) with the appropriate solvent (used during HPLC validation) and added to HPLC vials for analysis. The analysis was performed in triplicate to determine a good average EE%. The EE% of each vesicle system was calculated according to the Equation B.1 in Section B.2.3.6.

C.3 Results and discussion

C.3.1 TEM

The TEM characterisation of the vesicle systems were captured with a Gatan bottom mount digital camera. The images illustrated by Figure C.1 showcase only selected images of the vesicles formation in the prepared dispersions of liposomes and proliposomes without the API. As seen in Figures C.1.a to C.1.d, all the vesicles possess a spherical shape and range between different nano- and micrometres, as proposed by literature (Elzainy *et al*., 2005:282). The surface of the liposome particles in Figures C.1.a and C.1.b seem to be more rigged and irregular compared to the liposomes formed through proliposomes (Figures C.1.c and C.1.d). The proliposomes formed a more smooth membrane, as seen in Figures C.1.c and C.1.d. It is also clear that a membrane has formed to give structural integrity to the vesicles, distinctly illustrated by the dark regions caused by the osmium that roots within the lipid layer of the vesicles. These results prove the formation of liposome vesicles in the vesicle and provesicle dispersions and their strong structural durability.

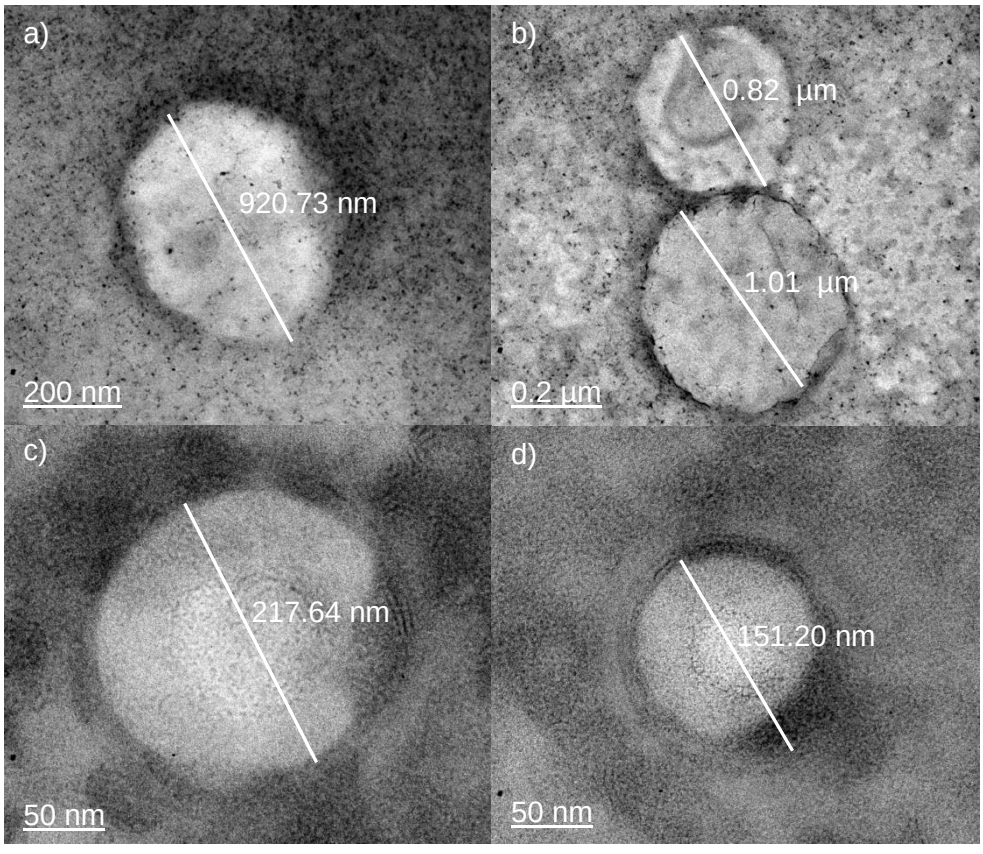

Figure C.1: Micrographs of (**CL2**) and (**CPL2**) captured on the TEM at 200 kV: a) a single liposome, b) two liposome vesicles that formed, c) and d) are both liposomes that formed from proliposomes.

C.3.2 Droplet size and distribution

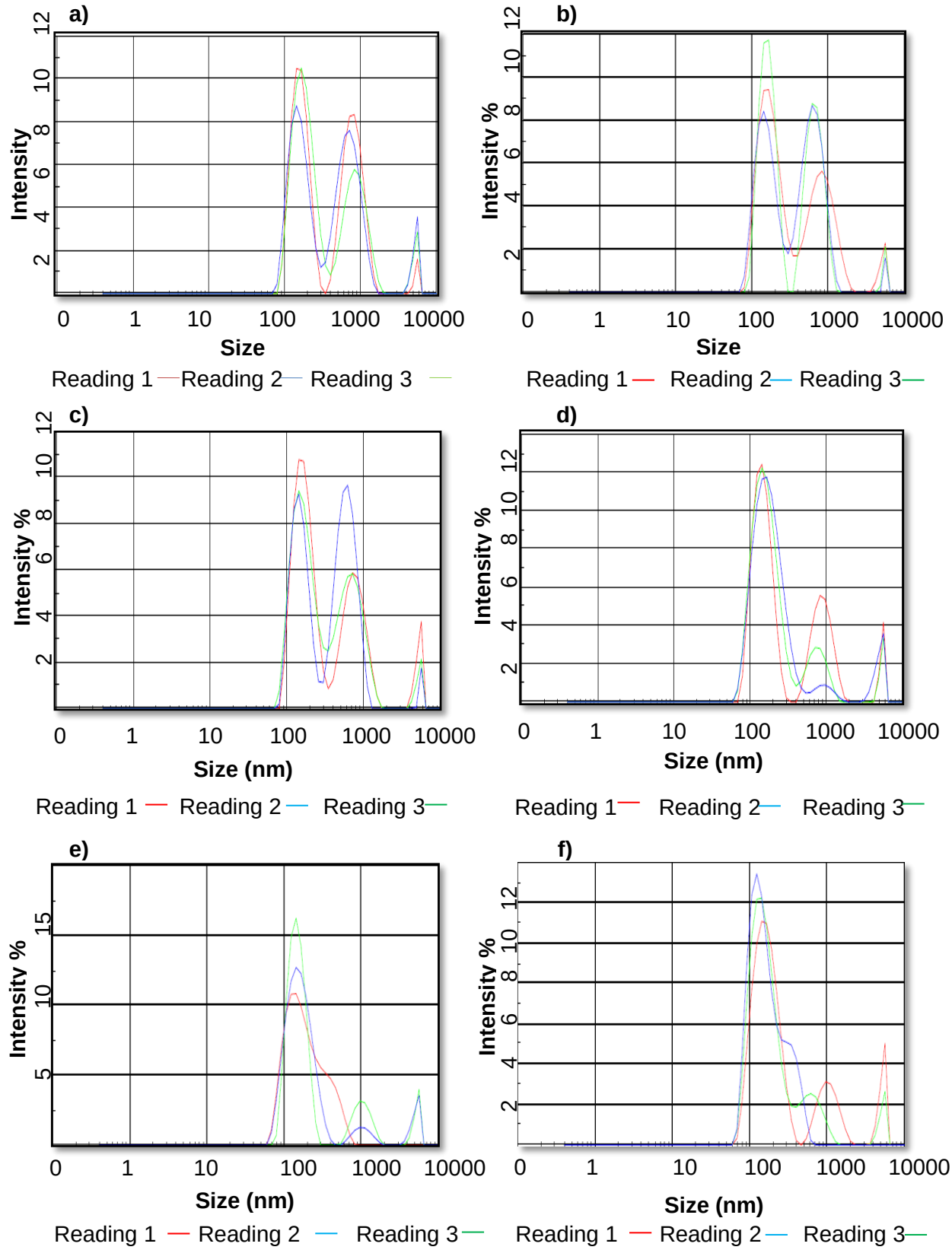

Figure C.2: Average particle size of the final dispersions; a), b), c) illustrate the (**CL2**) (n = 3) measurements and d), e), f) illustrate the (**CPL2**) (n = 3) measurements.

The samples prepared from each vesicle (n = 3) and provesicle (n = 3) system were analysed in triplicate and during each test, the average droplet size was measured by taking three readings per sample to ensure a good quality result. Figure C.2 shows the readings of the average particle size of (**CL2**) and (**CPL2**), respectively.

Figure C.2 presents a graph of each vesicle systems' droplet size distribution. All graphs illustrated 'good' quality results. Figures C.2.a to C.2.c represents the (**CL2**) dispersions and Figures C.2.d to C.2.f represents the (**CPL2**) dispersion. Each individual graph shows the three readings recorded per sample; the experiment was performed in triplicate, thus three graphs per vesicle system. Shah *et al*., (2014:65) explains that a PdI of 0.5 and less would show a level of heterogeneity in the dispersions, but is still within acceptable limits for a quality dispersion. The (**CL2**) and (**CPL2**) graphs all have three distinct curves illustrating the heterogeneity of the dispersions, as a large difference is found in the sizes of particles throughout. The largest curves in each graph are relatively narrow and predominantly fall within the droplet sizes that were measured, compared to the two other smaller peaks. This indicates that the particles are in close range to each other, even though there is a large distribution of particles found in each dispersion. The micrographs captured on the TEM provide an illustration of the variety of particle sizes found in each dispersion, and support the PdI and average droplet sizes found for each, respectively. This supports the large PdI value, expressing an increased level of heterogeneity. This proves that the PdI and average droplet size of (**CL2**) and (**CPL2**) are acceptable to be used for further studies.

C.3.3 Zeta potential

Figure C.3 illustrates the vesicle and provesicle systems' zeta-potential. As previously mentioned in Section B.3.1.3, the ideal zeta-potential for a possibly stable formulation must be more positive than + 30 mV or more negative than - 30 mV. Both dispersions illustrated a highly positive zeta-potential, which corresponds with the range proposed by literature, showing a good stability for each dispersion. The (**CL2**) exhibited a higher zeta-potential than the (**CPL2**), 36.80 ± 3.24 mV and 27.97 ± 4.90 mV, respectively. The zeta-potential of both systems indicates the dispersions are stable and will not experience aggregation or flocculation during the following studies, due to the repelling action exhibited by the particles, with an increase in surface charge of the particles (Sutradhar *et al*., 2013:1). Even though (**CPL2**) exhibited a lower zeta-potential than + 30 mV, it is still accepted as sources differ in ranges. The lowest acceptable range is a zeta-potential value more negative than - 25 mV and more positive than + 25 mV (Mothilal *et al*., 2014:451).

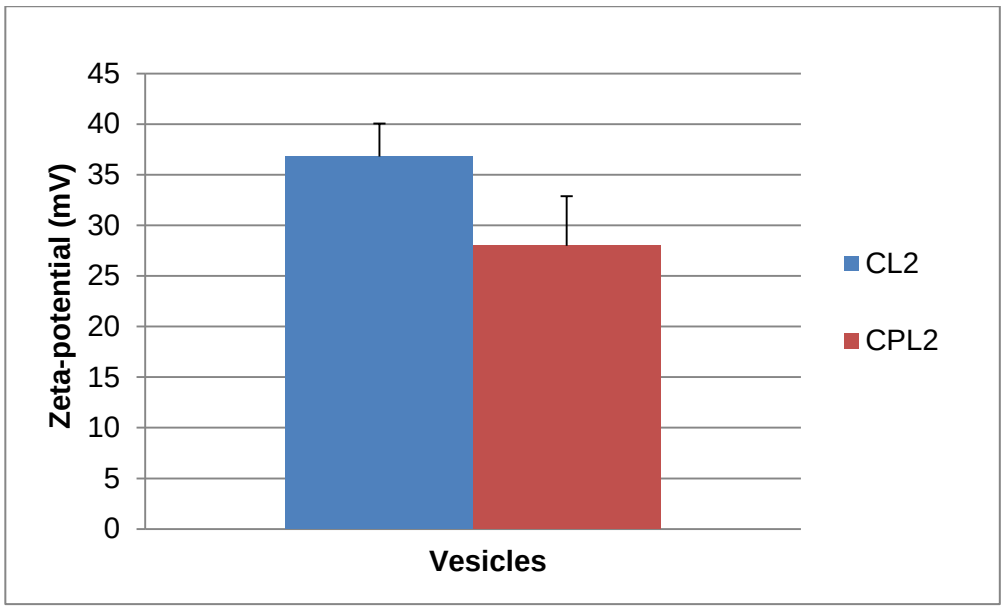

Figure C.3: The average zeta-potential of the (**CL2**) (n = 3) and (**CPL2**) (n = 3)

C.3.4 pH

In Table C.1, the pH measurement of the vesicle and provesicle dispersions are shown. The pH values of the (**CL2**) and the (**CPL2**) were both close to the pH of the hydration buffer (acetate buffer of pH 5) that was used during the preparation. This indicates no significant difference between the final dispersions surface charges. An ideal pH range for the topical delivery of a drug, according to Naik *et al.* (2000:319), is between 5 and 9. Even though the (**CL2**) are slightly below the lower limit for topical delivery, it is still within acceptable range. The (**CPL2**) displayed a more elevated pH than the (**CL2**), but both dispersions were within close proximity to each other. Therefore, the pH of the (**CL2**) and the (**CPL2**) are acceptable, deeming the dispersions usable and sufficiently stable for topical delivery.

Table C.1: Average pH of the final dispersions

Dispersions	Average pH
(**CL2**)	4.87
(**CPL2**)	5.24

C.3.5 Viscosity

Table C.2 shows the speed in rpm, average torque (%) and the average viscosity values (cP) of the (**CL2**) and the (**CPL2**). The rpm and average torque percentage are almost equivalent for both the vesicle and provesicle systems. The highest viscosity was displayed by the (**CL2**) (43.79 ± 0.133 cP). The dispersions exhibited a relatively high viscosity, which is advantageous for topical delivery, making both systems ideal for application to the skin, as the dispersion

would not disperse as easily. This improves the exposure time of CLF on the skin for maximum topical effect.

