

Investigation of the stabilising effects of niosomes on the amorphous forms of roxithromycin

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“Only those who will risk going too far can possibly find out how far one can go.”

T.S. Elliot

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Abstract

The first aim of this research study was to determine the effect of the excipients used in the typical niosome preparation on the solid-state nature of three solid-state forms of roxithromycin. The second aim was to ascertain which niosome had the highest concentration of roxithromycin delivered topically to the target-site, the epidermis-dermis (ED) of the skin.

To investigate this, roxithromycin, the active pharmaceutical ingredient (API) in this study, was used to prepare the two amorphous forms by the well-known quench cooling of the melt method and recrystallisation of the crystalline raw material (**RM**) from chloroform. It resulted in the formation of the quench cooled (**QC**) and chloroform desolvated (**CD**) amorphous forms. These solid-state forms were characterised in terms of x-ray powder diffraction (XRPD), differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR) to determine the degree of crystallinity. To explore the effects of the excipients, niosomes were prepared containing five different ratios of cholesterol and Span[®] 40, with each of the three solid-state forms incorporated in the five formulas resulting in 15 niosome systems. Lipid films (precursors of niosomes) were prepared and the physical stability was investigated, which led to the discovery that the preparation method rendered **RM** into an amorphous habit, it also showed the amorphous **QC** and **CD** remained amorphous.

Niosomes were prepared and characterised by means of morphology (microscopy), droplet size and distribution, zeta-potential, pH and drug entrapment efficiency (EE%) to establish if the vesicles had ideal physicochemical properties to be delivered topically to the skin. The characterisation revealed acceptable results and the study progressed towards release studies. Membrane release studies were conducted to evaluate if the API was being released from the vesicle systems and the results obtained showed that the API was released from all the niosomes. Skin diffusion studies were performed to determine if the API was delivered topically and/or transdermally, where it was noted that 4 niosomes were delivered transdermally and therefore into the systemic circulation, while 6 niosomes were found in both the stratum corneum-epidermis and the ED.

The aims of this study were reached because the preparation method of the niosomes rendered the crystalline form of the API into an amorphous habit and prevented the amorphous forms from recrystallising. Quantifiable concentrations of the API were delivered to the ED, resulting in successful topical drug delivery. From the three solid-state forms used during this study, niosomes containing the **QC** amorphous form displayed the best results. It was found the solid-state forms as well the excipients had an influence on the diffusion into and through the skin and it was noticed that specific areas could be targeted using certain excipients.

Keywords: Roxithromycin, amorphous, excipients, niosomes, lipid films, topical drug delivery

Uittreksel

Die eerste doel van hierdie navorsingstudie was om die effek te bepaal van die tipiese hulpstowwe wat gebruik word in die bereiding van niosome op die aard van die soliede fase van drie soliedefasevorme van roksitromisien. Die tweede doel was om vas te stel watter niosoomstelsel die hoogste konsentrasie van roksitromisien topikaal aan die teikenarea, die epidermis-dermis (ED) van die vel, toedien.

Om dit te ondersoek, is roksitromisien, die aktiewe farmaseutiese bestanddeel (AFB) in die studie, gebruik om die twee amorge vorme te berei deur die bekende blusverkoeling van die smeltselmetode en die herkristallasie van die kristallyne rou materiaal (**RM**) uit chloroform. Dit het gelei tot die vorming van die blusverkoelde (**BV**) en die chloroform gedesolueerde (**CD**) amorge vorme. Die soliedefasevorme is met x-straalpoeierdiffraksie (XSPD), differensiële skandering kalorimetrie (DSK) en Fourier-transform infrarooispektroskopie (FTIR) gekarakteriseer om die graad van kristalliniteit te bepaal. Om die effekte van die hulpstowwe te bestudeer, is niosome berei wat vyf verskillende verhoudings van cholesterol tot Span® 40 bevat, elk met die drie soliedefasevorme geïnkorporeer in die vyf formules wat dus 15 niosoomstelsels tot gevolg het. Lipiedfilms (voorgangers van niosome) is berei en die fisiese stabiliteit is ondersoek, wat gelei het tot die ontdekking dat die bereidingsmetode die **RM** in 'n amorge staat lewer. Dit het ook getoon dat die **BV** en **CD** amorge vorme in die amorge staat behou is.

Niosome is berei en gekarakteriseer in terme van morfologie (mikroskopie), druppelgrootte en verspreiding, zetapotensiaal, pH en geneesmiddelinsluiteffektiwiteit (IE%) om vas te stel of die vesikels ideale fisies-chemiese eienskappe vir topikale aflewering aan die vel het. Die karakterisering het aanvaarbare resultate gelever en die studie het tot vrystellingstudies gevorder. Membraanvrystellingstudies is uitgevoer om te bepaal of die AFB uit die vesikelstelsel vrygestel word en die resultate toon dat die AFB wel uit al die vesikels vrygestel is. Veldiffusiestudies is uitgevoer om te bepaal of die AFB topikaal en/of transdermaal afgelewer is, waar gevind is dat 4 niosome transdermaal afgelewer is en dus die sistemiese sirkulasie bereik het, terwyl 6 niosome in beide die stratum corneum-epidermis en ED gevind is.

Die doel van hierdie studie is dus bereik want die bereidingsmetode van niosome het die kristallyne vorm van die AFB in 'n amorge staat gelever en verhoed dat die amorge vorme herkristalliseer. Kwantifiseerbare konsentrasies van die AFB is aan die ED afgelewer, wat gevolglik gelei het tot suksesvolle topikale geneesmiddel aflewering. Van die drie soliedefasevorme gebruik in hierdie studie, het niosome wat die **BV** amorge vorme bevat, die beste resultate getoon. Daar is ook gevind dat die soliedefasevorme, sowel as die hulpstowwe, 'n invloed het op diffusie in en deur die vel en dit is waargeneem dat spesifieke areas geteiken kan word deur sekere hulpstowwe te gebruik.

Sleutelwoorden: Roksitromisien, amorfe vorme, hulpstowwe, niosome, lipiedfilms, topikale geneesmiddelaflewering

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Appendix A: Method validation for high-performance liquid chromatographic analysis of roxithromycin

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Appendix C: Formulation and characterisation of niosome vesicle systems for topical delivery

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Abbreviations

%RSD	Percentage relative standard deviation
AIDS	Acquired immune deficiency syndrome
API	Active pharmaceutical ingredient
APVMA	Australian Pesticides and Veterinary Medicines Authority
ASD	Amorphous solid dispersion
ATL	Analytical Technology Laboratory
BP	British Pharmacopeia
CD	Chloroform desolvate amorphous form of roxithromycin
CD1	Niosomes containing 2% CD together with cholesterol and Span [®] 40 in the ratio of 0.5:1.0
CD2	Niosomes containing 2% CD together with cholesterol and Span [®] 40 in the ratio of 1.0:1.0
CD3	Niosomes containing 2% CD together with cholesterol and Span [®] 40 in the ratio of 2.0:1.0
CD4	Niosomes containing 2% CD together with cholesterol and Span [®] 40 in the ratio of 2.0:1.5
CD5	Niosomes containing 2% CD together with cholesterol and Span [®] 40 in the ratio of 2.0:2.5
CHCl ₃	Chloroform
CH ₃ OH	Methanol
CoA	Certificate of analysis
D	Diffusion coefficient
DCP	Dicetyl phosphate
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
ED	Epidermis-dermis
EE%	Entrapment efficiency

FDA	Food and Drug Administration
FT-IR	Fourier-transform infrared spectroscopy
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
H ₃ PO ₄	Phosphoric acid
HSM	Hot stage microscopy
HCl	Hydrochloric acid
HLB	Hydrophilic-lipophilic balance
HPLC	High-performance liquid chromatography
HRTEM	High-resolution transmission electron microscopy
ICH	International Conference of Harmonisation
IR	Infrared
J _{max}	Maximum flux
KBr	Potassium bromide
KH ₂ PO ₄	Potassium di-hydrogen orthophosphate
LAMB	Laboratory for Applied Molecular Biology
LLOQ	Lower limit of quantification
LOD	Limit of detection
Log D	Octanol-buffer distribution coefficient
Log P	Octanol-water partition coefficient
LUV	Large unilamellar vesicles
MeOH	Methanol
MLV	Multi-lamellar vesicles
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NH ₄ OH	Ammonia

NH ₄ H ₂ PO ₄	Ammonium di-hydrogen phosphate
NWU	North-West University
OED	Oxford English Dictionary
OH	Hydroxyl
P	Partition coefficient
PBS	Phosphate buffer solution
PdI	Polydispersity index
PVDF	Polyvinylidene fluoride
QC	Quench cooled amorphous form of roxithromycin
QC1	Niosomes containing 2% QC together with cholesterol and Span [®] 40 in the ratio of 0.5:1.0
QC2	Niosomes containing 2% QC together with cholesterol and Span [®] 40 in the ratio of 1.0:1.0
QC3	Niosomes containing 2% QC together with cholesterol and Span [®] 40 in the ratio of 2.0:1.0
QC4	Niosomes containing 2% QC together with cholesterol and Span [®] 40 in the ratio of 2.0:1.5
QC5	Niosomes containing 2% CQ together with cholesterol and Span [®] 40 in the ratio of 2.0:2.5
R ²	Correlation coefficient
RM	Raw material/monohydrate form of roxithromycin
RM1	Niosomes containing 2% RM together with cholesterol and Span [®] 40 in the ratio of 0.5:1.0
RM2	Niosomes containing 2% RM together with cholesterol and Span [®] 40 in the ratio of 1.0:1.0
RM3	Niosomes containing 2% RM together with cholesterol and Span [®] 40 in the ratio of 2.0:1.0
RM4	Niosomes containing 2% RM together with cholesterol and Span [®] 40 in the ratio of 2.0:1.5

RM5	Niosomes containing 2% RM together with cholesterol and Span [®] 40 in the ratio of 2.0:2.5
RPM	Revolutions per minute
SCE	Stratum corneum-epidermis
SD	Standard deviation
STD	Standard
SUV	Small unilamellar vesicles
TDL	Transdermal laboratory
TEM	Transmission electron microscopy
T _g	Transition temperature
T _K	Kauzmann temperature
T _m	Melting point
USP	United States Pharmacopeia
UV	Ultraviolet
XRPD	X-ray powder diffraction

Chapter 1

Introduction, aims and objectives

1.1 Introduction

Acne is a well-known skin disease that affects more than 80% of teenagers and young adults (Krautheim & Gollnick, 2004:398). Several factors are responsible for the formation of acne, one of which is the presence of the bacteria *Propionibacterium acnes* (Ramanathan & Hebert, 2011:332). Roxithromycin, as an active pharmaceutical ingredient (API), is a macrolide antibiotic that could be used in the topical treatment of acne, where it may reduce the population of *P. acnes* in the pilosebaceous duct and have mild anti-inflammatory effects (Katsambas *et al.*, 2004:440). Topical delivery of a drug to the skin is considered to be one of the most relevant routes for effectively treating skin diseases (More *et al.*, 2016:196). During this study, roxithromycin was investigated as a possible topical product. There is currently no topical formulation of this API available on the market, which in turn renders this research novel.