Table C.2: Viscosity results of the final dispersions

Dispersion	Speed (rpm)	Average torque (%)	Average viscosity (cP)
(**CL2**)	35	51.09	43.79 ± 0.133
(**CPL2**)	35	53.17	31.90 ± 0.118

C.3.6 Encapsulation efficiency

The results for the EE% of the vesicle and provesicles in the final dispersions are shown in Table C.3. The EE% of the (**CL2**) was relatively high (84.15 ± 4.200%) compared to that of the (**CPL2**) (64.99 ± 0.024%). This illustrates the successful encapsulation of CLF in the hydrophobic regions of the liposomes. This EE% of the API in the vesicle and provesicle system is ideal for topical drug delivery, as a higher drug concentration is preferred during topical delivery studies. The EE% of both dispersions is relatively close to the literature that Patel and Misra (1999) proposed.

Table C.3: EE% results of the final dispersions

Dispersion	Average EE%
(**CL2**)	84.15 ± 4.200
(**CPL2**)	64.99 ± 0.024

C.4 Conclusion

The characterisation of the final dispersions was performed to determine the physical properties associated with the liposomes and proliposomes. It is evident spherical vesicles formed successfully and that the droplet size and distribution of these dispersions were acceptable, even though there were larger particles and an increased level of heterogeneity for both dispersions. The zeta-potential of both dispersions were predominantly positive, assuring that no flocculation or agitation would occur and deemed the dispersion as stable. In conjunction with the zeta-potential, the pH was also measured to ensure none of the constituents used during the preparation altered the surface charge of the particles. No unwanted interactions occurred and the pH represented the hydration buffer that was used during the preparation of both dispersions. The successful encapsulation of CLF into the hydrophobic layer of the liposomes was indicated with the high EE% of both dispersions. These characteristics emphasise the properties linked to the (**CL2**) and the (**CPL2**), thus providing insight into the use of these dispersions for further studies.

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APPENDIX D

Diffusion studies of clofazimine

D.1 Introduction

The development of topical drug delivery formulations is of noticeable importance, as this represents one of the most innovative fields in the delivery of drugs. Amongst others, it aims to improve the solubility of poorly soluble drugs, e.g. clofazimine, to enhance penetration into the skin (Bartosova & Bajgar, 2012:4675). According to Kalepu and Nekkanti (2015:443), approximately 40% of marketed drugs experience a level of poor solubility, indicating the need to evaluate possible delivery improvement. This creates space and the opportunity to achieve improved approaches for the delivery of an API topically.

A diffusion method approach using Franz diffusion cells was used in this study to investigate the dermal absorption of the (**CL2**) and (**CPL2**) dispersions on excised human skin. This technique is ideal to control experimental conditions and to anticipate and evaluate dermal penetration of the human skin. This approach also has the added advantage of excluding human volunteers and being able to create reproducible results easily. The only major disadvantage in using this approach is that the peripheral blood flow, being emulated by a buffer equal to the pH of blood (pH of 7.4) may not reach the desired sink conditions (Bartosova & Bajgar, 2012:4673).

For the diffusion studies, both human skin and membranes (non-rate limiting) were subject to the dispersions, respectively. The formulation occurred as explained in Section B.4, creating a vesicle and provesicle system containing the API to investigate the following aims:

- Solubility and log D of CLF.
- Release of API from the vesicle after 6 h of exposure to a membrane.
- Determine after 12 h of exposure to the skin if the drug-vesicle system diffuses through the skin or accumulates in the skins layers.
- Effectiveness of the vesicles system for the topical delivery of CLF.

The objective of this study was to determine if CLF, being insoluble, could be delivered topically by means of using a vesicle carrier system, i.e. liposomes and proliposomes, to improve solubility and increase its ability to cross into the dermal layers of the skin. The aspiration of this study was to enhance the solubility of CLF so that it may concentrate within the dermal layers of the skin, thus possibly providing an additional route for skin lesion treatment in conjunction with the general treatment regime for MDR-TB.

D.2 Methods

D.2.1 Chromatographic conditions set for HPLC analysis of samples

A validated HPLC method for CLF was developed at the ATL of the NWU (Appendix A). The validation was performed to indicate an appropriate and reliable method to ascertain the amount of clofazimine present in the samples. The analysis was performed in a controlled laboratory at a fixed temperature of 25 °C.

An Agilent 1100 series HPLC, equipped with an Agilent 1100 isocratic pump, auto sampler, ultra violet (UV) detector with a ChemStation Rev. A.10.02 data acquisition and analysis software package was used during this study for analysis of the samples (Agilent Technologies, Palo Alto, CA). A Venusil C_{18} 150 x 4.6 mm column with particle size of 5 µm (Agela Technologies, Newark, DE) was fitted on the system and represented the stationary phase on the HPLC.

The mobile phase was prepared in a 70:30 ratio of acetonitrile and 0.005 M octane sulphonic acid - Na in HPLC water. Orthophosphoric acid (H_3PO_4) was used to adjust the pH to 3.5. The flow rate was set to 1 ml/min with a selected injection volume of 50 µl for the membrane and skin diffusion studies. The optimal wavelength for the detection of CLF was set at 284 nm; the retention time of CLF, after a runtime of 10.0 min, was ± 5.2 min.

D.2.2 Standard preparation

Before each new set of samples were analysed, a standard curve was procured. The standard prepared for the membrane release study consisted of 20 mg CLF (raw) dissolved in a solvent (50:50 ratio of methanol and HPLC water with 1% glacial acetic acid) and made up to volume in a 100 ml volumetric flask; 1 ml was transferred to a HPLC vial and injected at different volumes to establish a multi-point calibration curve that ranged between 20.6 µg/ml and 206.0 µg/ml.

The standards prepared for the skin diffusion studies consisted of 2.5 mg and 5.0 mg of CLF dissolved in the abovementioned solvent and was made up to volume in a 100 ml volumetric flask, respectively. 1 ml of each was transferred to separate HPLC vials and injected at different volumes to establish two separate multi-point calibration curves, which ranged between 0.5 µg/ml and 10.0 µg/ml and 5.2 µg/ml and 52.0 µg/ml for each standard.

D.2.3 Preparation of donor and receptor phases

Two vesicle systems were prepared for the membrane release and skin diffusion studies. The donor phase consisted of the (**CL2**) and (**CPL2**) dispersions, respectively. The preparation of the donor phase dispersions was performed according to the preparation discussed in

Section B.6 for both systems. The sample for the donor phase was prepared twice to accumulate a total volume of 20 ml, to be divided between twelve Franz cells per experiment. Table D.1 shows the amount of each ingredient used in preparing the 2% dispersions.

Table D.1: Formula for (**CL2**) and (**CPL2**) dispersions used during the membrane release and skin diffusion studies

Ingredients	(CL1)	(CPL2)
Clofazimine	200 mg	200 mg
Cholesterol	200 mg	200 mg
Phosphatidylcholine (PC)	1 570 mg	1 570 mg
α-Tocopherol	16 µl	16 µl
Methanol/chloroform (1:2)	10 ml	10 ml
Acetate buffer (pH 5)	10 ml	10 ml
Sorbitol	-	4 g

The receptor phase used for the membrane release and the skin diffusion studies were different due to CLF being virtually insoluble in aqueous solvents. According to Walters (2002:202), for topical studies the receptor phase is usually an aqueous medium, e.g. PBS (pH 7.4), but compounds with an aqueous solubility of less than 0.01 mg/ml need supplementary solubilisers to retain sink conditions. CLF's solubility increases in acidic environments, thus an alternative receptor phase solubiliser, ethanol, was used, i.e. 100% HPLC-grade ethanol for the membrane release studies and a combination (1:9) of HPLC-grade ethanol and PBS (pH 7.4) for the skin diffusion studies (Baik & Rosania, 2012:1). As the membrane release studies are purely to determine the release of CLF from the vesicle systems through a synthetic membrane, the use of 100% HPLC-grade ethanol is an acceptable receptor phase solvent. The maximum percentage ethanol allowed for transdermal and topical studies is 10%, thus a 1:9 ratio of HPLC-grade ethanol:PBS (pH 7.4) was used during the diffusion experiments. PBS (pH 7.4) was prepared by dissolving 0.06 M KH_2PO_4 in distilled water and adjusting the pH accordingly. Between experiments, the PBS (pH 7.4) was kept in a refrigerator at 2 – 5 °C. In advance of each experiment, the receptor solution was mixed accordingly and placed in a water bath at a fixed temperature of 37° C.

D.2.4 Aqueous solubility of CLF

CLF's solubility in PBS (pH 7.4) was tested to determine to what degree the API was soluble in PBS. An excess amount of CLF was used during this aqueous solubility experiment and was dissolved in 7 ml of PBS (pH 7.4), which was used as a solvent to dissolve the excess of CLF to create a saturated solution in a polytop. The polytop containing the excess amount of CLF in

PBS (pH 7.4) was placed in a water bath set at 32 °C (emulating the temperature of the skin) for 24 h; during this period, the samples were agitated and investigated at regular intervals to ensure the solution remained saturated at all times. Following the 24 h waiting period, the test tubes were removed and the samples filtered through 0.45 µm polyvinylidene difluoride (PVDF) filters. The filtered solution was then transferred into vials and analysed on the HPLC in triplicate. A standard curve was also obtained by preparing a standard solution of 5 mg CLF in 100 ml of solvent and injected at different volumes on the HPLC.

D.2.5 *n-Octanol*-buffer distribution coefficient of CLF

To determine in what medium CLF is more suitably soluble, the log D of CLF in octanol (lipophilic phase) and PBS (aqueous phase) was determined. In order to determine the log D of CLF, a solution consisting of two co-saturated phases, i.e. PBS (pH 7) and *n*-octanol, was prepared. Over a 24 h period, the co-saturated solution was established by vigorously mixing the two phases until equilibrium was reached. Following equilibration of the mixture, it was added to a separation funnel and left to separate for 24 h. 20 ml of each of the co-saturated solution components was extracted into polytops, i.e. 20 ml of *n*-octanol and 20 ml of PBS. A pre-weighed mass of 200 mg CLF was added to the 20 ml *n*-octanol phase; 3 ml of each solution was then transferred into a test tube followed by an equal volume of the extracted PBS phase. The prepared solution containing the two phases was then placed into a mechanical shaker bath for an overnight period, which caused a disrupting effect in the solution. After the overnight period, 1 ml of the *n*-octanol-phase was extracted, placed in a polytop and diluted with 10 ml of methanol. 1 ml of the diluted *n*-octanol solution and 1 ml of PBS extracted from the separate polytops were transferred in vials for HPLC analysis to determine the concentration of the API in each separate phase. A standard curve was also obtained by preparing a standard solution of 5 mg CLF in 100 ml of solvent and injected at different volumes on the HPLC. The experiment was performed in triplicate.

In order to determine the distribution coefficient, Equation D.1 was used. This equation divides the ratio of drug in the *n*-octanol phase by the aqueous phase (PBS).

$$\text{Log D} = \frac{\text{concentration in n-octanol}}{\text{concentration in PBS}} \qquad \textbf{Equation D.1}$$

D.2.6 Membrane release studies

To determine the successful release of the API from the vesicle systems, a membrane release study was performed. Two separate release studies were conducted, one for the (**CL2**) and the other for the (**CPL2**). Twelve Franz cells, two of which contained placebo dispersions, were used in each release experiment.

The donor phase and receptor phase were prepared as explained in Section D.2.3. The donor phase was placed in a water bath at a temperature of 32 °C and the receptor phase in a water bath of 37 °C; the 32 °C temperature represents the temperature of the skin and the 37 °C temperature represents the temperature of blood in the body. The assembly of the Franz cells involved the addition of Dow Corning® (Sigma-Aldrich, Germany) high vacuum grease to both the receptor (containing a magnetic stirrer bar) and donor phase components. PVDF hydrophilic membrane filters, with pore sizes of 0.45 µm, were cut into small circles (15 mm in diameter) and placed between the receptor and donor phases, followed by the secure assembly of the Franz cell unit with a horseshoe clamp to prevent any leakage. 2 ml of HPLC-grade ethanol was then injected into the receptor compartment and 1 ml of the dispersion was injected into the donor compartment. To avoid any loss of the dispersion from the donor compartment, Parafilm® was used to cover the opening. After the Franz cells were fully assembled, they were placed onto a Variomag ® magnetic stirrer plate in a pre-heated water bath set at 37 °C. After every hour for a 6 h period, the receptor phase contents were extracted into vials and injected by HPLC to determine the concentration of the released amount of CLF through the membrane. After the extraction from each Franz cell, an equal volume of the pre-heated HPLC-grade ethanol solution was used to replace the previously extracted contents.

D.2.7 Human skin preparation for diffusion studies

The skin was obtained from patients after plastic surgery had been performed by qualified surgeons. Full-thickness skin was obtained from the abdomen of Caucasian woman and frozen upon arrival at the bio-safety laboratory of the North-West University at - 20 °C for a maximum of 24 h (ethical approval reference number: NWU-00114-11-A5). The full-thickness skin was subject to inspection and alteration to ensure the selected skin was suitable for the required studies. The skin was dermatomed with a Zimmer™ electric dermatome after being inspected for any lesions or stretch marks, as the diffusion in these areas will differ. The electric dermatome was used to cut pieces of skin at a continuous thickness of 400 µm and placed on Whatman® filter paper. The combined skin and filter paper were wrapped in aluminium foil and stored at - 20 °C in a freezer. The subcutaneous fat left over after processing was duly discarded.

D.2.8 Skin diffusion

The topical delivery of CLF was determined and evaluated by studying its passive diffusion topically. The skin penetration study consisted of 12 Franz cells, with two cells containing placebo dispersions as controls and the rest containing the prepared vesicle system consisting of the API. This study was performed twice, once for the (**CL2**) and once for the (**CPL2**) dispersions.

The method used for the skin diffusion studies is similar to the procedure used in Section D.2.6 for the membrane diffusion study. The only difference is that instead of the PVDF membrane filters, dermatomed skin was used. The previously prepared skin on the Whatman® filter paper was cut into circles with a diameter of 15 mm and placed on the receptor compartment of the Franz cell. The skin was placed with the stratum corneum facing upwards toward the donor compartment followed by the rest of the Franz cell assembly, as explained in Section D.2.6. The conditions were kept constant to those created during the membrane release study. Over a 12 h period, at 2 h intervals, the receptor phase was extracted, placed in HPLC vials and replaced with the pre-heated HPLC-grade ethanol:PBS (7.4) (1:9). The extracted receptor phase from each Franz cell was analysed as described in Section D.2.1 by HPLC. These results were used to determine if CLF had diffused into the receptor phase illustrating transdermal diffusion.

D.2.9 Tape stripping

The tape stripping method was used during this study to determine the localisation and extent of transport of the API. This method determines the extent to which CLF concentrates in each layer of the skin, i.e. the stratum corneum-epidermis (SC.E) and the epidermis-dermis (ED.D). The tape stripping method gives an acceptable indication of the topical delivery of CLF after a 12 h diffusion period.

After completion of the diffusion studies, the skin samples were carefully removed from the Franz cells and placed on a piece of Parafilm® that was fixed onto a solid surface. The diffusion area of each skin sample was then diligently marked, as they comprise the only sections that can be used for tape stripping. The skin samples were immediately gently cleaned with clean tissue paper, where after the marked diffusion areas of each sample were placed on 3M Scotch® Magic™ tape. After removal, the first pieces of tape were disposed of in the designated biosafety bin. The following 15 pieces of 3M Scotch® Magic™ tape were applied to the skin and removed consecutively to extract the SC.E, until the skin had a gleaming appearance. Each set of 15 tape strips were placed in a polytop containing 5 ml HPLC-grade ethanol and stored in a fridge overnight at 4 °C. Lastly, the areas of excess skin where no diffusion occurred were removed and disposed of accordingly. After the tape stripping procedure, the skin samples, i.e. ED.D, from each Franz cell where diffusion occurred, were cut into small pieces and placed in polytops containing 5 ml HPLC-grade ethanol and stored in a fridge overnight at 4 °C. After an 8 h period in the freezer, the ethanol was removed from each polytop and filtered through PVDF filters into HPLC vials for analysis (Pellett *et al.*, 1997:91). This method was performed for the liposome and proliposomes diffusion study.

D.2.10 Data and statistical analysis of release and diffusion studies

The membrane release and skin diffusion data were quantitatively calculated according to the respective objective. The data from the membrane release studies reflected the cumulative amount of CLF per area unit (µg/cm^2) plotted against time (h) for the dispersions, respectively. The slope generated from the data was used to represent the average flux (µg/cm^2.h) of each. The average percentage API released from the vesicle systems was also determined for the membrane release studies. The skin diffusion results, i.e. transdermal and tape stripping respectively, were processed to reflect the percentage API that diffused into the receptor phase and the overall amount of CLF per area unit (µg/cm^2) over a period of 12 h. The tape stripping data reflected the separate concentrations (µg/ml) of CLF in the SC.E and ED.D layers to evaluate the success in which the API was topically delivered from vesicle systems, i.e. liposomes and proliposomes to the differentiating skin layers.

The above-mentioned data attained from the membrane release, skin diffusion and tape stripping studies were statistically analysed using the Statistica (StatSoft Inc., 2016) software programme. Two-way analysis of variance (ANOVA) tests were performed for the skin diffusion studies to determine if any statistical difference existed between the dispersions and the targeted skin layers. The statistical difference between the dispersions in each individual skin layer, i.e. SC.E and ED.D respectively, were also statistically analysed according to parametric t-tests and non-parametric tests. The same statistical approach was rendered for the membrane release studies to determine the statistical difference between the dispersions' flux values. The significance level for the ANOVA, t-tests and non-parametric tests was accepted when the p-value was smaller than 0.05 ($p < 0.05$).

D.3 Results and discussion

D.3.1 Aqueous solubility of CLF

CLF is deemed as practically insoluble in water at pH 7, according to Lu *et al.* (2011:5189). The results regarding CLF's solubility in PBS (pH 7.4), at 32 °C, was inconclusive. Lu *et al.* (2011:5187) used a detection limit of 0.01 g/100 ml also conveyed as 0.1 mg/ml. It was found the aqueous solubility of CLF was so little that even with a detection limit that small it was not possible to detected CLFs concentration to calculate its level of solubility. The almost complete insolubility of CLF in similar mediums is also supported by Bevan and Lloyed (2000:1785), Bolla and Nangia (2012:6250), Ganesh *et al*. (2013:198) and Narang and Srivastava (2002:1002), as they also expressed the extremely low solubility of CLF due to its highly hydrophobic nature, expressing the difficulty in determining the aqueous solubility of the API. Using literature, the solubility of CLF (0.000225 mg/ml) was obtained from Pubchem (2015). For drug compounds

to penetrate into the skin, the preferred aqueous solubility is 1 mg/ml or larger. This indicates CLF would not be able to permeate into the skin with ease, as a solubility of 0.00025 mg/ml would be highly undesirable for topical drug delivery (Naik *et al*, 2000:319).

D.3.2 Octanol-buffer distribution coefficient of CLF

The successful permeation of a drug molecule into the hydrophilic and lipophilic layers of the skin is largely determined by the log P value. A log P between 1 and 3, according to Naik *et al*., (2000:319), is ideal for drug molecules, since this enhances the chances of permeation into the skin. Instead of using the log P value for this study, the log D was determined as it provided a better distribution indication of the API in the more lipid or aqueous phase. As stated before, CLF is a highly lipophilic compound (Ganesh *et al.,* 2013:198) and in this study, it was determined that CLF has a log D of 4.60. Baik *et al.* (2013:1218) indicated that at a physiological pH, the log D of CLF ranges between 5 and 7. The small difference in the experimental and literature log D values may be due to experimental conditions not being exact. It is evident from these results that even with a high lipophilic nature (log D of 4.60) the permeation of CLF into the skin could prove troublesome and possibly concentrate in the more lipophilic area of the skin, i.e. stratum corneum (Naik *et al*, 2000:319).

The experimental log D value indicates the high lipophilic nature of CLF and this would illustrate the inverse relationship with aqueous solubility, i.e. high lipophilicity and low aqueous solubility of CLF in an aqueous medium.

D.3.3 Membrane release studies

The release of the API from the vesicle system was determined by means of membrane release studies, the results of which, as shown in Table D.2, indicate the successful release of CLF through the PVDF membranes from the (**CL2**) and (**CPL2**) dispersions.

Table D.2: Average flux (µg/cm^2.h), medium flux (µg/cm^2.h) and average %CLF released from (**CL2**) and (**CPL2**) after a 6 h membrane release study

Formulation	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)	Average %API released
(**CL2**)	158.78 ± 17.95	163.61	2.608 ± 0.30
(**CPL2**)	158.96 ± 19.20	181.52	2.895 ± 0.30

Figures D.2 and D.4 illustrate the membrane release data of (**CL2**) and (**CPL2**), respectively, which depicts the cumulative amount of CLF per area plotted against time (6 h) for each individual Franz cell.

The slope of the linear curve, when the average cumulative amount per area applied (of all the Franz cells used together) is plotted against time (6 h), represents the average flux (Figures D.1 and D.3). The median flux represents the middle point of the slopes obtained from all the Franz cells used during a 6 h membrane release study.

Usually the average, instead of median, is used to represent flux especially when there are no outliers in the data present. The absence of outliers prevents any effect on the average value. The median value represents a more reliable flux value when outliers are present and subsequently is more resistant in order to centre the data (Schmidlkofer, 2013:14). The average flux data for both dispersions illustrate a linear distribution from each Franz cell and illustrates the true flux of the API across the membrane.

The average flux for the (**CL2**) and (**CPL2**) dispersions are 158.78 ± 17.95 µg/cm^2.h and 158.96 ± 19.20 µg/cm^2.h, respectively. The median flux for the (**CL2**) and (**CPL2**) dispersions are 163.61 µg/cm^2.h and 181.52 µg/cm^2.h, respectively. The median flux was slightly higher than the average flux, but did not differ by much and therefore the release from the vesicle systems is closely relatable. Therefore, the average flux data would be sufficient and a reliable representation of the APIs release from both vesicle systems.

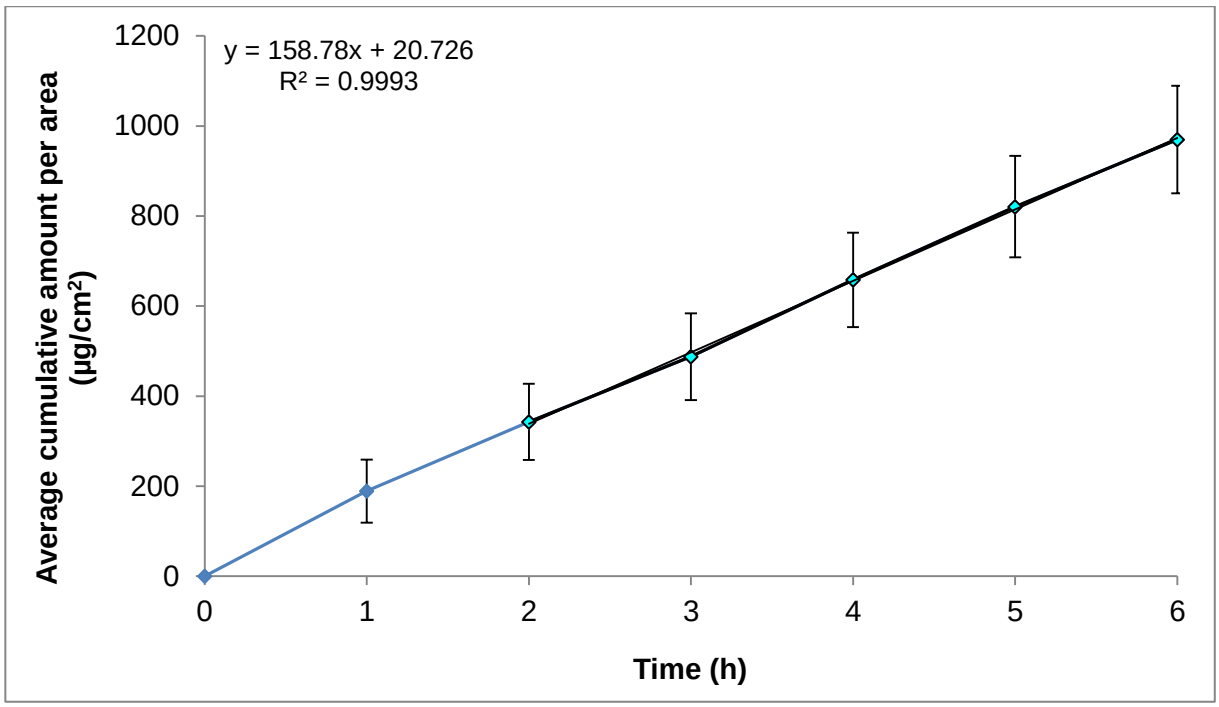

Figure D.1: Average cumulative amount of CLF per area that diffused through the membrane after the administration of the (**CL2**) as a function of time (6 h). The average flux was calculated from the graphs slope (n =10).

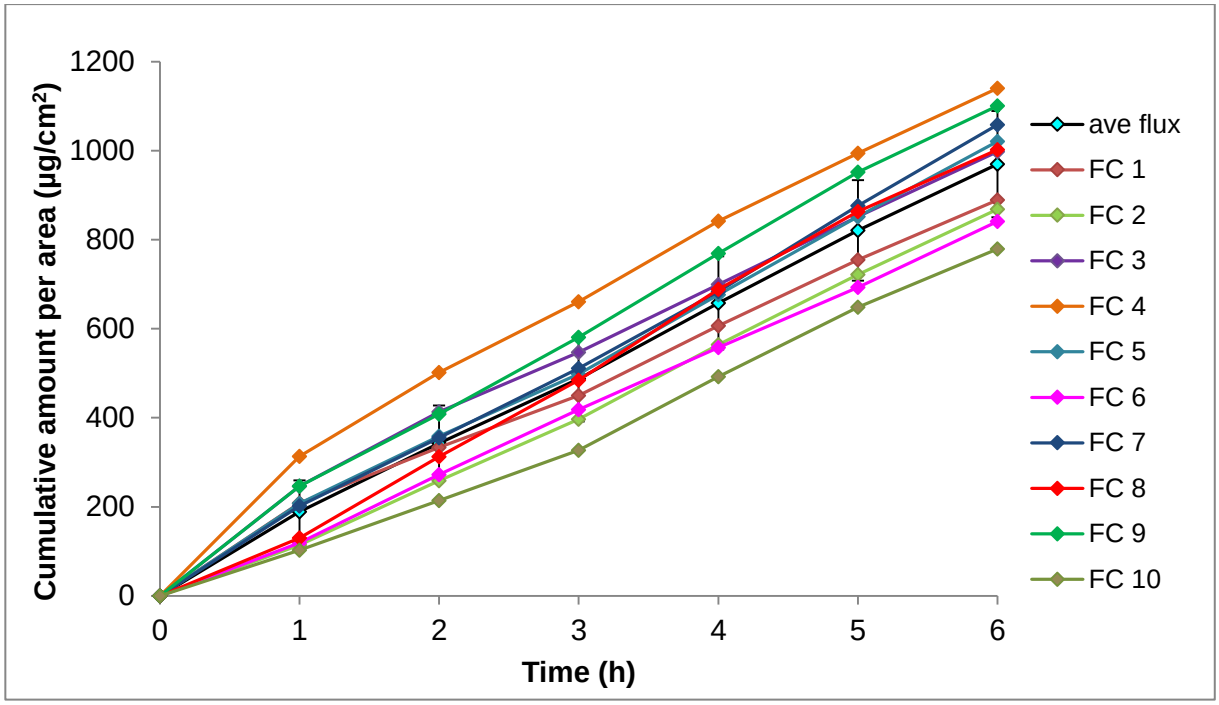

Figure D.2: Cumulative amount of CLF per area that diffused through the membrane after the administration of the (**CL2**) as a function of time (6 h) for each individual Franz cell, illustrating average flux (n = 10)