The skin is classified as the largest organ in the human body and its primary function is protection (Menon, 2002:3-4). The skin has 3 main layers, i.e. the epidermis, dermis and hypodermis, with the stratum corneum that forms the outermost layer of the epidermis, acting as the rate-limiting barrier to drug penetration (Foldvari, 2000:418). The structure of the stratum corneum has been studied thoroughly and can be described in terms of the “bricks and mortar” model (Washington *et al.*, 2001:183). Transport across the stratum corneum is mostly by means of passive diffusion and is dependent on the affinity of a drug to the lipophilic environment (Jepps *et al.*, 2013:154). The viable epidermis is a hydrophilic environment and therefore, if a drug wants to be delivered topically, it has to be soluble in both the lipophilic and hydrophilic environments (Perrie *et al.*, 2012:393).

For an API to permeate into the skin, it needs to have certain favourable physicochemical characteristics, which include molecular weight, aqueous solubility, melting point and lipophilicity (Benson & Watkinson, 2012:16). Roxithromycin has a molecular weight of 837.06 g/mol (Merck, 2017), which is larger than the ideal molecular weight of less than 500 Da (g/mol) (Uzor *et al.*, 2011:681). This could limit the permeation of the API into the skin, since the size does not comply with the size of an ideal topical API. The ideal aqueous solubility that an API should possess for transdermal delivery is > 1 mg/ml (Naik *et al.*, 2000:319), roxithromycin is very slightly soluble in water with a solubility of 0.0335 mg/ml at 25 °C (Aucamp *et al.*, 2013:26). A melting point of < 200 °C is ideal for passive transdermal delivery (Naik *et al.*, 2000:319), making roxithromycin an ideal candidate with regards to melting point, considering it has a melting point between 116 and 122 °C (Aucamp *et al.*, 2012:469). The

lipophilicity can be determined in terms of the *n*-octanol-water partition coefficient (log P) value of the API and describes the partitioning of the API between the oil and water phases (N'Da, 2014:20786). The favourable log P values for dermal absorption are between 2 and 3 (Akhlaq *et al.*, 2014:178). Roxithromycin has a log D value of 1.52 (Csongradi, 2015:178), which qualifies it as an excellent candidate.

Due to the poor aqueous solubility of roxithromycin, two amorphous solid-state forms were used because these forms have less structured molecular packing than the crystalline form, giving them altered properties including increased solubility (Biradar *et al.*, 2006:22). During previous experiments performed by Aucamp *et al.* (2013) and Milne *et al.* (2016), the increased solubility of the amorphous forms of roxithromycin was proved. It should be noted that these studies (combining different solid-state forms and topical drug delivery) have not been performed extensively, which makes this an innovative study.

Niosome vesicle systems are of great importance as a drug delivery system, given that they are able to entrap a broad selection of substances including lipophilic, hydrophilic and amphiphilic drugs (Rahimpour & Hamishehkar, 2012:145). It has also been reported that niosomes increase the time the drug stays in the stratum corneum and epidermis, while reducing absorption into the systemic circulation (Uchechi *et al.*, 2014:212), which is a typical feature of topical delivery. Niosomes were used to encapsulate roxithromycin because it enhances skin penetration (Kumar & Rajeshwarrao, 2011:214) and could help overcome the larger size of the molecules that are not optimal for transdermal drug delivery.

This study can be seen as a continuation of a previous study conducted by Csongradi (2015), as the results obtained led to some questions about the amorphous forms of roxithromycin and their stability during the preparation of the niosome vesicle systems.

1.2 Problem statement

The amorphous forms of APIs are unstable and can easily crystallise to the thermodynamically more stable solid-state form. During a previous study by Csongradi (2015), a distinctive increase was observed in the concentration of roxithromycin that was delivered to the epidermis/dermis by the niosomes containing the amorphous forms. This leads to the hypothesis that the amorphous API entrapped in the lipophilic bilayer remains in the amorphous state, probably due to the formation of an amorphous solid dispersion containing the amorphous API in combination with one or more of the excipients used during niosome preparation.

1.3 Aims and objectives

The aim of this study was to determine which excipient(s) in the niosome formula had a stabilising effect on the two amorphous forms of roxithromycin, preventing them from

transforming to the crystalline form. In order to investigate this, five formulas with different ratios of the excipients, encapsulating the three solid-state forms, were investigated to determine which excipient or combination of excipients was responsible for this effect, hence 15 niosome dispersions were prepared. The second aim was to establish which of the 15 niosomes would have the highest concentration of the API delivered topically.

The objectives of this research study were:

- The development and validation of a high performance liquid chromatography (HPLC) method to determine the concentration of roxithromycin and the niosome excipients throughout all objectives of this study.
- Preparation of the quench cooled (**QC**) and chloroform desolvate (**CD**) amorphous forms of roxithromycin.
- Solid-state characterisation of crystalline roxithromycin raw material (**RM**) and the two amorphous forms (**QC** and **CD**). The solid-state characterisation will only entail x-ray powder diffraction (XRPD), differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR).
- Preparation of the lipid films (precursors to niosomes) containing all three roxithromycin solid-state forms (crystalline and two amorphous forms) separately with all five ratios of Span[®] 40 and cholesterol. Testing of crystallisation of amorphous roxithromycin in these combinations using XRPD, DSC and FTIR.

If any instability, pertaining to a particular drug/excipient concentration ratio, was identified during the previous objective those combinations would be eliminated. All roxithromycin/excipient combinations that proved stable would be used during subsequent objectives:

- Preparation of the niosome vesicle systems containing **RM**, **QC** amorphous or **CD** amorphous forms of roxithromycin separately in the five ratio concentrations.
- The characterisation of the vesicle system with and without roxithromycin in terms of morphology, droplet size and distribution, zeta-potential, pH, and drug entrapment efficiency (EE%).
- Determining the release of roxithromycin from the niosomes through membrane release studies.
- Determining the topical and/or transdermal delivery of roxithromycin from the niosome formulation by performing Franz cell skin diffusion studies followed by tape stripping, respectively.

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Chapter 2

Topical delivery of niosome encapsulated solid-state forms of roxithromycin

2.1 Introduction

Topical (dermal) and transdermal drug delivery systems have recently experienced increased attention because of the many advantages over other drug delivery routes, some of which include the large surface area of the skin offering various sites of administration, avoiding first-pass metabolism, controlled and constant blood levels and improved patient compliance due to easier medication regimes, non-invasive and painless administration (Sharma *et al.*, 2013:286). Barry (2001:101) noted that the transdermal route was the most innovative research area for drug delivery, competing with oral routes of drug administration. Despite all these advantages, the skin and its natural barrier function still poses a problem for formulators to develop a successful transdermal drug delivery system. To clarify, topical delivery can be described where the drug is intended for a local effect in the skin, in contrast with transdermal delivery, which is intended for systemic effects with the skin only as a point of entry (Goyal *et al.*, 2016:77).

The skin is the largest organ in the body and forms a distinctive and interesting interface between humans and the outside environment (Hadgraft, 2004:291). The skin provides a flexible and self-renewing barrier to the outside world, protecting the body from any external influences as well as preventing loss of water and other components (Kielhorn *et al.*, 2006:10). The skin consists of three main layers that include the epidermis, dermis and hypodermis (Sharma *et al.*, 2013:287). According to Foldvari (2000:418), the structure of the stratum corneum, the outermost layer of the skin and part of the epidermis, forms the rate-limiting barrier to drug penetration through the skin.

Various strategies have been implemented over the years to improve delivery of drugs transdermally with greater skin permeability and can be divided into active and passive methods. Active methods involve enhancing delivery by means of physical or mechanical methods, thus using external energy as a driving force or reducing the barrier of the stratum corneum. Passive methods entail the optimisation of drug formulations or vehicles, such as penetration enhancers or vesicles, i.e. liposomes (Brown *et al.*, 2006: 176-177). Honeywell-Nguyen and Bouwstra (2015:68-69) describe vesicles as colloidal particles that are filled with water and might be used in one or more of four ways: (1) it can act as a penetration enhancer, (2) act as a carrier to deliver an entrapped drug to the skin, (3) serve as a sustained release depot for topical drugs and (4) serve as a rate-limiting membrane for transdermal drugs. The focus of this study is niosome vesicle systems, which will be explored further to determine their stabilising effect on amorphous forms of roxithromycin.

Niosomes are hydrated vesicle systems formed from non-ionic surfactant(s), able to encapsulate various compounds with different solubilities. Niosomes made their first appearance in the 1970's in the cosmetic industry as a product in cosmetic formulations invented by L'Oreal, where after they were intensely studied as an alternative to liposomes (Bagheri *et al.*, 2014:1671). Niosomes and liposomes are very similar systems, but the former is composed of non-ionic surfactant(s), while phospholipids are used to form the latter. The non-ionic surfactant is said to give niosomes higher chemical stability and are stable towards degradation. Niosomes are less expensive to formulate, and storage and handling are easier than with liposomes (Tangri & Khurana, 2011:47).

In this research study, we focused on the API, roxithromycin, which is classified as a semi-synthetic macrolide antibiotic and is a 14-membered lactone ring derivative of erythromycin (Bryskier, 1998:1). Roxithromycin has a log D value of 1.52 (Csongradi, 2015:178), which is very favourable for transdermal drug delivery, however it has an aqueous solubility of 0.0335 mg/ml at 25 °C (Aucamp *et al.*, 2013:26), which is poorly soluble. The poor water solubility can be improved by using the more soluble amorphous form of roxithromycin (Biradar *et al.*, 2006:22). Two amorphous forms of roxithromycin, namely the non-crystalline quench cooled form and the chloroform desolvated form, which are more soluble than the stable crystalline solid-state form, were used. The API was encapsulated into a niosome vesicle system to help improve the solubility problems and topical drug delivery (Kumar & Rajeshwarrao, 2011:209).

Acne vulgaris is a common and chronic skin disorder of the pilosebaceous unit affecting almost 80% of adolescents and young adults (Krauthaim & Gollnick, 2004:398). Clinical features include seborrhoea (excess grease), open and closed comedones (non-inflammatory lesions), nodules, papules and pustules (inflammatory lesions) and different degrees of scarring (Williams *et al.*, 2012:361). The four components involved in acne formation are increased sebum production, inflammation, unusual desquamation of keratinocytes and the presence of the bacteria *Propionibacterium acnes* (Ramanathan & Hebert, 2011:332). Many skin diseases like acne are typically found in the deeper dermal layers of the skin and conventional dosage forms are unable to treat these conditions effectively, as they have poor retention in the skin (More *et al.*, 2016:197). Topical roxithromycin could be used to treat mild acne, their target site being the skin, specifically in the follicles, where they decrease the population of *P. acnes* and therefore could reduce acne (Krauthaim & Gollnick, 2004:401). Topical treatment could thus be used in combination with systemic treatment to achieve a synergistic therapeutic effect, which helps to treat acne faster, since the topical preparation can reach the skin layers sooner than the systemic preparation would.

2.2 Niosomes

Niosomes are microscopic lamellar vesicular structures, very similar to liposomes but instead of using phospholipids, they are composed of non-ionic surfactant and cholesterol (Rajera *et al.*, 2011:945). The non-ionic surfactant that constitutes amphiphilic molecules forms the vesicle bilayer with two distinct areas, a hydrophilic head with no charge groups and a lipophilic tail. Therefore, the surfactant has both water-soluble and oil-soluble components (Pandey *et al.*, 2014:2). The bilayer encloses the aqueous compartment and the hydrophilic heads remain in contact with the aqueous medium, whereas the lipophilic tails are orientated away from it (Rahimpour & Hamishehkar, 2012:143). Due to this unique structure of niosomes (Figure 2.1), they are able to encapsulate both hydrophilic and lipophilic substances, hydrophilic drugs are entrapped in the internal aqueous compartment and the lipophilic or amphiphilic drugs are entrapped in the lipid bilayer (Honeywell-Nguyen & Bouwstra, 2015:68).

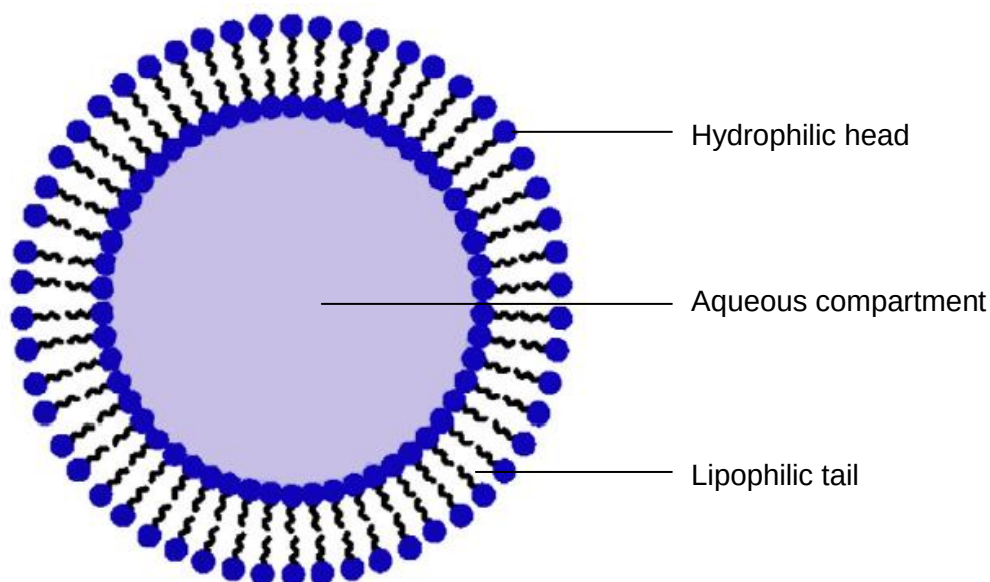


Figure 2.1: Schematic presentation of a niosome (Adapted from Moghassemi & Hadjizadeh, 2014:24).

2.2.1 Formulation aspects

It is important to know the basic components of a formulation before preparation as it affects the properties of the niosomes. These components include non-ionic surfactant, cholesterol and additive excipients.

2.2.1.1 Non-ionic surfactant

Several types of surfactants exist, but non-ionic surfactants are most commonly used when preparing vesicles (Kumar & Rajeshwarrao, 2011:210). They are preferred over cationic, anionic and ampholytic surfactants due to their ability to form stable, compatible formulations that are less irritating and relatively non-toxic. Non-ionic surfactants exert functions as wetting agents, penetration enhancers and solubilisers (Mahale *et al.*, 2012:48). When choosing which surfactant to use, it is important to note the hydrophilic-lipophilic balance (HLB) as this is a good indicator if vesicles will form and plays a role in controlling entrapment efficiency. The HLB range for non-ionic surfactant is 0.0 – 20.0, with lipophilic surfactant < 9.0 and hydrophilic surfactant > 11.0 (Moghassemi & Hadjizadeh, 2014:25). A surfactant with an HLB value of 8.6 produces niosomes with the highest entrapment efficiency, values between 14.0 and 16.0 will not form niosomes and for values > 6.0, cholesterol needs to be added to form a vesicle with a bilayer (Kumar & Rajeshwarrao, 2011:210). Span[®] 40 (Sorbitan monopalmitate) was the surfactant used during this study and has an HLB value of 6.7 (Bayindir & Yuksel, 2010:2052) and therefore cholesterol needed to be added to form bilayered vesicles.

2.2.1.2 Cholesterol

In addition to surfactant, another main component used in the preparation of niosomes is cholesterol. Cholesterol is a steroid derivative and an important component in cell membranes, which affects permeability through the bilayer and its fluidity (Sankhyan & Pawar, 2012:21). It also affects other properties of niosomes such as entrapment efficiency, stability and rigidity, amongst others (Rajera *et al.*, 2011:945). According to Kumar and Rasjeshwarrao (2001:210), aggregation of surfactants can be subdued by adding cholesterol to the formulation, it permits hydrophobic surfactants to form vesicles and it stabilises the bilayer. Cholesterol is also an amphiphilic molecule and as a result, orientates its aliphatic chain to the hydrocarbon chain of the surfactant and its hydroxyl (-OH) group towards the aqueous phase (Sankhyan & Pawar, 2012:21).

2.2.1.3 Additive excipients

Other excipients often used are charged molecules, which increases the stability of the vesicle by preventing aggregation by means of electrostatic repulsion. An example of a substance with a charge inducer role is dicetyl phosphate (DCP), which transmits a negative charge on the niosomes surface therefore stabilising the bilayers (Rajera *et al.*, 2011:946).

2.2.2 Types of niosomes and methods of preparation

2.2.2.1 Types of niosomes

There are different types of niosomes which can be divided into three groups based on their size, namely small unilamellar vesicles (SUV, 10 – 100 nm), large unilamellar vesicles (LUV, 100 – 3000 nm) and multi-lamellar vesicles (MLV) that presents with more than one bilayer (Moghassemi & Hadjizadeh, 2014:28).

2.2.2.2 Methods of preparation

Niosomes are generally prepared by the hydration of non-ionic surfactant with the hydration medium, but there are a few variations to this method depending on the use of the niosomes. The preparation methods influences the size of the vesicles and their distribution, number of bilayers, the permeability of the membrane and the entrapment efficiency of the aqueous phase (Chandu *et al.*, 2012:27). Various methods can be used to prepare SUV, LUV and MLV (Karim *et al.*, 2010:375-376).

To prepare SUV, the following methods can be used:

- Sonication
- Micro-fluidisation
- The “bubble” method
- Proniosome technology

The following methods can be used to prepare LUV:

- Reverse phase evaporation method
- Ether injection method

While the following methods can be used to prepare MLV:

- Thin film hydration method
- Trans-membrane pH gradient drug uptake process
- Freeze and thaw method
- Dehydration rehydration method

The method chosen during this study was an adapted version of the hand shaking/thin film hydration method.

2.2.3 Advantages and disadvantages of niosomes

2.2.3.1 Advantages

Niosomes, as vesicle systems for topical drug delivery, offer many advantages as listed below (Rahimpour & Hamishehkar, 2012:145):

1. They are biodegradable and non-immunogenic.
2. They have low toxicity and are compatible with biological systems.
3. They can be used in the delivery of a wide variety of drugs because they can entrap lipophilic, hydrophilic as well as amphiphilic substances.
4. They can be used in sustained or targeted release systems.
5. They can increase the drug permeation through the skin.
6. Characteristics such as size, shape, components, fluidity of the niosome and others can be controlled.
7. Raw materials are easy to obtain.
8. They exhibit better patient compliance than conventional oily preparations.
9. Handling and storage are easy.
10. They are stable and osmotically active.

2.2.3.2 Disadvantages

According to Chandu *et al.* (2012:26), the disadvantages of niosomes are as follows:

1. Fusion
2. Aggregation
3. Physical instability
4. Leaking of the drug that is entrapped
5. Hydrolysis of the drug restricting the shelf life

2.2.4 Factors affecting physicochemical properties of niosomes

2.2.4.1 Membrane additives

The stability of niosomes is affected by the amount of additives used, as well as the surfactant and the drug. By manipulating membrane properties with different additives, the stability and permeability of niosomes can be altered (Tangri & Khurana, 2011:47), for example, the addition

of cholesterol increases the rigidity and decreases the permeability of drugs through the membrane (Das & Ram, 2013:3).

2.2.4.2 Amount and type of surfactant

The general amount of surfactant/lipid used to prepare niosomes is 10 – 30 mM (1.0 – 2.5% w/w). By changing the surfactant to water ratio at the time of the hydration, it may affect the microstructure of the system and thus the properties of the system (Mahale *et al.*, 2012:48). To prevent vesicle aggregation, components may be added to stabilise the system by repulsive steric or electrostatic forces, thus preventing the formation of aggregates (Kumar & Rajeshwarrao, 2011:212).

2.2.4.3 Cholesterol content

Cholesterol has a dual action; firstly, it can increase the chain order of liquid bilayers and secondly, it can decrease the chain order of gel bilayers. The gel phase can be changed to the liquid phase at high cholesterol concentrations (Chandu *et al.*, 2012:26). By adding cholesterol to the formulation, it increases the entrapment efficiency and hydrodynamic diameter of the niosomes. It has been shown that with an increase of cholesterol, the release rate of encapsulated drug is reduced and as a result, the rigidity of the bilayer increases (Tangri & Khurana, 2011:52).

2.2.4.4 Effect of the encapsulated drug

The physicochemical properties of the encapsulated drug, such as chemical structure, molecular weight, lipophilicity, hydrophilicity and the HLB value of the drug, can affect the encapsulation of the drug in niosomes (Rajera *et al.*, 2011:947). The encapsulation of the drug may cause an increase in vesicle size due to the interaction of the drug particles with the head groups of the surfactant, which may result in a charge increase on the polymer, causing repulsion of the surfactant leading to bigger vesicles (Das & Ram, 2013:3). According to Karim *et al.* (2010:378), amphiphilic drugs decrease leakage from the niosome vesicle and increase encapsulation, hydrophilic drugs increase leakage from the vesicles and decrease stability, whereas hydrophobic drugs decrease leakage from vesicles, stabilise the vesicles and improve transdermal drug delivery.

2.2.4.5 Temperature of hydration

An important factor in the preparation of niosomes is the temperature of the hydration medium, as this affects the size and shape of the vesicles (Mahale *et al.*, 2012:47). It is essential that the temperature should be higher than the gel to liquid phase transition temperature of the system, where the gel transition temperature of Span[®] 40 is 46 – 47 °C (Kumar & Rajeshwarrao,

2011:947). The change in temperature influences the arrangement of surfactant into vesicles and affects changes in vesicle shape and additionally, the time of hydration and the volume of the hydration medium influence the vesicle structure (Mahale *et al.*, 2012:48). Fragile niosomes may be formed due to incorrect selection of these factors.

2.2.5 Applications of niosomes

Niosome vesicle systems have been of great importance during recent years, as they have significant potential as a drug delivery system for various routes of administration. Niosomes have many advantages, one being they can encapsulate different types of drugs, as well as proteins, genes and vaccines (Moghassemi & Hadjizadeh, 2014:24). One of the major applications is in the transdermal/topical delivery of drugs. According to Kumar and Rajeshwarrao (2011:214), niosomes are the best vesicle system for transdermal drug delivery, since they enhance penetration through the skin and act as reservoir to prolong the drug. Niosomes attempt to achieve targeted drug delivery to localise the drug at the site of administration, thereby enhancing the therapeutic activity of the drug and reducing systemic toxic effects (Karim *et al.*, 2010:375). Niosomes are also used to solve problems including instability, insolubility and the fast degradation of drugs (Rajera *et al.*, 2011:945). Surfactants act as penetration enhancers, as they have the potential to solubilise the lipids of the stratum corneum and therefore enhance the transport of the drug across the skin (Pandey *et al.*, 2014:2).