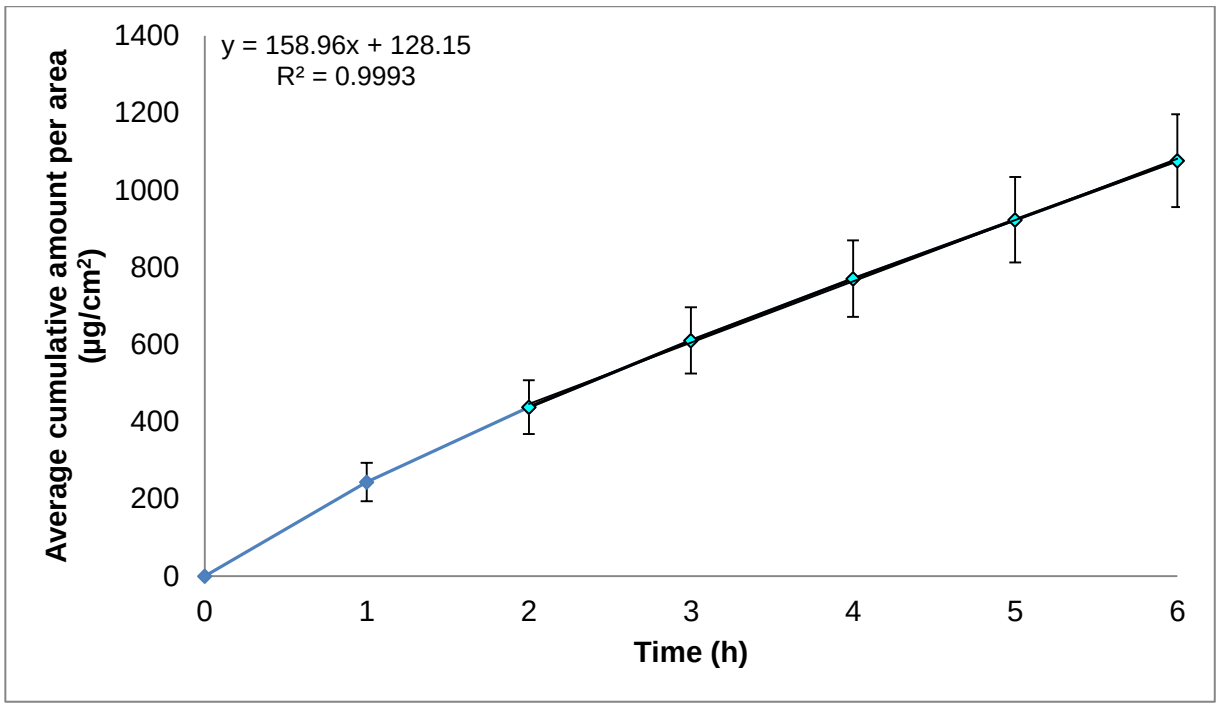

Figure D.3: Average cumulative amount of CLF per area that diffused through the membrane after the administration of the (**CPL2**) as a function of time (6 h). The average flux was calculated form from the graphs slope (n =10).

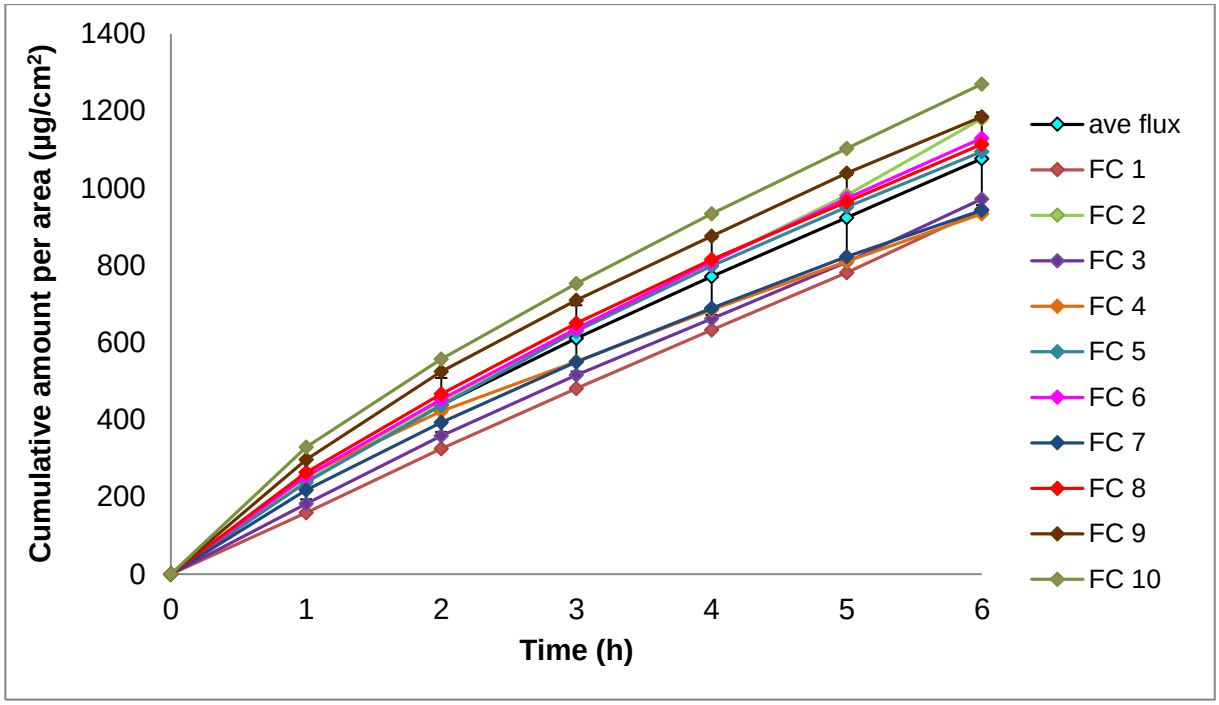

Figure D.4: Cumulative amount of CLF per area that diffused through the membrane after the administration of the (**CPL2**) as a function of time (6 h) for each individual Franz cell, illustrating average flux (n = 10)

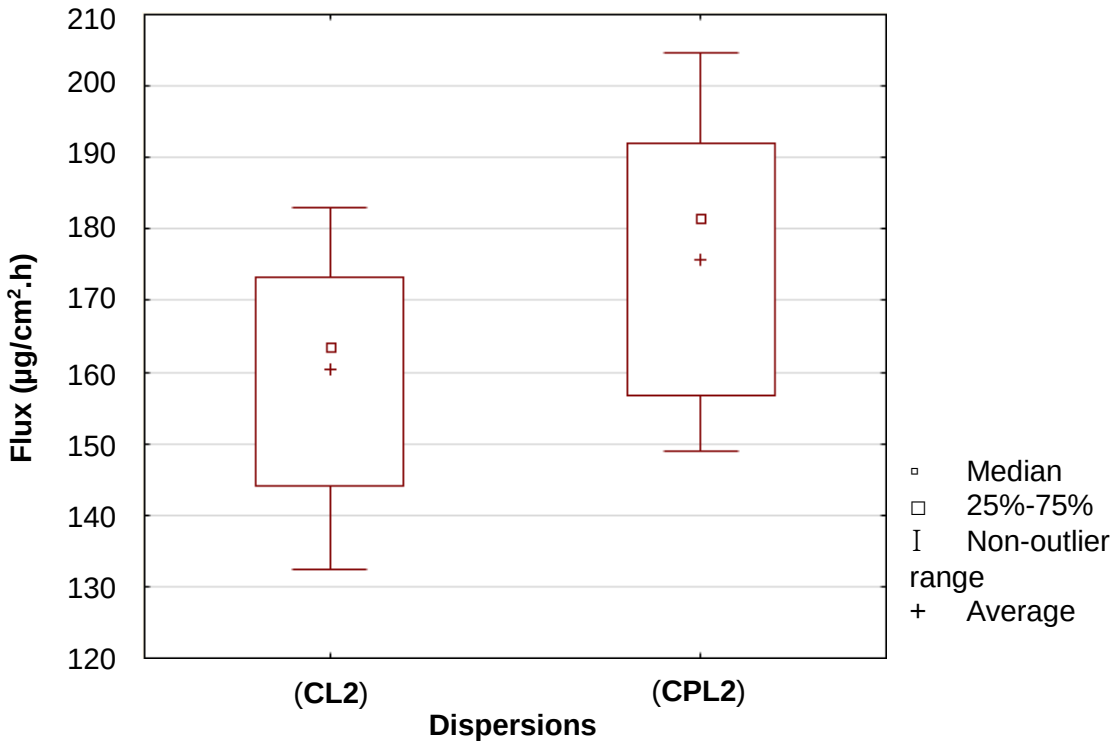

Figure D.5: Box-plot representing the flux (µg/cm^2.h) of (**CL2**) and (**CPL2**) present in the receptor phase during the 6 h membrane release studies. The median and average concentrations are respectively shown by the small square and plus symbols.

The box-plots in Figure D.5 display the flux (µg/cm^2.h) of the API that diffused into the receptor phase over the 6 h membrane release study from both dispersions, respectively. The small square and plus symbols illustrate the median and average concentrations, respectively. The individual box-plots illustrate that the proliposomes had a higher average flux value than the liposomes, but the data from each box-plot overlap, showing the close relationship between the two dispersions.

The membrane release study was performed to identify the successful release of CLF from both vesicle systems. It is important to note that these results are completely independent from the skin diffusion study results. The more suitable system from the membrane study has no direct correlation to being the ideal vesicle system for the skin diffusion study, as these results only depict the efficiency of API release from the vesicles. The results from the membrane release study reveal that both vesicle systems, i.e. liposomes and proliposomes, successfully released CLF through the membranes over 6 h.

D.3.4 Franz cell diffusion studies

The transdermal and topical delivery of CLF was determined by skin diffusion studies of the vesicle systems followed by the tape stripping, respectively. The goal of the diffusion study using human skin was to determine the transdermal or topical delivery of CLF, as this is used to investigate an additional route to deliver CLF cutaneously. The tape stripping and skin diffusion study indicates if the API was more concentrated in the SC.E, ED.D or the extracted receptor phase representing the blood plasma. The (**CL2**) and (**CPL2**) dispersions' skin diffusion results are discussed in the following section.

D.3.4.1 Transdermal diffusion

The receptor phase of both the (**CL2**) and (**CPL2**) dispersions showed no detection of CLF during the 12 h skin diffusion, respectively. This was also to be expected as CLF is a highly lipophilic drug. It should be kept in mind that the transdermal delivery of CLF was not the focus of this study, but rather the topical delivery, i.e. SC.E and/or ED.D of the API. Thus, the obtained results from the receptor phase supported the favourable outcome for topical delivery of CLF.

D.3.4.2 Tape stripping

The average API concentrations in the SC.E and ED.D are illustrated in Figures D.6 to D.11 and presented in Table D.3 for both the (**CL2**) and (**CPL2**) dispersions. The development of skin lesions from the *M. tuberculosis* initiate in the cutaneous tissue and support the region of topical delivery of CLF (Dos Santos *et al*., 2014:221). The SC.E possesses a more lipophilic character and is ideally suited for the topical delivery of lipophilic drugs (Naik *et al.*, 2000:319). Thus, the SC.E is the predicted area in which CLF are to concentrate. The ED.D (hydrophilic) is the targeted site for the cutaneous delivery of CLF, as the CTB lesions mostly reside in this layer (Yamaguchi *et al*., 2007:4391). Therefore, the (**CL2**) and (**CPL2**) dispersions should concentrate the API in the ED.D layer where minimal drug diffusion to the blood system would occur. The difficulty is the poor solubility and extremely high lipophilicity of CLF, which could limit the diffusion past the SC.E layer and rather concentrate in this layer (Naik *et al.*, 2000:319). The FC abbreviation in each graph refers to Franz cell.

Table D.3: Average concentration of CLF that remained in the SC.E and ED.D after the 12 h diffusion studies (n = 10)

Vesicle system	Average concentration in the SC.E (µg/ml)	Average concentration in the ED.D (µg/ml)
(**CL2**)	0.285 ± 0.199	0.079 ± 0.540
(**CPL2**)	0.523 ± 0.209	0.614 ± 0.320

D.3.4.2.1 Concentration in the SC.E

All 15 tape strips extracted from the skin, collected per Franz cell (n = 10), were analysed to determine the concentration of API found in the SC.E. The HPLC analysis results confirmed that both vesicle systems containing the API concentrated within this layer.

Figures D.6 and D.7 represent the concentration of CLF in the SC.E from the (**CL2**) and (**CPL2**) dispersions, respectively. The graphs indicate that all the Franz cells contained the API and no leakage occurred during either study. It is noticeable that CLF concentrated in the SC.E, indicating successful penetration into this lipophilic skin layer. The highest concentration for the (**CL2**) after the 12 h diffusion period was 0.578 µg/ml and an average concentration of 0.258 ± 0.199 µg/ml was established for the complete **(CL2)** tape stripping study. The highest concentration for the (**CPL2**) after the 12 h diffusion period was 1.012 µg/ml and an average concentration of 0.523 ± 0.209 µg/ml was established for the complete (**CPL2**) tape stripping study. The (**CPL2**) resulted in a higher average concentration of CLF than the (**CL2**). The outlier present in the (**CPL2**) box-plot in Figure D.8 does not influence this higher average concentration of the (**CPL2**), as the average concentration and median value are almost identical and closely related. The variation may be due to the stratification differences associated between the two different skin donors that were used (shortage of available skin from a single donor) (Margetts & Sawyer, 2007:171, Nava *et al*., 2011:965). The variation in the concentration of CLF is also due to the difference in the SC.E cohesiveness, as it possesses an inhomogeneous composition and continuous differentiation of corneocytes throughout this layer (Bolzinger *et al*., 2012:164). The comparative box-plots in Figure D.8 illustrate the higher average concentration of CLF in the SC.E by (**CPL2**) compared to (**CL2**). The penetration of CLF into this lipophilic layer was expected due to its high log P value and the results proved the successful accumulation of CLF in the SC.E by both dispersions (Naik *et al.*, 2000:319).

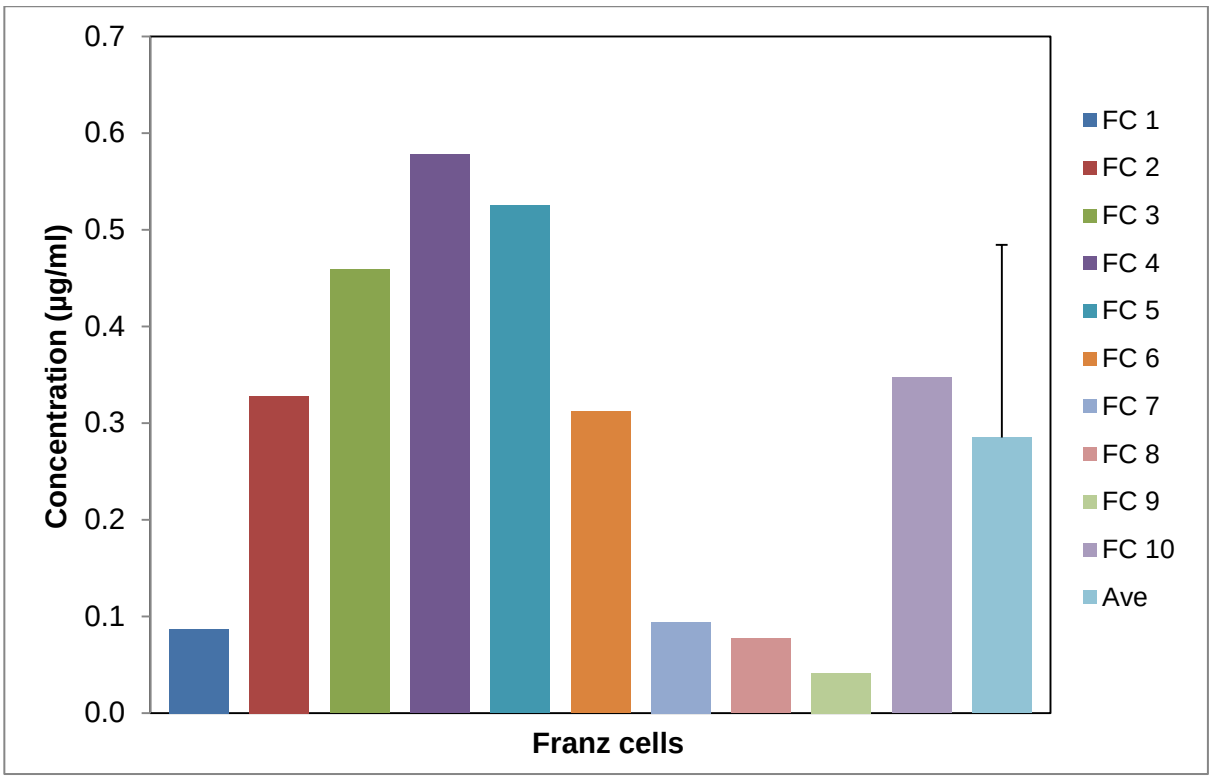

Figure D.6: SC.E data of (**CL2**) (n = 10)

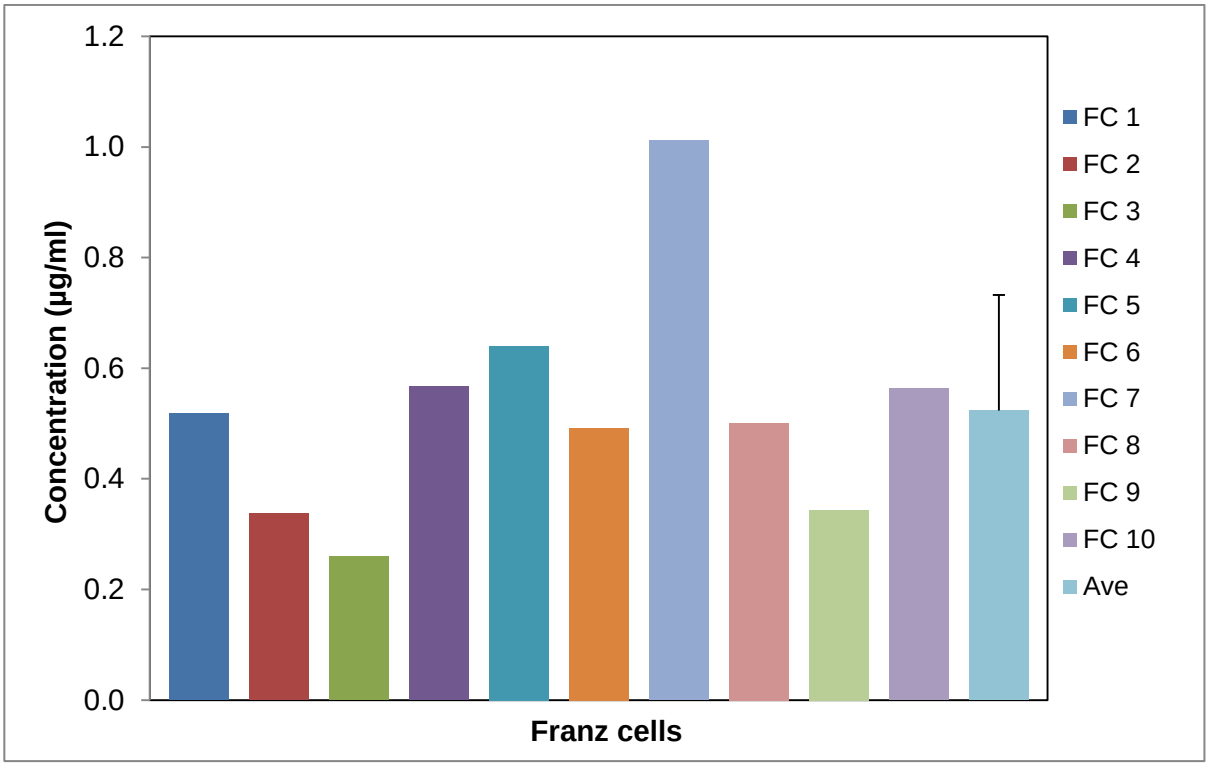

Figure D.7: SC.E data of (**CPL2**) (n = 10)

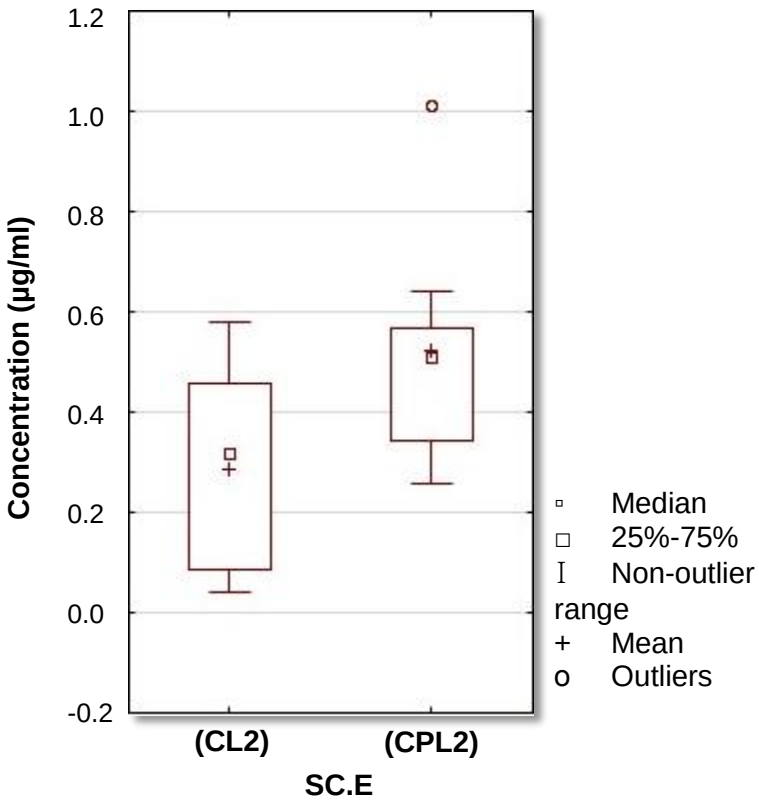

Figure D.8: Box-plot of the (**CL2**) and (**CPL2**) concentrations (µg/ml) found in the SC.E during tape stripping. The small box illustrates the median value and the plus symbol, the mean calculated from the data.

D.3.4.2.2 Concentration in the ED.D

After tape stripping, the diffusion area was cut into small pieces and left overnight in a solvent. The solvent was extracted and analysed to determine the concentration of CLF in the ED.D.

Figures D.9 and D.10 represent the ED.D data of each dispersion system and indicates that all the Franz cells contained the API and no leakage occurred in each respective experiment. From the results, it is clear that CLF also concentrated in the ED.D layer, indicating successful permeation through the SC.E. This absorption across the SC.E is atoned to the brick and mortar model, where CLF permeated into the ED.D layer through the corneocytes or the intercellular spaces along the lipid matrix of the SC.E (Schneider *et al*., 2009:198). The highest concentration for the (**CL2**) after the 12 h diffusion period was 0.205 µg/ml and an average concentration of 0.079 ± 0.540 µg/ml was established for the complete (**CL2**) study. The highest concentration for the (**CPL2**) after the 12 h diffusion period was 1.111 µg/ml and an average concentration of 0.614 ± 0.320 µg/ml was established for the complete (**CPL2**) study.

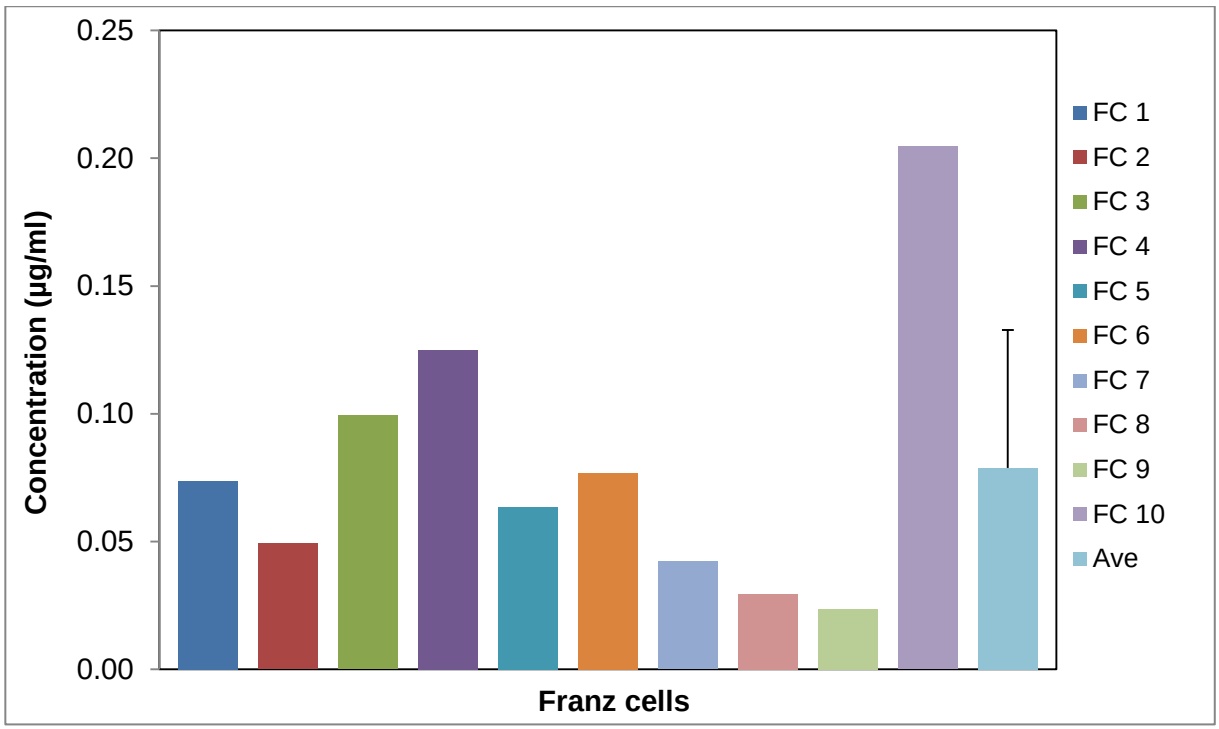

Figure D.9: ED.D data of (**CL2**) (n = 10)

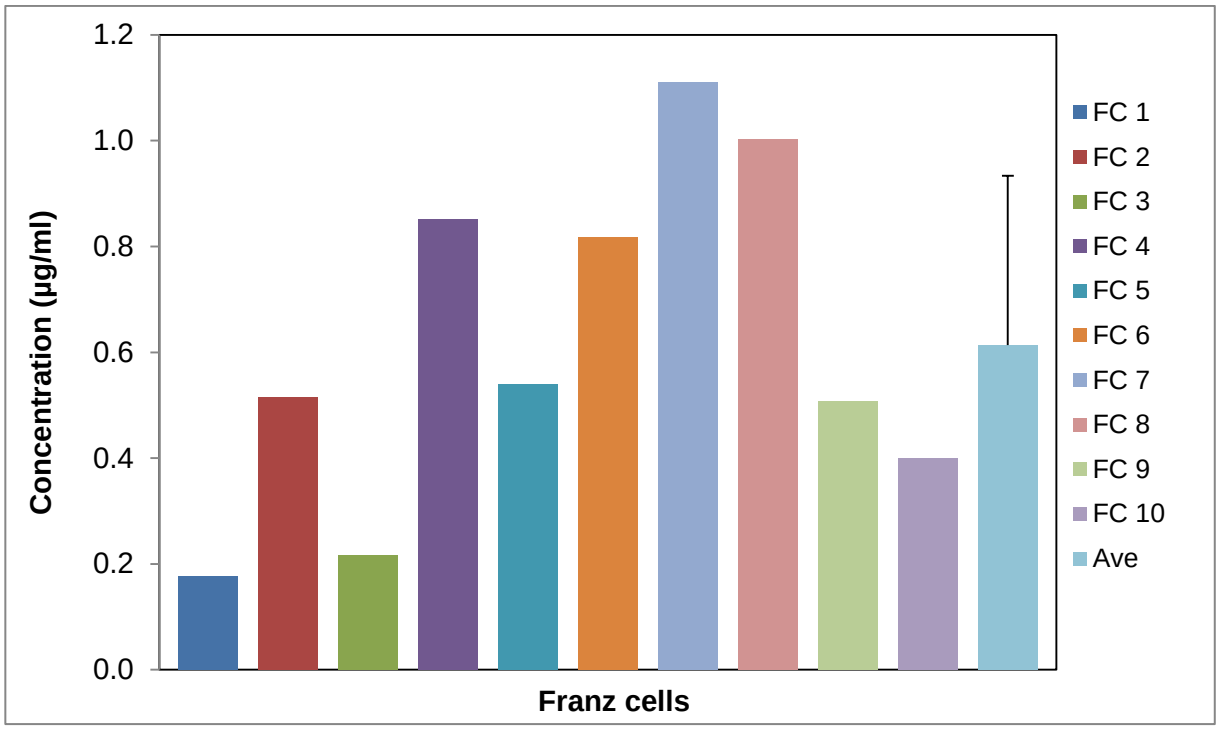

Figure D.10: ED.D data of (**CPL2**) (n = 10)