2.2.6 Mechanisms of niosomal skin delivery

Several mechanisms have been proposed for niosomes to follow during transdermal drug delivery including (Arul *et al.*, 2015:926; Rahimpour & Hamishehkar, 2012:146):

- Niosomes as a whole diffuse through the stratum corneum layer.
- Smaller vesicles reforms in the skin where the amount of water present in the skin is important.
- Lipophilic drugs cross the stratum corneum by means of aggregation, fusion and adhesion to the surface of the cells, resulting in a high thermodynamic activity gradient of the drug.
- Niosomes modify the stratum corneum by loosening the cells thus increasing permeation.
- Non-ionic surfactant itself acts as a penetration enhancer and can enhance the permeation of the drug through the skin.

2.3 Roxithromycin

Roxithromycin is classified as a semi-synthetic macrolide antibiotic and is a 14-membered lactone ring derivative of erythromycin. Roxithromycin has been modified by the replacement of the 9-keto group with an ether-oxime side chain (Bryskier, 1998:1). It shows a similar antimicrobial spectrum to erythromycin, but has a higher potency and a longer half-life, which enables fewer administrations. Roxithromycin also has a lower affinity for cytochrome P450 enzymes and consequently, has less undesired interactions (Ostrowski *et al.*, 2010:83).

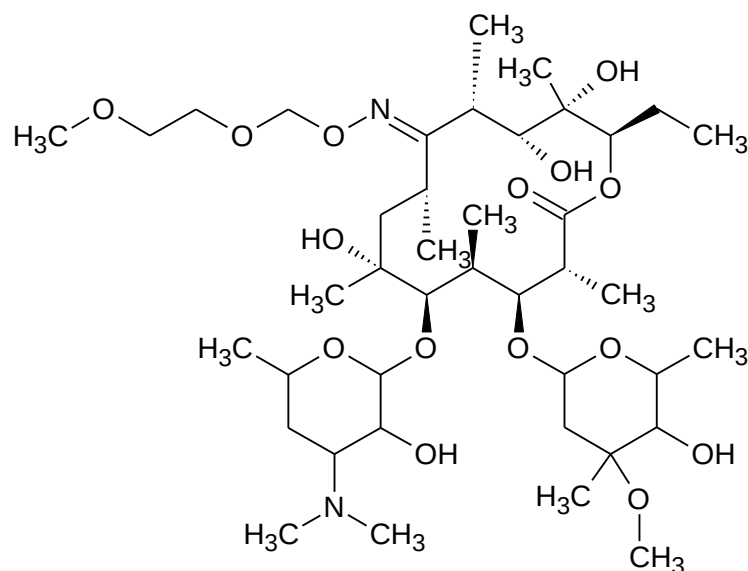


Figure 2.2: Structure of roxithromycin

2.3.1 Mechanism of action of roxithromycin

Roxithromycin binds reversibly to the 50S ribosomal subunit of susceptible organisms, thereby inhibiting protein synthesis, and prevents bacteria from growing by inhibiting translocation of peptidyl-tRNA peptides (MacDougall & Chambers, 2011). At low concentrations, roxithromycin is bacteriostatic, meaning it prevents the growth of bacteria, whereas at high concentrations it is bactericidal, meaning it kills bacteria (Medsafe, 2016).

2.3.2 Physicochemical information

The physicochemical properties of roxithromycin are summarised in Table 2.1.

Table 2.1: Physicochemical properties of roxithromycin

Molecular formula (BP, 2016)	Monohydrate: C ₄₁ H ₇₆ N ₂ O ₁₅
Molecular weight (BP, 2016)	Monohydrate: 837.06 g/mol
Content (BP, 2016)	96.0 – 102.0% (anhydrous substance)
Appearance	White or almost white crystalline powder
Description (BP, 2016)	Roxithromycin is a semi-synthetic macrolide antibiotic, with similar composition, structure and mechanism of action to erythromycin.
Solubility (BP, 2016)	Very slightly soluble in water. Slightly soluble in dilute hydrochloric acid. Freely soluble in acetone, alcohol and methylene chloride.
Melting point (Aucamp <i>et al.</i> , 2012)	116 – 122 °C
Log D (octanol/buffer) (Csongradi, 2015)	Log D = 1.52
pKa (Babić <i>et al.</i> , 2007; Qiang & Adams, 2004)	9.17
Storage (Medsafe, 2016)	Store in a cool, dry place below 25 °C, in an airtight container away from sunlight and moisture.

2.3.3 Clinical uses of roxithromycin

Roxithromycin has similar antibacterial activity to erythromycin and other macrolides and is active against gram-positive cocci and bacilli, as well as gram-negative cocci and some bacilli (Bryskier, 1998:1). Macrolides are mainly used to treat respiratory tract infections caused by *Streptococcus pyogenes*, *S. pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenza*, *Chlamydia pneumoniae* or *Mycoplasma pneumoniae*. Macrolides has also been used to treat diseases caused by *Legionella pneumophila* (Legionnaire's disease), *Borrelia burgdorferi* (Lime's disease), *Neisseria gonorrhoea* (Gonorrhoea), *Chlamydia trachomatis* and *Mycobacterium avium* complex in acquired immune deficiency syndrome (AIDS) patients. Macrolides has also been used to treat skin and soft tissue infections caused by *S. pyogenes* and *Propionibacterium acnes* (Katz & Mankin, 2009:532-534). In this study, we will focus on the activity of roxithromycin against *P. acnes*, as it is the bacteria mostly responsible for acne formation (Krauthaim & Gollnick, 2004:401).

2.3.4 Adverse effects and contra-indications of roxithromycin

According to Markham and Faulds (1994:317), large studies have illustrated that the general incidence of adverse effects are around 4% during roxithromycin therapy, with gastrointestinal symptoms being the most common, accounting for 75 – 80% of these. Gastrointestinal symptoms include nausea, vomiting, abdominal pain, flatulence and diarrhoea. Hypersensitivity reactions such as a rash, pruritus and angioedema may occur. Other adverse effects possibly associated with roxithromycin may include headache, malaise, tinnitus, dizziness, pancreatitis, paraesthesia and taste and/or smell disturbances (MIMS, 2016:278). Safety and tolerance in children and the elderly were very good and the drug appears to have fewer adverse effects than erythromycin (Markham & Faulds, 1994:317; Young *et al.*, 1989). Due to the many adverse effects that may occur with systemic absorption after oral administration, using the topical route of administration, where there are only local effects, may reduce the occurrence of adverse effects and increase patient compliance.

Roxithromycin is contra-indicated in the following conditions (MIMS, 2016:278):

- Macrolide antibiotic hypersensitivity.
- Concomitant treatment with vasoconstrictive ergot alkaloid derivatives.
- Concomitant treatment with pimozide/terfenadine/astemizole and cisapride.
- Acutely impaired hepatic function.

2.4 Solid-state properties of APIs or excipients

Solid-state properties are an important field of study in the pharmaceutical industry, as the majority of drugs and excipients exist as solids (Buckton, 2013:132). Different solid-state forms have substantial differences in physical properties due to the variation in their crystal packing (Vippagunta *et al.*, 2001:4). Many drugs like roxithromycin display very poor aqueous solubility and it is considered more cost effective to improve the poor physical properties than to develop a new drug in entirety (Biradar *et al.*, 2006:22). As a result, ways to overcome the poor solubility of drugs and improve bioavailability are considered key within the pharmaceutical industry. One of the most promising ways to achieve this is the conversion of the crystalline form of the drug to the amorphous form (Laitinen *et al.*, 2013:65).

2.4.1 Classification, structure and stability

Solids can broadly be classified into two categories, namely the crystal state and the amorphous form based on the molecular packing (Florence & Attwood, 2015:8). The crystalline solids are the solid-state which occur most frequently, and which can be subdivided further, as shown in Figure 2.3.

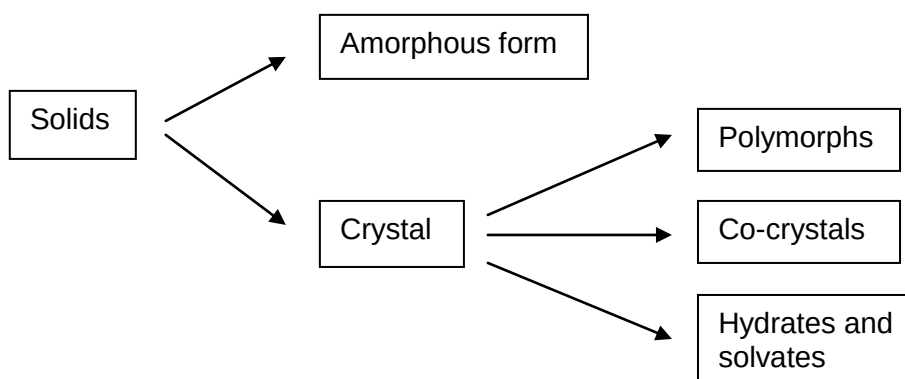


Figure 2.3: Classification of solids (Adapted from Florence and Attwood, 2015:8).

Crystalline solids have a well-defined molecular structure (structural unit) with the presence of short-range and long-range ordering of the molecules. The amorphous form does not have a distinctive molecular structure, because it lacks the long-range packing of molecules, as seen in Figure 2.4 (Yu; 2001:30).

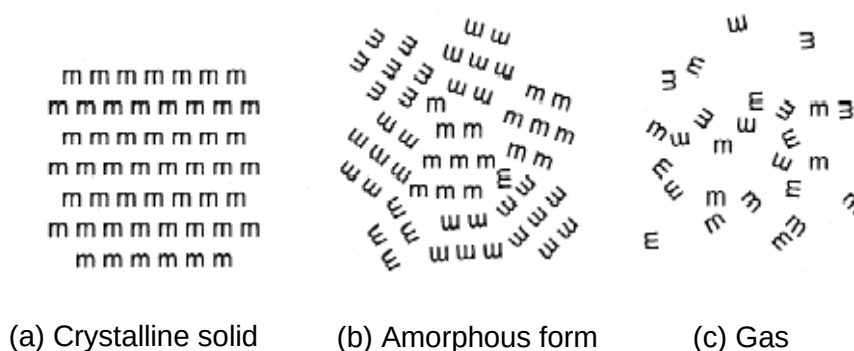


Figure 2.4: Schematic representation of solid-state forms with (a) crystalline solid, (b) amorphous solid and (c) gas (Adapted from Yu, 2001:30).

Amorphous forms have the highest internal energy of a solid-state form, therefore have better thermodynamic properties and enhanced molecular motion compared to the crystalline form. These properties lead to higher dissolution rates and greater solubility, but because of the high energy amorphous forms possess, they tend to be less stable (Laitinen *et al.*, 2013:67). By having this higher free energy, it is anticipated the amorphous form would show greater chemical reactivity, which can lead to some tendency to spontaneously convert back to the more thermodynamically stable crystalline form during storage and handling. From a pharmaceutical point of view, we would like to prevent this conversion back to the more stable crystalline form, as the amorphous form with better thermodynamic properties leads to enhanced bioavailability of the drug (Hancock & Zografi, 1997:2), which is one of our main goals. The amorphous form of drugs is combined with excipients that stabilise the amorphous

form and prevent recrystallisation during storage and dissolution. This combination is known as an amorphous solid dispersion (ASD) (Van den Mooter, 2012:81).

2.4.2 Thermal behaviour

The term phase transition describes the transformation of a thermodynamic system from one state of matter to another by means of heat transfer. It commonly describes the transition between solid, liquid and gaseous states (Buckton, 2013:127). These possible transitions are very important as different phases have different chemical, physical and mechanical properties, affecting the properties of the final pharmaceutical product (Zhang *et al.*, 2004).