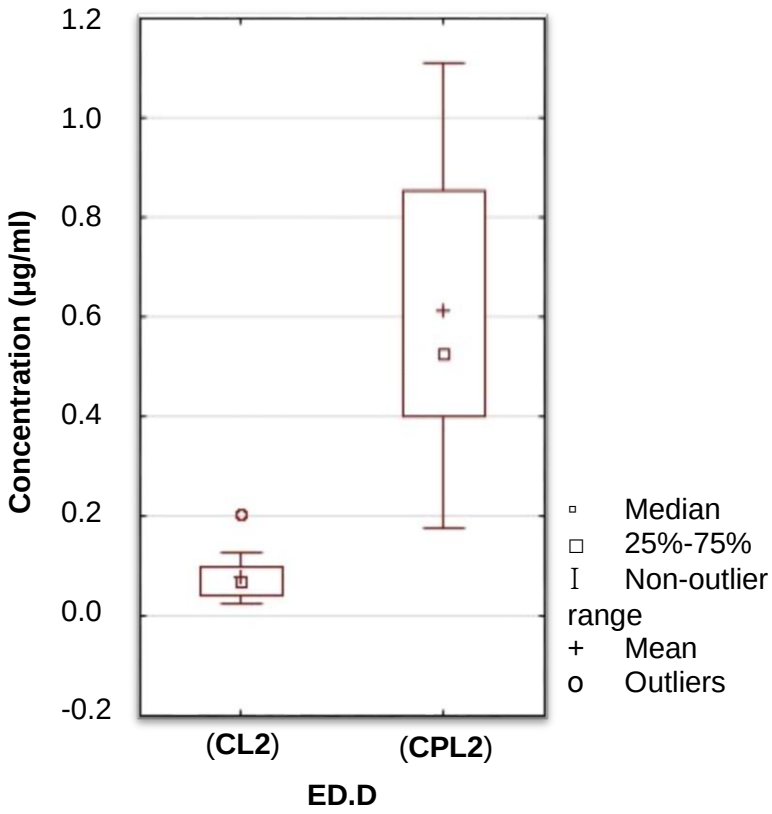

Figure D.11: Box-plot of the (**CL2**) and (**CPL2**) concentrations (µg/ml) found in the ED.D after tape stripping. The small box illustrates the median value and the plus symbol the mean calculated from the data.

A more successful permeation occurred using the (**CPL2**) vesicle system than the (**CL2**) vesicle system, and these results can be confirmed by the box-plots in Figure D.11, as there is large difference between the dispersions average concentrations of CLF in the ED.D layer. The difference in concentrations can be accredited to the expected variation from different skin donors, as the integrity of the skin is easily reshaped (Margetts & Sawyer, 2007:171). These results, which are confirmed by the box-plots (Figure D.11), indicate the successful permeation of CLF into this hydrophilic layer. The hydrophilic nature of the ED.D and the lipophilic nature of CLF stand in contrast to the expected results due to the different polarities. This indicates that the two vesicle systems may have theoretically improved the solubility of CLF by concentrating the lipophilic API in a hydrophilic ED.D skin layer.

D.3.4.3 Statistical analysis of diffusion studies

A t-test was performed to determine if there was any statistical difference between the average flux of the (**CL2**) and (**CPL2**) dispersions over the PVDF membranes. The Statistica (StatSoft Inc., 2016) software was used for data analysis. Non-parametric tests were also performed, but illustrated similar results, thus only using the parametric t-test was sufficient to show any statistical significance in the data. Figure D.5 illustrates the average flux (µg/cm^2.h) between

(**CL2**) and (**CPL2**) dispersions (p = 0.08). The p-values concluded that both dispersions exhibited no statistical significant difference at a 5% level. The box-plots, shown in Figure D.5, of the (**CL2**) and (**CPL2**) flux values illustrate the close relationship between the releases of CLF from both systems.

The SC.E and ED.D data after the 12 h diffusion studies were statistically analysed according the t-test, non-parametric tests, ANOVA tests and the Statistica (StatSoft Inc., 2016) software. The non-parametric tests showed similar statistical results to the parametric t-test and were excluded. The skin used in this study was taken from two different donors (due to the shortage of skin, and experiments were done according to skin availability). As stated above, the use of different skin donors can influence the data due to the integrity differences between donors and may evidently influence the statistical results (Margetts & Sawyer, 2007:171, Nava *et al*., 2011:965).

A two-way analysis of variance (ANOVA) test was conducted to determine the statistical significance of the dispersion and the skin layer effects, and for the interaction effect between them. There was a statistical significant difference found between the data of the dispersions (p = 0.000002), but no statistical significance was found between the two skin layers (p = 0.4) data. Thus, there was an expressive difference between the (**CL2**) and (**CPL2**) concentrations. A statistical significance was also found in the interaction effect of the dispersions and the skin layers (p = 0.04). Due to this significant interaction effect, it was decided to compare the dispersions within each skin layer, as well as the comparison of skin layers within the dispersions.

T-tests were performed in the following two combinations: (**CL2**) versus (**CPL2**) in the SC.E and the ED.D, respectively and secondly, the (**CL2**) and (**CPL2**) individually in both skin layers. The (**CL2**) (p = 0.02) and (**CL2**) (p = 0.00006) showed a statistical significance between their concentrations in both the SC.E and the ED.D layers. The (**CL2**) (p = 0.006) represented a statistical significance in both layers, whereas the (**CPL2**) (p = 0.46) were statistically the same and represent no difference between the two layers.

D.4 Conclusion

Due to the cutaneous lesion development of CTB, the ED.D was the preconceived and ideal target area for CLF to have its anti-mycobacterial effect (Dos Santos *et al*., 2014:221). The highly lipophilic properties of CLF destined it to accumulate only within the lipophilic region of the skin, i.e. SC.E. Therefore, implementing the use of liposomes ((**CL2**)) and proliposomes ((**CPL2**)) to alter the solubility of the API for further passive diffusion into the intended target area was approached. The successful release of CLF from the liposomes and proliposomes

were illustrated by the membrane release results, as the (**CPL2**) represented a slightly larger release but was statistically indifferent to that of the (**CL2**) release. The delivery of CLF into the SC.E and ED.D and not into the receptor phase demonstrates that the topical, rather than transdermal, delivery of the API was a success. This proved that both the liposomes and proliposomes effectively penetrated the API through the SC.E (defensive barrier preventing drug absorption) and into the hydrophilic ED.D. Even though there was successful penetration of CLF from the proliposomes into the ED.D, an almost equal amount accumulated in the SC.E. This supported the predicted outcome of CLF accumulating in the predominantly lipophilic SC.E due to the APIs physicochemical properties. Vesicle systems have the ability to improve the therapeutic index and bioavailability of drug absorption. Liposomes, furthermore, have the multifunctional capabilities of a) acting as drug carrier by transporting the encapsulated drug molecules across the skin barrier, b) being penetration enhancers, c) serving as storing places for active compounds to be released and d) serving as rate-limiting membrane barriers for the controlled release of APIs (Honeywell-Nguyen & Bouwstra, 2005:67-68; Pierre & Costa, 2011:609). This was seen in the presence of small amounts of the lipophilic CLF in the hydrophilic ED.D, since both vesicle systems improved the solubility of the API to a certain extent.

As literature states, CLF is incredibly insoluble in an aqueous medium; the study in determining the solubility of CLF in PBS (pH 7.4) yielded no different result. Thus, for the purpose of this study, the aqueous solubility of CLF was retained from a literature source, i.e. Pubchem (2015), stating that CLF has a solubility of 0.000225 mg/ml. Also determined was the log D to confirm the lipophilic character of CLF, which was found to be 4.60 and indicates that CLF might be too lipophilic and have difficulty crossing the stratum corneum, preferring to reside within this layer (Naik *et al*., 2000:319). In comparison, the (**CL2**) and (**CPL2**) both yielded low concentrations of CLF in the SC.E and ED.D, indicating topical delivery, which correlates well with the aim of the study. The results accumulated throughout this study fit the scope of improving the APIs solubility using vesicle systems and provide evidence to support the topical delivery of CLF.

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APPENDIX E

***In vitro* cytotoxicity studies of clofazimine**

E.1 Introduction

Investigating the cytotoxicity profile of pharmaceutical actives using *in vitro* skin models is a valuable tool in assessing any possible irritation or relatable skin damage as an effect thereof (Groeber *et al.*, 2011:364). In the past, testing of drugs' toxicity was conducted using animals as human surrogates. This approach has been denounced due to certain assumptions made regarding the effects low doses of drugs in humans have, e.g. by exposing animals to higher doses of the APIs than the expected standard level of exposure to humans and evidently making correlated assumptions. The use of animals as human surrogates does have ethical and economical limitations and consequently, provide a driving force for the use of non-animal models for testing drug toxicity (Holmes *et al*., 2010:15). Thus, as the understanding of animal models evolve and improve, the prediction regarding human safety and eventually the *in vitro* approach can possibly limit the need to use animal testing in the future (Chapman *et al*., 2013:89).

The use of cell cultures *in vitro* not only mimics a human skin model, but also produces reliable and reproducible data to obtain optimum results and exclude most concerns regarding animal drug testing (Groeber *et al.*, 2011:353, Jurisic & Bumbasirevic, 2008:51). There are also certain concerns in using an *in vitro* approach, e.g. it mostly portrays the effect an API has on a cellular level and does not account for any other extracellular actions that occur (e.g. the absence of absorption, excretion (pharmacokinetics), metabolism and distribution), which leads to the exclusion of crucial mechanisms (Hartung & Daston, 2009:234, Yoon *et al*., 2012:634). The expulsion of these mechanisms influences the interpretation of the data between *in vitro* and *in vivo* studies, as the context adhering to the results are not relatable to draw direct conclusions (Yoon *et al*., 2012:634). Thus, the outcome of this experiment does not give any relatable information regarding toxicity of CLF *in vivo,* rather only *in vitro* and merely provides preliminary results, which may propagate further interest in future studies.

The human skin consists of a variety of cell types, which include fibroblasts, keratinocytes and melanocytes, each with their own distinctive role (López-García *et al*., 2014:44). For the investigation into skin irritants, the use of a keratinocyte cell line is a biologically relevant target as they have a similar nature to that of a live human organism (Ölschläger *et al*., 2009:147, Wilheim *et al*, 2001:713). Consequently, investigating the cell death caused by CLF involved the *in vitro* cytotoxicity determination of the free drug, (**PL2**) and (**CL2**) dispersion on HaCaT

cells lines. The cell death caused by CLF was evaluated according to a biochemical approach using a colorimetric method (Fukutomi *et al*, 2011:4001).

During the *in vitro* experiments, LDH release tests was performed and evaluated to determine the level of HaCaT cell death related to the use of a vesicle, i.e. liposome system (Fotakis & Timbrell, 2006:171). Two vesicle samples with increasing concentrations were evaluated, i.e. 1) (**PL2**) and 2) (**CL2**) and the API, respectively.

E.2 Materials and method

E.2.1 Equipment and materials

The Laboratory for Applied Molecular Biology (LAMB), at the North-West University (Potchefstroom), provided the following equipment for the cell culture preparation and LDH release experiment: laminar flow hood (high flow minimum 20 min), CO_2 incubator (humidified, 37 °C and 5% CO_2), water bath (37 °C), pipettor, light microscope, haemocytometer and an aspirator. Table E.1 shows the constituents used for the preparation of the cell culture and LDH release experiments.

Table E.1: List of products used for cells culture preparation and LDH release study

Product	Brand	Catalogue number	Lot number
Dulbecco's modified eagle's medium (DMEM) with high glucose, 4.0 mM L-glutamine, sodium pyruvate	HyClone™	SH30243.FS	AB216032
MEM Non-Essential Amino Acid (MEM NEAA) (100%)	HyClone™	SH30238.01	AAB199680
L-Glutamine	HyClone™	SH30034.02	-
Penicillin/Streptomycin (Pen/Strep)	Lonza™	DE17-602E	5MB068
Trypan Blue solution (0.4%)	Sigma-Aldrich®	T8154	RNBC9030
Phosphate Buffered Saline (1x)	HyClone™	SH30256.01	AAD201744
Trypsin-Versene® EDTA	Lonza™	BE17-161F	5MB168
Foetal Bovine Serum (FBS)	HyClone™	SV30160.03	RZM35923
75 cm^2 cell culture Flask	Corning®	43146RU	24315044
96-Well plate	TPP®	92096	20150301
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega™	-PRG1780	-

E.2.2 Cell line

An HaCaT (human, adult, low calcium, high temperature) cell line was used in this study, due to ease of preparation and its closeness to ordinary phenotype, making this spontaneously immortalised cell line an ideal keratinocyte model (Ölschläger *et al*., 2009:147). Keratinocytes have a defining role in the skin, taking on the part of being a tissue regenerator of the epidermis (e.g. wound healing), making them suitable during *in vitro* models for evaluating the toxicity of an API (Deyrieux & Wilson, 2007:77, Lopez-Garcia *et al*., 2010:2846, Ölschläger *et al*., 2009:147). HaCaT cells have been used broadly in epidermal pathophysiology and homeostasis studies (Seo *et al*., 2012:171) and are ideal for cell viability/death studies due to their human skin origin, which makes these cells closely comparable to normal skin components (Sorrell & Caplan, 2004:667).

E.2.3 Cell culture cultivation

The epidermal cell line used to determine the cytotoxicity (cell death) of the API was the HaCaT cell line. The procedure followed for preparation of the cells cultures was adapted from a standard seeding of mammalian cells, SOP (Gouws, 2014), used in the LAMB - CTC lab. The HaCaT cells were sustained with a culture growth medium, i.e. DMEM (or high glucose medium), in a humidified incubator at 37 °C and 5% CO_2. The growth medium was enriched with the following supplements: 10% FBS, 1% Pen/Strep, 4 mM L-glutamine and 1% MEM NEAA (Ahn *et al*., 2012:21).

The HaCaT cells were cultivated in a 75 cm^2 flask with the growth medium (DMEM or high glucose medium); the culture media was replaced every other day with fresh growth media to provide the cells with a continuous supply of nutrients. When a confluence of 80% was reached, the cells were passaged to new flasks at smaller dilutions to enable continuous cell growth. The following procedure was employed for the HaCaT cell culture using Trypsin for seeding (Gouws, 2014):

- All items, including the laminar flow work area, were cleaned with 70% ethanol.
- This was followed by decanting the preheated growth medium from the flask.
- 10 ml of a preheated buffer was added to the flask and decanted to remove the buffer from the flask.
- Trypsin-Versene (3 ml) was added to the flask and closed, where after the solution was distributed equally in the flask before placing it back into the CO_2 incubator.
- After 10 to 15 min in the incubator (37 °C), the solution was checked for detachment every 2 min by gently shaking the flask.

- After the detachment of most cells, a serological pipette was used to pipet the cells to observe (under a microscope) if a single cell suspension had developed.
- To the flask, 6 ml preheated growth medium was added and pipetted carefully.
- The suspension containing the cells was then transferred into a 50 ml tube and pipetted vigorously.
- The cell suspension was then diluted to obtain the required concentration and the volume was transferred into a new 75 cm flask.
- The flask was then placed in a CO_2 incubator and maintained until needed for the experiment.

E.2.4 Seeding of cells for toxicity assay

A Trypan Blue dye exclusion approach was used to quantify the amount of cells present in the culture by visualising the morphology of the cells. This method was used to determine the total number of cells present in the cell suspension to, in turn, calculate the amount of the suspension needed to acquire a total of 20 000 cells/well in a 96-well plate. During this assay, dead (non-viable) cells accept the dye, whilst live (viable) cells do not (Chang *et al*., 2011:204). When this assay yields cell viability of at least 95%, the cells can be used for further experimental work. The following procedure was adhered to procure a counting mixture for the haemocytometer:

- A cover slide was positioned in the middle of the haemocytometer.
- In a microcentrifuge tube, Trypan Blue 0.4% (w/v) and Phosphate Buffered Saline, at volumes of 25 µl and 15 µl, were combined, respectively.
- The cell suspension (10 µl) was then combined with the Trypan Blue-Phosphate Buffered Saline mixture (35 µl) and mixed well.
- The counting mixture was then incubated at room temperature for 3 min.
- This was followed by pipetting the mixture and extracting 10 µl.
- The extracted counting mixture was then placed on the edge of the cover slip and gently discharged (Repeated for the other half of the counting chamber).

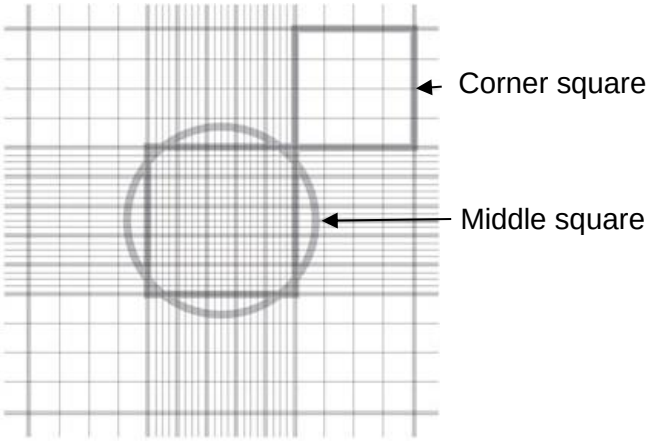

Figure E.1: Illustration of a standard haemocytometer chamber adapted from Sigma-Aldrich (2016).

The etched counting chamber (9 mm^2), as shown in Figure E.1, provides nine large squares, but only the live cells in the corner and middle squares where counted giving a total of five squares per counting chamber side, thus 10 squares per haemocytometer in total. The quantification of cells was done by counting the live (clear, round) cells.

The total number of cells present in the suspension was calculated according to Equation E.1:

$$\frac{\text{Live cells in squares (n = 10)}}{10} \times (5 \times 10^4) \times \text{Total volume of cell suspension} \qquad \textbf{Equation E.1}$$

Equation E.2 was then used to calculate the %cell viability:

$$\%\text{viable cells} = \frac{\text{Unstained cells (n)}}{\text{Total number of cells}} \times 100 \qquad \textbf{Equation E.2}$$

E.2.5 Stock and dispersion preparation

A stock solution, i.e. free drug sample, was prepared to use as a reference regarding the cytotoxicity of the API without the vesicle system. The stock solution (20 mg/ml) was prepared by dissolving 200 mg CLF (raw) in 10 ml solvent (50:50 ratio of methanol and HPLC water with 1% glacial acetic acid). A range of concentrations was acquired by diluting the stock solution in the growth medium to acquire 0.1, 0.2 and 0.4 mg/ml concentrations for each respective well. Each sample was prepared in triplicate and repeated twice.

The preparation of the (**PL2**) and (**CL2**) dispersion was done according to the procedure explained in Section B.2.2.3 and B.2.2.4, respectively. Table E.2 shows the amount of each ingredient needed to prepare 10 ml of each dispersion. The dispersions were diluted to 0.1, 0.2 and 0.4 mg/ml samples in the growth medium, in correlation to the free drug concentrations, to

establish a comparable concentration range between the dispersion samples and the stock samples. Each sample was prepared in triplicate and repeated twice.

Table E.2: Formula for (**PL2**) and (**CL2**) dispersions

Ingredients	(PL2)	(CL2)
CLF	-	200 mg
Cholesterol	200 mg	200 mg
PC	1540 mg	1570 mg
α-Tocopherol	16 µl	16 µl
Methanol/chloroform	10 ml	10 ml
Acetate buffer (pH 5)	10 ml	10 ml

E.2.6 Determining cell death using LDH assay

A colorimetric method was used to establish cell viability. A CytoTox 96® Non-Radioactive Cytotoxicity Assay Detection Kit was used to evaluate the LDH release. The LDH release measurements were conducted with: 1) a negative control (wells containing only culture medium), 2) positive control (medium representing total cell damage of 100% LDH release), 3) a medium containing the placebo dispersion ((**PL2**)) samples, 4) a medium containing the dispersion with API ((**CL2**)) samples and 5) free drug sample (API only) (Ahn *et al*., 2012:21). A range of concentrations from the stock solution (free drug), (**PL2**) and (**CL2**) were prepared to ensure several concentrations levels were tested.

The following cytotoxicity protocol was performed to determine the %cell deaths caused by the samples:

- Firstly, a 96-well assay plate was set up containing the growth medium and HaCaT cells.
- The selected sample wells were then made up to volume (100 µl) with the respective stock samples (0.1, 0.2, 0.4 mg/ml), (**PL2**) (0.1, 0.2, 0.4 mg/ml) samples and (**CL2**) (0.1, 0.2, 0.4 mg/ml) samples.
- The plate was then incubated at 37 °C for 12 h.
- 45 min before the incubation period was over, 10 µl of the 10X Lysis Solution was added to the positive control wells to enable maximum LDH release, which is needed to determine the %cytotoxicity of all the tested samples.
- Into a new 96-well flat clear bottom plate, 50 µl aliquots containing the samples were transferred.

- To each sample aliquot, 50 µl of the CytoTox 96® Reagent was added and covered with foil, followed by incubating the plate at room temperature for 30 min.
- After incubation, 50 µl of the Stop Solution was added to each sample well.
- Lastly, before 1 h had passed after adding the Stop Solution, the absorbance (OD) was recorded at 490 nm using the SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA).

The cytotoxicity of CLF was determined by evaluating the leakage of LDH into the culture media (Fotakis & Timbrell, 2006:171). Firstly, from the acquired sample well values, the average values of the growth medium background were subtracted accordingly. Secondly, Equation E.3 was used to quantify the %cytotoxicity.

$$\%\text{Cytotoxicity} = \frac{\text{Experimental LDH release (OD490)}}{\text{Maximum LDH release (OD490)}} \times 100 \qquad \textbf{Equation E.3}$$

E.3 Results and discussion

E.3.1 Cell death

The free drug, (**PL2**) and (**CL2**) dispersions' toxicity was examined by evaluating the cell death of the HaCaT cells using an LDH release assay; LDH is a cytoplasmic enzyme present in most cells and upon cell damage, is released into the extracellular medium. The release of LDH from the damaged HaCaT cells was detected by an LDH assay, which estimates the determinant enzyme of cell damage (Perche *et al*., 2012:99). The detection of LDH leakage in the cell culture media is done in the presence of a tetrazolium salt in the assay. Firstly, the released LDH causes the conversion of enzymes, i.e. nicotinamide adenine dinucleotide (NAD), into its reduced form nicotinamide adenine dinucleotide (NADH), when lactate is oxidised to pyruvate. With each newly synthesised NADH molecule, in conjunction with an electron acceptor, the tetrazolium salt in the assay is converted to formazan, which elicits a colour change in proportion to the amount of LDH released. The intensity of the formazan product illustrates the level of cell damage that has occurred (Chan *et al*., 2013:66).

The reference value for cell viability, as mentioned by López-García *et al*. (2014:45), was adapted to reflect cell death, thus a strong cytotoxicity ranged between 100% and 80%, a moderate cytotoxicity between 80% and 60%, a weak cytotoxicity 60% and 40% and below 40% is considered as non-cytotoxic. Figure E.2 provides the %cell damage from each sample at predetermined concentration ranges.

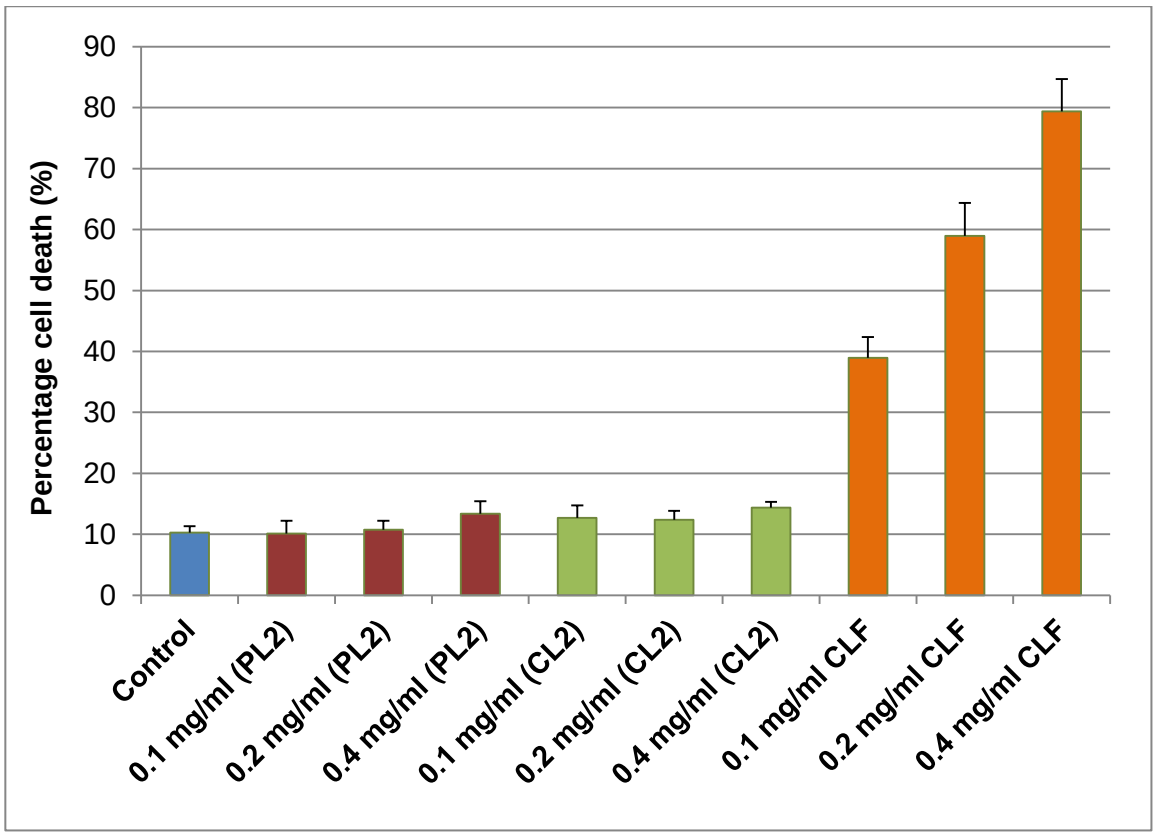

Figure E.2: Percentage cell death of the HaCaT cells after exposure to the (**PL2**) and (**CL2**) dispersions and the API (stock solution) at concentrations of 0.1, 0.2 and 0.4 mg/ml

The graph in Figure E.2 illustrates the effect the (**PL2**), (**CL2**) and API (free drug) had on the HaCaT cells. The control (representing only the growth medium) had a %cell death of 10.25 ± 1.09% and was used as a reference of a non-cytotoxic sample. Generally, liposomes consist of natural phospholipids, which evidently make them non-toxic and pharmacologically inactive, and a non-cytotoxic effect would be expected (Sercombe *et al*., 2015:2). The 0.1, 0.2 and 0.4 mg/ml concentrations of the (**PL2**) dispersions had a %cell death of 10.11 ± 2.09%, 10.72 ± 1.48% and 13.37 ± 2.07%, respectively, illustrating no cell damage had occurred and supported by literature (Sercombe *et al*., 2015:2). Similar, but slightly larger percentages were calculated for the (**CL2**) dispersion, since it had a %cell death of 12.71 ± 2.05%, 12.38 ± 1.46% and 14.35 ± 0.96% for the 0.1, 0.2 and 0.4 mg/ml concentrations, showing no significant cell damage occurred. It is clear the 0.1, 0.2 and 0.4 mg/ml concentrations of the (**PL2**) and (**CL2**) dispersions were non-cytotoxic, according to López-García *et al*. (2014:45) guidelines, indicating the dispersions, regardless of the concentrations, exhibited almost no cell death. Even though the (**CL2**) dispersions did experience a slightly higher level of cell damage, it is not of significant value in terms of toxicity. Clearly the vesicle dispersion containing the API ((**CL2**)) did not have the expected increased level of cytotoxicity compared to the placebo vesicle

dispersions containing no API ((**PL2**)), even when differentiating between the ranges of concentrations. Liposomes exhibit a protective effect on encapsulated drugs by preventing the influence of naturally occurring processes (e.g. metabolised before reaching target site) on the drug (Bozzuto & Molinari, 2015:979), evidently safeguarding against biodegradation (Sercombe *et al*., 2015:2). One possible reason the (**PL2**) and (**CL2**) dispersions yielded closely relatable cell death percentages was because of the protective property the liposomes had on CLF. Another possible reason could be the hydrophobic nature of liposomes, as it is inhomogeneous with the aqueous (hydrophilic) growth medium and causes the vesicle to retain the API leading to the non-cytotoxic results (Sercombe *et al*., 2015:1).