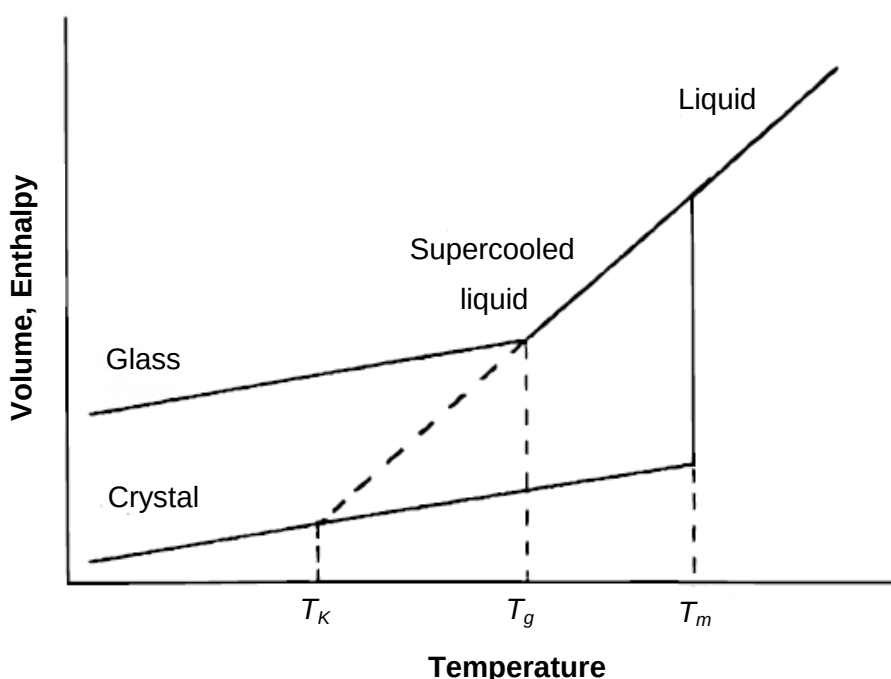


Figure 2.5: Schematic representation of the thermodynamic phase transitions (Adapted from Hancock & Zografi, 1997:2).

One big difference between the crystal and amorphous form is that crystals have melting points (where the crystal breaks up) and amorphous forms do not (Buckton, 2013:132). An amorphous form is produced by rapidly cooling a liquid below its melting point (T_m), this amorphous state is regarded as an equilibrium “supercooled” liquid (Einfalt *et al.*, 2013:306-307), or also referred to as a “rubbery state”. By cooling the supercooled liquid further, a change in slope is usually seen at a characteristic temperature identified as the glass transition temperature (T_g) (Laitinen *et al.*, 2013:66). The T_g represents the point where a big change in the mobility of the molecules in the glass occurs (Buckton, 2013:132). At temperatures below the T_g , the material is in a thermodynamically unstable glass state, “kinetically frozen”. The

Kauzmann temperature (T_K) is assumed to mark the lower limit of the experimental T_g , indicating the point where entropy of the system reaches zero (Hancock & Zografi, 1997:2).

2.5 The skin and topical/transdermal drug delivery

2.5.1 Structure and function of the skin

The skin, integument, is classified as the largest organ in the body and is derived from the Latin word '*integere*', which literally means to cover (Venus *et al.*, 2010:471). It is made up of various different types of cells that contribute to its structure and work together to exhibit three main functions: protection, regulation and sensation (Menon, 2002:3-4). The skin is a multi-layered system and consists of three anatomical layers (Figure 2.6); from the outside to inside layers are the epidermis, dermis and the hypodermis (Alexander *et al.*, 2012:27).

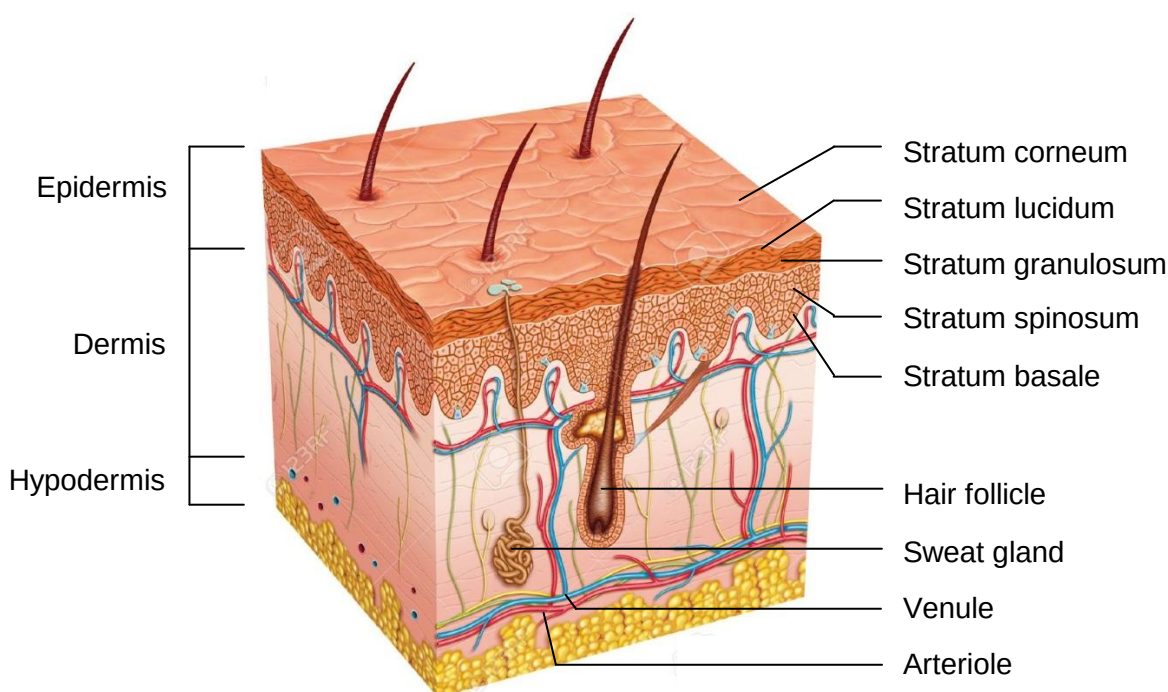


Figure 2.6: Structure of the skin (Adapted from 123RF Stock Photos, 2016).

2.5.1.1 The epidermis

The epidermis is a constantly renewing epithelium and consists of the outermost layer, the stratum corneum (non-viable epidermis) and the viable epidermis (Benson & Watkinson, 2012:4). The main function of the epidermis is to prevent the loss of water and other components from the body to the environment, as well as protecting the body from the outside surroundings (Baroni *et al.*, 2012:257).

2.5.1.1.1 Stratum corneum

The stratum corneum forms the outermost layer of the epidermis and consists of 10 – 15 layers of corneocytes (dead keratinocytes), forming a 10 – 20 µm thick surface layer (Foldvari, 2000:417). The stratum corneum is the primary protective barrier of the skin and because it is the main barrier to drug absorption, the structure has been studied intensively and can be described by the “bricks and mortar” model (Washington *et al.*, 2001:183). Keratin-filled corneocytes form the bricks, while the lipid layer between the cells is considered the mortar. Corneocytes are transformed from keratinocytes that exit in the basal to the granular layer of the epidermis (Wickett & Visscher, 2006:98-99). The stratum corneum is lipophilic and the intercellular lipid layers form a pathway through which drugs must diffuse for them to reach their target area. Thus, lipophilic drugs will cross more readily, while hydrophilic drugs are less permeable (Williams, 2013:680).

2.5.1.1.2 Viable epidermis

The viable epidermis constitutes the first layers of living cells and comprises of keratinocytes and dermal fibroblasts (Goyal *et al.*, 2016:77). It consists of four layers; progressing inwards is the stratum lucidum, stratum granulosum, stratum spinosum and above the dermis is the stratum basale (Venus *et al.*, 2010:471). The viable epidermis is a hydrophilic environment, creating an effective barrier against lipophilic substances (Jepps *et al.*, 2013:155).

2.5.1.2 The dermis and hypodermis

The dermis is the middle and largest skin layer and contains blood and lymphatic vessels, nerve endings, connective tissue, hair follicles and sweat glands (Foldvari, 2000:418). The dermis supports the epidermis and the dermal-epidermal junction acts as a physical barrier for large molecules (Sharma *et al.*, 2013:287-288). The hypodermis, also known as the subcutaneous fat, is the deepest layer of the skin and comprises of loose connective tissue that can vary in thickness (Goyal *et al.*, 2016:77). It acts as an insulator against heat, thereby regulating temperature, and protects the body by acting as a shock absorber (Reddy *et al.*, 2014:1096).

2.5.2 Targeting the skin for drug delivery

Thought must be given to the intended target site, as three main targets for topical and transdermal drug delivery exists (Benson & Watkinson, 2012:14). Firstly, the surface of the skin is the target for locally acting substances including cosmetics, disinfectants and insect repellents. Secondly, the skin layers are targeted during topical delivery and treat microbial infections, inflammation and skin diseases, including acne (Jhawar *et al.*, 2013:47). Lastly, the transdermal delivery of the drug (where the systemic circulation acts as a target site) is considered as an alternative for conventional systemic administrations (Morrow *et al.*, 2007:37).

2.5.3 Advantages and limitations of topical drug delivery

The topical and transdermal drug delivery systems are a novel and innovative strategy to deliver a drug to the body through the skin. These systems, like any other, have advantages as well as limitations, with the former outweighing the latter.

2.5.3.1 Advantages

The advantages of topical and transdermal drug delivery systems are (Brown *et al.*, 2006:177; Ghulaxe & Verma, 2015:38; Kumar & Philip, 2007:634):

1. The large surface area of the skin ($1 - 2 \text{ m}^2$) provides various sites available for drug absorption.
2. Applications are non-invasive and convenient.
3. Easy to use without the need for trained staff members to administer the drug, which reduces the costs.
4. Hepatic first-pass metabolism is avoided.
5. Steady infusion of a drug over a longer period, lowering peak plasma levels, which lead to reduced side effects.
6. Drugs with a short half-life, poor oral availability and narrow therapeutic index can be administered via this route.
7. In the event of toxicity, easy termination of this route exists.
8. Direct access to the target area or the diseased area in the case of treatment of skin disorders.
9. Serves as an alternative route for oral administration where the patient is unconscious or nauseated.
10. Improved patient compliance and acceptance.
11. Drugs can be given via the transdermal route to patients with gastrointestinal problems, as there is no direct contact between the drug and the intestinal tract.

2.5.3.2 Limitations

The limitations of topical and transdermal drug delivery systems are (Brown *et al.*, 2006:177; Ghulaxe & Verma, 2015:38; Kumar & Philip, 2007:634):

1. The skin provides a barrier to absorption and thus only drugs with specific properties can overcome this function and be absorbed.

2. Due to this barrier function, only very potent drugs with small concentrations can be used via this route.
3. A drug must have a molecular weight less than 500 Da to permeate through the skin, as the diffusion is inversely related to size.
4. Hydrophilic drugs pass very slowly or cannot permeate through the skin, thus negatively affecting bioavailability.
5. Adverse effects such as itching, erythema, oedema, etc., may be experienced with this route.
6. Drugs fail to reach systemic circulation if they have a very high or low partition coefficient.

2.5.4 Mechanisms of skin penetration

There are three potential pathways a molecule can follow to cross the epidermis, namely: 1) through the intercellular lipids, 2) by the transcellular route, and 3) via the skin appendages (the transappendageal route) (Figure 2.7). Penetration through the skin usually occurs by a combination of these pathways and is not always particular to one specific route (Benson & Watkinson, 2012:14).