The free drug samples showed different results than the vesicle samples, as the values for the %cell death were 38.97 ± 3.41%, 58.98 ± 5.39%, 79.36 ± 5.32% for the 0.1, 0.2, 0.4 mg/ml concentrations, respectively, illustrating a substantial increase in the level of cell death. A direct equivalence was found between the concentration of the API and the level of cell death. Thus, as the concentration of the free drug increases exponentially, the %cell death also increases proportionally. The 0.1, 0.2 mg/ml samples were both close to the range of weak cytotoxicity and the 0.4 mg/ml sample in range of being strongly cytotoxic. A correlation can be seen when the vesicle system was absent, as a dramatic increase in cytotoxicity was observed. Metha (1996:1896) performed similar *in vitro* studies on CLF and liposomes encapsulating CLF, only on different cell lines (Red blood cells and macrophages), and yielded matching results to this study. The results from the Metha (1996:1896) study illustrated that for red blood cells, the free drug exerted a strong toxicity (40 – 50% cell lysis) compared to the liposome system (10 – 30%) and for the macrophages cell line, the liposome system illustrated a significantly lower cytotoxicity than the free drug. This study reveals the consistent level of improvement liposomes provide to decrease the level of toxicity of CLF *in vitro*.

It is evident the %cell death differed during the absence of a vesicle system as this resulted in a stronger level of cell death in the HaCaT cells. Regardless of concentration, the (**PL2**) and (**CL2**) dispersion samples had closely relatable results representing a non-cytotoxic result. The free drug samples experienced stronger levels of cytotoxicity, which may be atoned to the protective barrier the liposomes provide and possibly, the absence of a hydrophilic vesicle increased the level of API exposure to the more aqueous medium. The results were also supported by literature, which illustrates a correlation between the improvements liposomes provide, against the toxicity of the free drug CLF.

E.4 Conclusion

Even though the *in vitro* results cannot be compared to *in vivo* studies, the results are of great value for future studies. The samples containing the liposome system ((**PL2**) and (**CL2**)) exhibited a non-cytotoxic result, while the free drug (stock solution) samples experienced a weak cytotoxicity for the lower concentrations and a strong cytotoxicity for the highest concentration. This was attributed to the protective barrier liposomes provide, shielding CLF from biodegradation. This protective effect was confirmed by literature, as different cell lines yielded similar results, which illustrates that encapsulating CLF in liposomes improves the protective effect the vesicle has on the API for toxicity *in vitro*.

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APPENDIX F

Authors Guidelines: Drug Delivery

F.1 Aims and scope

Drug Delivery serves the academic and industrial communities with peer reviewed coverage of basic research, development, and application principles of drug delivery and targeting at molecular, cellular, and higher levels. Topics covered include all delivery systems including oral, pulmonary, nasal, parenteral and transdermal, and modes of entry such as controlled release systems; microcapsules, liposomes, vesicles, and macromolecular conjugates; antibody targeting; protein/peptide delivery; DNA, oligonucleotide and siRNA delivery.

F.2 Instructions for authors

F.2.1 About the journal

Drug Delivery is an Open Access, international, peer reviewed journal, publishing high-quality, original research.

Open Access (OA) means you can publish your research so it is free to access online as soon as it is published, meaning anyone can read (and cite) your work. The standard article publishing charge (APC) for this journal is: £1000/$1600/€1335. Depending on your location, these charges may be subject to local taxes.

Please note that this journal only publishes manuscripts in English.

This journal accepts the following article types: original papers, reviews and book reviews.

F.2.2 Peer review

Taylor & Francis is committed to peer-review integrity and upholding the highest standards of review. Once your paper has been assessed for suitability by the editor, it will then be single blind peer-reviewed by independent, anonymous expert referees.

F.2.3 Preparing your paper

All authors submitting to medicine, biomedicine, health sciences, allied and public health journals should conform to the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, prepared by the International Committee of Medical Journal Editors (ICMJE).

F.2.3.1 Structure

Your paper should be compiled in the following order: title page; abstract; keywords; main text: introduction, methods, results, discussion and conclusion; acknowledgments; declaration of interest statement; references; appendices (as appropriate); table(s) with caption(s) (on individual pages); figures; figure captions (as a list).

F.2.3.2 Word limits

Please include a word count for your paper.

A typical paper for this journal should be no more than 30 pages; this limit includes tables; references; figure captions; with no more than 6 figures and tables.

F.2.3.3 Style guidelines

Please refer to these style guidelines when preparing your paper, rather than any published articles or a sample copy.

Font: Times New Roman, 12 point, double-line spaced. Use margins of at least 2.5 cm (or 1 inch).

Title: Use bold for your article title, with an initial capital letter for any proper nouns.

Abstract: Indicate the abstract paragraph with a heading or by reducing the font size. Check whether the journal requires a structured abstract or graphical abstract by reading the Instructions for Authors. The Instructions for Authors may also give word limits for your abstract.

Keywords: Please provide keywords to help readers find your article. If the Instructions for Authors do not give a number of keywords to provide, please give five or six.

Headings: Please indicate the level of the section headings in your article:

- First-level headings (e.g. Introduction, Conclusion) should be in bold, with an initial capital letter for any proper nouns.
- Second-level headings should be in bold italics, with an initial capital letter for any proper nouns.
- Third-level headings should be in italics, with an initial capital letter for any proper nouns.
- Fourth-level headings should be in bold italics, at the beginning of a paragraph. The text follows immediately after a full stop (full point) or other punctuation mark.
- Fifth-level headings should be in italics, at the beginning of a paragraph. The text follows immediately after a full stop (full point) or other punctuation mark.

Tables and figures: Indicate in the text where the tables and figures should appear, for example by inserting [Table 1 near here]. The actual tables should be supplied either at the end of the text or in a separate file. The actual figures should be supplied as separate files. The journal Editor's preference will be detailed in the Instructions for Authors or in the guidance on the submission system. Ensure you have permission to use any tables or figures you are reproducing from another source.

Running heads and received dates are not required when submitting a manuscript for review; they will be added during the production process.

Spelling and punctuation: Each journal will have a preference for spelling and punctuation, which is detailed in the Instructions for Authors. Please ensure whichever spelling and punctuation style you use is applied consistently.

Please use American spelling consistently throughout your manuscript.

Please use single quotation marks, except where 'a quotation is "within" a quotation'. Please note that long quotations should be indented without quotation marks.

F.2.3.4 Formatting and templates

Papers may be submitted in any standard format, including Word. Figures should be saved separately from the text. To assist you in preparing your paper, we provide formatting templates.

Word templates are available for this journal. Please save the template to your hard drive, ready for use.

If you are not able to use the templates via the links (or if you have any other template queries) please contact authortemplate@tandf.co.uk

F.2.3.5 References

Please use this reference guide when preparing your paper. An EndNote output style is also available to assist you.

Harvard references are commonly used in the social sciences. Cited publications are referred to in the text by giving the author's surname and the year of publication, and are listed in a bibliography at the end of the text. This guide is based on the British Standards BS 5605:1990 Recommendations for citing and referencing published material and BS 1629:1989 Recommendations or references to published materials.

EndNote for Windows and Macintosh is a valuable all-in-one tool used by researchers, scholarly writers, and students to search online bibliographic databases, organize their references, and create bibliographies instantly. There is now an EndNote output style available if you have access to the software in your library (please visit http://www.endnote.com/support/enstyles.asp and look for TF-X Harvard).

F.2.3.5.1 How to cite references in your text

If the author's name occurs naturally in the sentence, the year is given in parentheses:

In a popular study Harvey (1992) argued…

If the name does not occur naturally in the sentence, both name and year are given in parentheses:

A more recent study (Stevens 1988) has shown…

If two or more references by the same author published in the same year are cited, distinguish these by adding a, b, c, etc. after the year:

Johnson (1994a) discussed…

If you want to include two or more references within the same parentheses, use chronological order and separate with commas:

…as discussed by several authors (Smith 1993, 2003, Brown 1995, Smith and Jones 1997, Green 2004,).

Three or more authors

If more than two authors, give the surname of the first author followed by et al.

Office costs amount to 20% of total costs in most businesses (Wilson et al. 1997).

A source quoted in another source

If you refer to a source quoted in another source, cite both in the text, but only list the work you read in the bibliography: A study by Smith (1960 cited Jones 1994) showed that…

Anonymous work

Use Anon in the text:

(Anon 1988)

F.2.3.5.2 How to organize the reference list

List references to all documents cited in the text, under the heading References. They are listed in alphabetical order of authors' names. If you have cited more than one item by a specific author, they should be listed chronologically (earliest first), and by letter (1993a, 1993b) if more than one item has been published during a specific year.

F.2.3.5.3 Book

Cutler, T., Williams, K., and Williams, J., 1986. Keynes, Beveridge and beyond. London: Routledge.

Four or more authors

Pearce, I.F., et al., 1976. A model of output, employment, wages and prices in the UK. Cambridge: Cambridge University Press.

Edited book

Chester, D.N., ed., 1951. Lessons of the British war economy. Westport, CT: Greenwood Press.

Corporate author

Name of issuing body, Year. Title of publication. Place: Publisher, Report Number (where relevant).

Unesco, 1993. General information programme. Paris: Unesco, PGI-93/WS/22.

Chapter

Author, A., 2006. Chapter title. In: B.B. Editor and C.C. Editor, eds. Title of book. Place: Publisher, page numbers.

Bantz, C.R., 1995. Social dimensions of software development. In: J.A. Anderson, ed. Annual review of software management and development. Newbury Park, CA: Sage, 502–510.

Place of publication

Give the city. If more than one city is listed, give the first one or the location of the publisher's head office. If the city is not well known, add a country, region or state. States should be denoted by a two-letter code, e.g. Hillsdale, NJ.

Publisher's name

Omit superfluous terms such as Publishers, Co, Inc., but retain the words Books or Press. Where the publisher is a university and the place or location is included in the name of the university, do not repeat the place of publication.

F.2.3.5.4 Internet

Web page, website, e-book

Holland, M., 2004. Guide to citing Internet sources [online]. Poole, Bournemouth University. Available from: http://www.bournemouth.ac.uk/library/using/guide_to_citing_internet_sourc.html [Accessed 4 November 2004]. If no author, reference by title.

F.2.3.5.5 Journal article

Evans, W.A., 1994. Approaches to intelligent information retrieval. Information processing and management, 7 (2), 147–168. Greenfield, J., 1990. The Sevso Treasure: the legal case. Apollo, 132 (3), 14–16.

Online journal

Korb, K.B., 1995. Persons and things: book review of Bringsjord on Robot-Consciousness. Psycoloquy [online], 6 (15). Available from: http://psycprints.ecs.soton.ac.uk/archive/00000462/ [Accessed 20 May 2004].

Volume and issue details can be omitted if you can't find them, and pages are often not given for electronic journals. Give the paragraph number if available.

F.2.4 Checklist: What to include

F.2.4.1 Author details

Please ensure everyone meeting the International Committee of Medical Journal Editors (ICJME) requirements for authorship is included as an author of your paper. Please include all authors' full names, affiliations, postal addresses, telephone numbers and email addresses on the cover page. Where available, please also include ORCiDs and social media handles (Facebook, Twitter or LinkedIn). One author will need to be identified as the corresponding author, with their email address normally displayed in the article PDF (depending on the journal) and the online article. Authors' affiliations are the affiliations where the research was conducted. If any of the named co-authors moves affiliation during the peer-review process, the new affiliation can be given as a footnote. Please note that no changes to affiliation can be made after your paper is accepted.

F.2.4.2 Abstract

- A non-structured abstract of no more than 250 words.
- You can opt to include a video abstract with your article.
- 5 – 10 keywords.

F.2.4.3 Funding details

Please supply all details required by your funding and grant-awarding bodies as follows:

- For single agency grants: This work was supported by the [Funding Agency] under Grant [number xxxx].
- For multiple agency grants: This work was supported by the [funding Agency 1]; under Grant [number xxxx]; [Funding Agency 2] under Grant [number xxxx]; and [Funding Agency 3] under Grant [number xxxx].

F.2.4.4 Disclosure statement

This is to acknowledge any financial interest or benefit that has arisen from the direct applications of your research.

F.2.4.5 Biographical note

Please supply a short biographical note for each author. This could be adapted from your departmental website or academic networking profile and should be relatively brief.

F.2.4.6 Geolocation information

Submitting a geolocation information section, as a separate paragraph before your acknowledgements, means we can index your paper's study area accurately in JournalMap's geographic literature database and make your article more discoverable to others.

F.2.4.7 Supplemental online material

Supplemental material can be a video, dataset, fileset, sound file or anything which supports (and is pertinent to) your paper. We publish supplemental material online via Figshare.

F.2.4.8 Figures

Figures should be high quality (1200 dpi for line art, 600 dpi for grayscale and 300 dpi for colour, at the correct size). Figures should be saved as TIFF, PostScript or EPS files.

F.2.4.9 Tables

Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text. Please supply editable files.

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Mrs. Van Niekerk is since 2008 a registered and accredited Language Editor for the Potchefstroom Campus of the North-West University. During this time she has done the language editing of hundreds dissertations in English as well as Afrikaans. She finishes her studies for a degree in Education in December 1964 and received her National Education Teacher's Diploma.

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