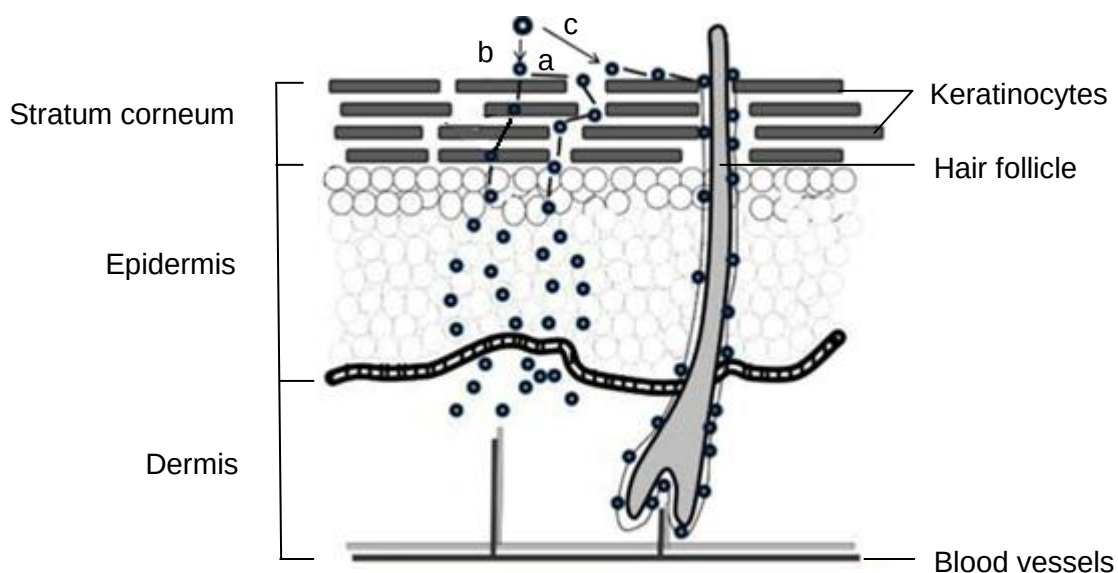


Figure 2.7: Penetration pathways: (a) intercellular, (b) transcellular and (c) transappendageal pathways (Adapted from Valenzuela & Simon, 2012:76).

2.5.4.1 Transcellular route

The transcellular pathway is where the drug moves across the stratum corneum, passing through the corneocytes, which contain an aqueous compartment surrounded by a lipid compartment through which the drug must move. The drug must partition into and diffuse through the aqueous and lipid compartments of the multiple bilayers before reaching its target area (Williams, 2013:679). This route of permeation is the most common pathway used for highly hydrophilic molecules (Morrow *et al.*, 2007:38-39).

2.5.4.2 Intercellular route

The intercellular pathway is where the drug diffuses through the lipid matrix, which surrounds the intercellular spaces of the keratinocytes (Valenzuela & Simon, 2012:75). The stratum corneum, comprising of its “bricks and mortar” model, yields a convoluted pathway through which the drug must permeate. The intercellular region consists of alternating bilayers and therefore the drug must partition into and diffuse through aqueous and lipid regions of the bilayer (Williams, 2013:679). Due to these obstacles, this pathway is the most common route for small, uncharged molecules to penetrate through the skin (Morrow *et al.*, 2007:38-39).

2.5.4.3 Transappendageal route

Skin appendages provide a direct pathway through which a drug can permeate into the skin, avoiding the barrier of the stratum corneum. The skin appendages include hair follicles, sebaceous glands and sweat ducts and are commonly referred to as the ‘shunt route’ (Mitragotri, 2003:79). Morrow *et al.* (2007:38) describes the limitations of this route as: 1) the small surface area of approximately 0.1% for the permeation of drug particles, 2) sweat from sweat ducts moves in the opposite direction of the permeating drug, thus restricting permeation, and 3) lipid-rich sebum from sebaceous glands functions as a barrier to hydrophilic drugs. Even though this route poses a few limitations, it is often the dominant pathway directly after application and may be important for ions and large polar molecules.

2.5.5 Transport through the skin and mathematical models of diffusion

A drug is absorbed through the skin mainly by means of passive diffusion and the active transport thereof plays no role. The drug is therefore absorbed according to the concentration gradient and moves from a high concentration to a low concentration (Jhawar *et al.*, 2013:50). When a drug is applied to the skin, the outside of the skin contains a high concentration of the drug, whereas the inside has a low concentration and as a result, the drug molecule diffuses through the skin to the area of low concentration.

Permeation of a drug across the skin (stratum corneum) follows Fick's First Law (Equation 2.1), which says that the rate of diffusion of molecules across an area of skin, is proportional to the measured concentration gradient (Williams, 2003:41-42). This equation can be used to identify parameters involved in diffusion of a drug across the skin (Uzor *et al.*, 2011:683).

$$J = \frac{dm}{dt} + \frac{DPC_0}{h}$$

Equation 2.1

Where:

- J = steady-state permeation or flux
- dm/dt = rate of skin permeation
- D = diffusion coefficient of the drug in the stratum corneum
- P = partition coefficient between the stratum corneum and the vehicle
- C₀ = applied concentration of the drug
- h = diffusional path length or the membrane thickness

For a topical/transdermal preparation to reach the systemic circulation, it needs to follow a few steps, which involve (Bhowmick & Sengodan, 2013:638):

1. Dissolution of the drug inside and release from the vesicle.
2. Partitioning in the stratum corneum (the outermost layer).
3. Diffusion through the stratum corneum.
4. Partitioning from the lipophilic stratum corneum into the more hydrophilic viable epidermis.
5. Diffusion through the viable epidermis into the deeper dermal layers.
6. Uptake into the capillary network and ultimately the systemic circulation.

2.5.6 Factors affecting skin permeation

To develop an effective transdermal drug delivery system, three factors namely the drug, skin and vehicles should be considered. These factors affecting skin permeation can be classified as biological factors and physicochemical factors (Reddy *et al.*, 2014:1097), the latter being the main factors focused on in this study, as they are easier to control than the biological factors.

2.5.6.1 Biological factors

Biological conditions of the skin change and affect the permeation of a drug by altering the properties of the skin and these factors include hydration of the skin, skin age and temperature, blood flow, anatomical location, skin condition (disease), skin metabolism and pigmentation (Jhawar *et al.*, 2013:49).

2.5.6.2 Physicochemical factors

Physicochemical factors form the primary factors that affect permeation of drugs, since it influences transport of the vehicles through the skin. Thus, for molecules to pass through the stratum corneum, it must possess favourable physicochemical properties (Hadgraft, 2004:292; N'Da, 2014:20785). Several of these factors will be discussed below.

2.5.6.2.1 Drug concentration

It was perceived that the drug flux increases when the drug concentration increases. Maximum flux ($J_{\max}$) is reached when the solution is saturated, because a higher concentration cannot be achieved (Akhlaq *et al.*, 2014:179). The flux is directly proportional to the concentration gradient across the barrier, because absorption of drugs is governed by passive diffusion. If the concentration of the drug over the barrier is higher, the concentration gradient will be higher and thus more diffusion will occur across the skin (Sharma *et al.*, 2011:75).

2.5.6.2.2 Diffusion coefficient

The diffusion coefficient (D) is defined as the amount of the drug that diffuses across the skin (stratum corneum) in an area per unit time and therefore describes the ease of diffusion through membranes (Williams, 2003:27). Permeation of a drug depends on its diffusion coefficient and when the temperature is constant, the diffusion coefficient of a drug depends on characteristics of the drug, the diffusion medium and the interaction between the two (Reddy *et al.*, 2014:1098).

2.5.6.2.3 Partition coefficient

Permeation of the stratum corneum requires partitioning of the drug into the membrane, where solubility in the membrane plays a limiting factor. The partition coefficient (P) is used to determine the solubility or diffusion of a drug in the aqueous and lipid areas of the membrane (Jhawar *et al.*, 2013:49). Therefore a drug must possess both aqueous and lipid solubility to achieve successful transdermal delivery resulting in an optimum partition coefficient (Perrie *et al.*, 2012:393). It is observed in Fick's First Law that a high partition coefficient results in an increased flux or increased skin permeation. The log P is used as an adequate description of the partitioning of a drug between the hydrophilic cells of the epidermis and the lipophilic stratum corneum. The lower the log P value, the more hydrophilic the compound (N'Da, 2014:20786). A log P value between 1 and 4 is optimal for dermal absorption, with values between 2 and 3 most favourable (Akhlaq *et al.*, 2014:178). A good indication if a drug is favourable for transdermal permeation, is the log D value (the log P value at a certain pH) of a compound. The log D value for roxithromycin is 1.52 (Csongradi, 2015:178), making it extremely favourable for dermal absorption and transdermal delivery.

2.5.6.2.4 Solubility and melting point

Sufficient aqueous solubility is required for a substance to permeate through the skin successfully (Akhlaq *et al.*, 2014:178). The permeant also needs to move through the lipophilic intercellular domains and as a result, both lipid solubility and high aqueous solubility are required to maximise flux (Benson & Watkinson, 2012:14). According to Naik *et al.* (2000:319), the ideal aqueous solubility should be >1 mg/ml and the melting point should be < 200 °C for passive transdermal drug delivery. Roxithromycin has a melting point between 116 to 122 °C (Aucamp *et al.*, 2012:469) and is in accordance with specifications, but the solubility is 0.0335 mg/ml at 25 °C (Aucamp *et al.*, 2013:26), which is described by the BP (2016) as very slightly soluble in water. The poor water solubility can be improved by using the more soluble amorphous forms of roxithromycin (Biradar *et al.*, 2006:22).

2.5.6.2.5 pH, pKa and ionisation

The pH of the stratum corneum is 4.2 to 5.6 and that of the viable epidermis is 7.3 to 7.4 (N'Da, 2014:20787). As reported by Sharma *et al.* (2011:75), the ideal pH for a saturated solution during topical delivery should be between 5 and 9, as drugs with pH values above or below these values would cause harm to skin. The pH of a drug plays an important role in determining the ionisation of the drug at the surface of the skin. It is said that unionised species have better absorption into the skin than ionised species (Jhawar *et al.*, 2013:49).

The ionisation of the drug can be determined using an adaption of the Henderson-Hasselbalch equation (Equations 2.2 and 2.3):

$$\% \text{ionised} = 100 / 1 + \text{anti-log} (\text{pKa} - \text{pH}) \quad \text{Equation 2.2}$$

$$\% \text{unionised} = 100 - \% \text{ionised} \quad \text{Equation 2.3}$$

Roxithromycin has a pKa value of 9.17 (Babić *et al.*, 2007; Qiang & Adams, 2004), and when the ionisation is determined at pH 7.4, the unionised species is 97.95%, suggesting the API would have good absorption into the viable epidermis.

2.5.6.2.6 Molecular size and shape

The size of the molecule is inversely related to the absorption of the drug and therefore small molecules will permeate faster than larger ones (Reddy *et al.*, 2014:1098). For a drug to be suitable for transdermal transport, Uzor *et al.* (2011:681) stated that its molecular weight should be less than 500 Da (500 g/mol). The molecular weight of roxithromycin is 837.06 g/mol (Merck, 2017), which is larger than the ideal and could limit the absorption of the drug into the skin. According to Billich *et al.* (2005:3157), a study was conducted on a molecule with a

molecular weight of more than 500 Da that resulted in permeation of the molecule across the skin by means of passive diffusion. According to Foldvari (2000:417), the 500 Da rule should be revised, since novel methods are emerging that are able to improve the permeation of larger molecules into and through the skin.

2.6 Conclusion

Acne is a common skin disease found in approximately 80% of adolescents and young adults and thus widely found globally. Roxithromycin, an antibiotic, was inspected during this study for the possible topical treatment of acne, as no topical form of the API is currently used to treat this skin disease. Roxithromycin is a promising contestant for topical delivery as most of its physicochemical properties are ideal for delivering the drug to the skin. One characteristic that is not ideal is the poor solubility of the drug. The amorphous forms of the API, **QC** and **CD** forms, were investigated during this study, as they often have better solubility.

Another important factor to note is the skin. The unique structure of the skin, especially the stratum corneum, makes the delivery of drugs locally to the skin very difficult. The different APIs were therefore encapsulated into niosome vesicle systems, which may help improve skin permeability thus enhancing bioavailability.

The aim was to formulate the different solid-state forms into niosomes containing varying ratios of cholesterol and Span[®] 40 to see which excipients had a stabilising effect on the amorphous forms and to determine which preparation had the best topical delivery of the API.

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Chapter 3

Article for publication in the European Journal of Pharmaceutical Sciences

Chapter 3 is written in article format for the purpose of publication in the *European Journal of Pharmaceutical Sciences*. The complete author guidelines for publishing are found in Appendix E. This chapter has been justified to improve reading and the article was written in UK English, according to the author guidelines.

Topical delivery of roxithromycin amorphous forms encapsulated into niosome vesicle systems

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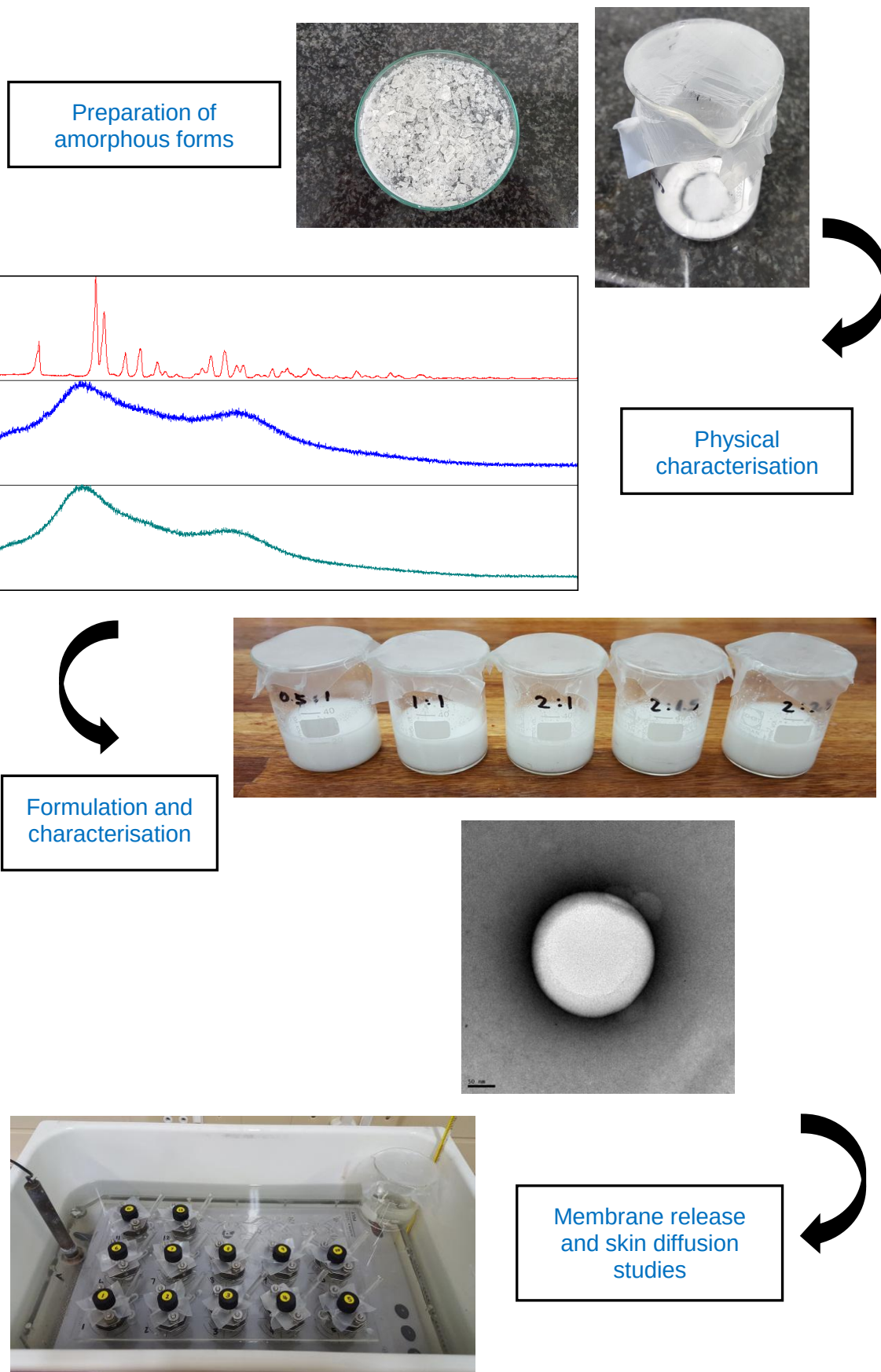
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Abstract

The purpose of this research study was to determine if the excipients (cholesterol and Span[®] 40) used during the formulation of niosome vesicle systems, had a stabilising effect on the amorphous forms of roxithromycin. This was investigated by formulating crystalline roxithromycin and the amorphous quench cooled and chloroform desolvated forms into niosomes containing five different ratios of the excipients. The three solid-state forms were characterised physically before the lipid films (precursors to niosomes) were examined for stability. During the stability investigation, it was found the preparation of the lipid films rendered the crystalline form of roxithromycin into an amorphous habit. It was also observed that the combination of the amorphous forms with cholesterol and Span[®] 40 resulted in the amorphous forms remaining stable throughout further preparation processes. The niosome vesicles were characterised to evaluate if they have ideal properties for topical drug delivery. Membrane release and skin diffusion studies were conducted to determine if roxithromycin was released from the niosomes and to see if transdermal and/or topical delivery was achieved. The membrane release studies revealed that roxithromycin was released from all the niosomal systems. The skin diffusion results obtained showed some transdermal delivery, as well as very good topical delivery of roxithromycin. It was concluded that the excipients, as well as the solid-state forms, influenced the diffusion of roxithromycin into the skin and that the quench cooled amorphous form displayed the best results during this study. Roxithromycin was successfully delivered to the target site in the epidermis-dermis, where it could possibly be used to treat acne topically.

Keywords: Roxithromycin, amorphous forms, excipients, niosomes, lipid films, topical drug delivery

Graphical abstract



1 **1 Introduction**

2 Acne is a well-known chronic skin disorder, affecting more than 80% of adolescents and young
3 adults (Krautheim & Gollnick, 2004). Acne can present with several clinical features including
4 inflammatory and non-inflammatory lesions, seborrhoea and different degrees of scarring
5 (Williams *et al.*, 2012). One of four components responsible for the formation of acne is the
6 presence of the bacteria *Propionibacterium acnes* (Ramanathan & Hebert, 2011).
7 Roxithromycin, a macrolide antibiotic, was investigated as the active pharmaceutical ingredient
8 (API) during this study, as evidence exists that it is active against *P. acnes*, but no topical
9 treatment of the API currently exists. Roxithromycin could be used to treat acne topically, as it
10 could reduce the population of *P. acnes* and have mild anti-inflammatory effects (Katsambas *et al.*,
11 2004). It is said that topical treatment is one of the most pertinent routes to treat skin
12 diseases effectively (More *et al.*, 2016).

13 When formulating a product for topical drug delivery, one obstacle that should be kept in mind is
14 the skin itself. The skin is classified as the largest organ, as it covers the entire human body
15 and comprises 16% of the total body weight (Venus *et al.*, 2010). The skin consists of three
16 main anatomical layers, i.e. the epidermis, dermis and hypodermis (Alexander *et al.*, 2012).
17 The stratum corneum, which forms the outermost layer of the epidermis, is known as the rate-
18 limiting barrier for penetration of the drug into the skin (Foldvari, 2000). The stratum corneum
19 with its unique structure has been studied intensively, and can be described in terms of the
20 “bricks and mortar” model (Washington *et al.*, 2001). The stratum corneum is lipophilic,
21 whereas the deeper epidermis, dermis and systemic circulation are hydrophilic environments
22 and therefore if topical drug delivery wants to be achieved, a drug must have adequate solubility
23 in both lipophilic and hydrophilic regions (Perrie *et al.*, 2012).

24 Certain ideal physicochemical properties are required for an API to permeate across the skin,
25 including melting point, aqueous solubility, lipophilicity and molecular weight (Benson &
26 Watkinson, 2012). A melting point of < 200°C and an aqueous solubility of > 1 mg/ml are ideal
27 for topical delivery (Naik *et al.*, 2000). Roxithromycin has a melting point between 116 and
28 122°C (Aucamp *et al.*, 2012), which is ideal, however, it has a solubility of 0.0335 mg/ml at 25°C
29 (Aucamp *et al.*, 2013), which is very slightly water-soluble. The API has an *n*-octanol-buffer
30 distribution coefficient (log D) of 1.52 (Csongradi, 2015), where values between 1 and 4 are
31 optimal for dermal absorption (Akhlaq *et al.*, 2014). A molecular weight of less than 500 Dalton
32 (g/mol) is favoured for topical delivery (Uzor *et al.*, 2011) and roxithromycin has a molecular
33 weight of 837.06 g/mol (Merck, 2017), which could limit the diffusion of the API into the skin.

34 Different solid-state forms of a given API vary significantly in terms of physical and chemical
35 properties, owing to their different crystal packing (Vippagunta *et al.*, 2001). A promising way of
36 overcoming the poor aqueous solubility roxithromycin displays is the conversion of the

crystalline form of the API to the more soluble amorphous forms (Laitinen *et al.*, 2013). Two amorphous forms of the API were used during this study, as previous experiments showed improved solubility of the amorphous forms of roxithromycin (Aucamp *et al.*, 2013; Milne *et al.*, 2016).

Niosome vesicle systems were used to encapsulate the API, as it enhances penetration of the drug into the skin (Kumar & Rajeshwarrao, 2011) and as a result could help overcome the size limitation of the molecules. Niosomes have many advantages, one being they can encapsulate various types of substances within its unique structure including lipophilic, hydrophilic and amphiphilic drugs (Rahimpour & Hamishehkar, 2012). Niosomes are also said to reduce absorption of the API into the systemic circulation, while increasing the drug to remain in the stratum corneum and epidermis (Uchechi *et al.*, 2014), making it an ideal vehicle for topical drug delivery.

The aims of this study were to determine if the excipients used to formulate the niosomes had a stabilising effect on the amorphous forms of the API, preventing them from crystallising back to the more stable crystalline form. Five formulas with different ratios of excipients were established, and the three solid-state forms were incorporated into these formulas, resulting in 15 niosome systems. The second aim was to determine which niosome had the highest concentration of the API delivered to the deeper epidermis-dermis target site. The objectives of this study were thus to prepare the amorphous forms and to physically characterise the different solid-state forms. The lipid films, precursors to niosomes, were prepared and characterised to determine the crystallinity and stability thereof. Niosomes were prepared and characterised to conclude if ideal physicochemical properties necessary for topical delivery were present. Membrane and skin diffusion studies were performed to determine if the API would be released from the vesicles, and the formula that resulted in the best topical delivery.

2 Materials and methods

2.1 Materials

Crystalline roxithromycin raw material was purchased from DB Fine Chemicals (Johannesburg, RSA). The components used to prepare the niosomes were cholesterol, Span[®] 40, chloroform, methanol and Milli-Q[®] water. Cholesterol was purchased from Sigma-Aldrich (Steinheim, Switzerland) and Span[®] 40 from Fluka (Steinheim, Switzerland). The chloroform and methanol used to prepare the niosomes were purchased from ACE Chemicals (Johannesburg, South Africa) and Milli-Q[®], double deionised high performance liquid chromatography (HPLC) grade water, used during all the experiments, was obtained from a Millipore[®] Milli-Q[®] water purification system (Bedford, USA). A phosphate buffer solution (PBS) (pH 7.4) was prepared using UnivAR[®] potassium phosphate (KH₂PO₄) and UnivAR[®] sodium hydroxide (NaOH)

purchased from Merck (Wadeville, South Africa). For the mobile phase, HPLC chromatography grade LiChrosolv[®] methanol (MeOH), supplied by Merck Millipore (Darmstadt, Germany), was used with the buffer solution that consisted of ammonium di-hydrogen phosphate (NH₄H₂PO₄) acquired from Saarchem (Krugersdorp, South Africa).

2.2 Methods

2.2.1 High performance liquid chromatography analysis

An HPLC analytical method for the quantification of roxithromycin was adapted and validated from a previously published method (Aucamp *et al.*, 2016). The analysis was performed in a controlled laboratory environment at the Analytical Technology Laboratory (ATL), North-West University (NWU), Potchefstroom Campus, South Africa. An Agilent[®] 1200 series HPLC system (Agilent Technologies, USA) and a Shimadzu (Kyoto, Japan) UFLC (LC-20AD) chromatographic system were used for concentration analysis. Data analysis was conducted using LabSolutions, LCsolution, Release 1.21 SP1 (Shimadzu, Kyoto, Japan). A Venusil XBP C₁₈ (2) reverse phase column (150 x 4.6 mm) with a 5 µm particle size was used (Agela Technologies, Newark, USA). The mobile phase consisted of 0.01 M NH₄H₂PO₄ buffer solution (pH adjusted to 7.0 with diluted ammonia), the buffer was mixed with methanol in the ratio 15:85. The mobile phase was filtered through a 0.22 µm nylon membrane filter (Membrane Solutions[®], Kent, USA) before use. A default injection volume of 50 µl was used with the flow rate set at 1 ml/min, a run time of 10 min, with roxithromycin having a retention time of ±5.0 min.

2.2.2 Preparation of the amorphous forms of roxithromycin

Two amorphous forms of roxithromycin, namely the quench cooled and chloroform desolvated forms, were prepared. The quench cooled form was prepared using the well-known quench cooling of the melt method. Crystalline roxithromycin raw material was evenly spread out on the surface of a glass Petri dish and subsequently melted in a laboratory oven (Binder, Tuttlingen, Germany) at a temperature of up to ±130°C, the resulting melt was removed from the oven and quenched on a cold granite surface, rendering the sample into a glass form (Aucamp *et al.*, 2012). The chloroform desolvated amorphous form was prepared by adding roxithromycin raw material to chloroform, heating the solution to approximately 60±3°C, until a saturated solution was reached. The solution was removed from the hot plate and left to cool to ambient temperature, and subsequently covered with Parafilm[®] (Beemis NA, Neenah, USA). After slow evaporation of the chloroform, a dense mass was formed and the sample was desolvated in an oven at 60±2°C, for 24 h (Aucamp *et al.*, 2012).

2.2.3 Preparation of niosomes

The niosome vesicles were prepared using an adapted version of the thin-film hydration method (Chandu *et al.*, 2012). The cholesterol, surfactant and API was dissolved in chloroform/methanol solution, stirred and heated above 50°C, which is the transition temperature of Span® 40 (Kumar & Rasjeshwarrao, 2001). After all the organic solvents evaporated, a thin-film formed at the bottom of the beaker. The film was hydrated using Milli-Q® water, stirred and heated for 30 min at a temperature of 50°C to form a milky suspension. Subsequently the solution was left to cool to room temperature (25±2°C) after which it was sonicated on ice for 2 min, using an ultrasonicator (Model UP200St, Hielscher Ultrasonics, Teltow, DE). The niosome dispersion was left to stand for 24 h at room temperature, to allow for optimal swelling of the vesicles (Muzzalupo *et al.*, 2011). The three solid-state forms of the API were used to formulate the niosomes in five different ratios of cholesterol and Span® 40, forming fifteen dispersions. The raw material form will be referred to as **RM**, while the quench cooled and chloroform desolvated forms will be known as **QC** and **CD**, respectively. The ratios of cholesterol and Span® 40 respectively, are as follow: dispersion 1 (0.5:1.0% w/w), dispersion 2 (1.0:1.0% w/w), dispersion 3 (2.0:1.0% w/w), dispersion 4 (2.0:1.5% w/w) and dispersion 5 (2.0:2.5% w/w).

Table 1

2.3 Physical characterisation

2.3.1 X-ray powder diffraction

X-ray powder diffraction (XRPD) patterns were obtained using a PANalytical Empyrean diffractometer equipped with a PIXcel^{3D} detector (PANalytical, Almelo, Netherlands). Sufficient sample was evenly distributed on a zero background holder. The measurement conditions were: target, Cu; voltage, 40 kV; current, 30 mA; divergence slit, 2 mm; anti-scatter slit, 0.6 mm; detector slit, 0.2 mm; monochromator; scanning speed, 2°/min (step size, 0.025°; step time, 1.0 sec).

2.3.2 Differential scanning calorimetry

A Shimadzu (Kyoto, Japan) DSC-60 instrument was used to record the differential scanning calorimetry (DSC) thermograms. Samples (3-5 mg) were accurately weighed and sealed in aluminium crimp cells with unpierced lids and heated between 25 to 200°C with a heating rate of 10°C/min and a nitrogen gas purge of 35 ml/min. The onset temperatures of the thermal events were reported so that reporting would be standard throughout the study and especially because of the fact that glass transition temperatures (T_g) were investigated.

2.3.3 Fourier-transform infrared spectroscopy (FTIR)

IR-spectra were recorded using a Shimadzu IR Prestige-21 spectrophotometer (Kyoto, Japan) over a range of 400-4000 cm^{-1} . Potassium bromide was used as a background. The samples were dispersed in a matrix of powdered potassium bromide by means of light grinding. IR-spectra were measured using a diffuse reflectance cell.

2.4 Characterisation of the niosomes

2.4.1 Transmission electron microscope

The morphology of the vesicles were studied using an FEI Tecnai G2 20S-Twin 200 kV high resolution transmission electron microscope (HRTEM) (Czech Republic, EU), fitted with an Oxford INCA X-Sight EDS System at the Electron Microscopy Laboratory of the NWU, Potchefstroom, South Africa. Vesicles with no encapsulated API were used to avoid damaging the microscope. The samples were roughly diluted 10x with distilled water and a drop of sample was stratified onto a copper carbon-coated 300 mesh grid and left to dry for 5 min. Osmium tetroxide was used to stain the lipid layers of the vesicles, making them more visible. The samples were left to dry for 15 min, before being viewed through the microscope.

2.4.2 Zeta-potential, droplet size and polydispersity index

The zeta-potential, droplet size and polydispersity index (Pdl) of the niosomes were determined using a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments Ltd, Worcestershire, UK). A drop of each sample was diluted 25x with Milli-Q[®] water, thereafter approximately 2 ml was injected into a clear disposable zeta cell and measured in triplicate.

2.4.3 pH

The pH of each dispersion was measured at ambient temperature using a Mettler Toledo[®] pH meter (Mettler Toledo, CU) equipped with a Mettler Toledo[®] InLab[®] 410 electrode (Mettler Toledo, CU).

2.4.4 Entrapment efficiency

The entrapment efficiency (EE%) of roxithromycin was determined by means of HPLC analysis. The dispersions were centrifuged using an Optima L-100 XP ultracentrifuge (Beckman Coulter, USA), with a 50.2 Ti Rotor, at 25 000 rpm for 30 min, at a temperature of 25°C. The samples were centrifuged at the Laboratory for Applied Molecular Biology (LAMB) of the NWU, Potchefstroom, South Africa. Two phases formed, namely the supernatant and the pellet, after which the supernatant was extracted using a syringe and transferred to HPLC vials. Equation 1

was adapted from Xiang *et al.* (2009) and used to calculate the amount of API entrapped inside the niosomes.

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

2.5 Diffusion studies

2.5.1 Preparation of the receptor phase

The receptor phase was made up of PBS (pH 7.4), prepared by weighing 6.5 g KH_2PO_4 and dissolving it in 250 ml of Milli-Q[®] water, then weighing 1.5 g of NaOH and dissolving it in 400 ml of Milli-Q[®] water. The NaOH was added to the KH_2PO_4 while stirring the solution and filled up to 1 000 ml with Milli-Q[®] water. The pH was measured and adjusted to 7.4 with phosphoric acid (H_3PO_4).

2.5.2 Preparation of the donor phase

The donor phase consisted of the niosome dispersion entrapping the three solid-state forms of roxithromycin. During the membrane release and skin diffusion studies, API loaded niosomes, as well as niosomes containing no API (placebo), were analysed. The dispersions were prepared using the method as discussed in Section 2.2.3. Freshly prepared samples were used for every experiment and the dispersions were placed in a water bath set at 32°C approximately 1 h before commencement of the diffusion study.

2.5.3 Membrane release studies

Membrane release studies were performed on all fifteen niosomes and each study consisted of 12 Franz cells, with two of the twelve containing the placebo, serving as a control group. The receptor phase and donor phase were placed in water baths 1 h before commencement of the diffusion study at temperatures of 37°C and 32°C, respectively. The Franz cells comprising of a donor and receptor compartment, were greased on the even sides with Dow Corning[®] (Auburn, USA) high vacuum grease. A magnetic stirring rod was placed inside the receptor compartments and a hydrophilic polyvinylidene fluoride (PVDF) membrane (Pall[®] Life Sciences, Michigan, USA), with a pore size of 0.45 μm and diameter of 25 mm, was placed between the receptor and donor compartments. The Franz cells were sealed with vacuum grease on the sides and clamped tightly with horseshoe clamps to prevent leakage. The receptor compartment was filled with 2 ml of preheated (37°C) PBS (pH 7.4), ensuring that no air bubbles formed. Each donor compartment was filled with 1 ml of preheated (32°C) niosomes and subsequently covered with a piece of Parafilm[®] (Neenah, USA) and a cap to prevent any loss of the formulation during the experiment. The 12 Franz cells on a tray were placed in a water bath (Grant Instruments, Cambridgeshire, UK) set at 37°C, onto a Variomag[®] (Variomag,

Daytona Beach, USA) magnetic stirrer plate, with the receptor compartments fully submerged in the water. The entire receptor phase was extracted and refilled with fresh preheated PBS (pH 7.4) hourly for 6 h. The extracted receptor phase was placed into vials and analysed on the HPLC.

2.5.4 Skin preparation

Female Caucasian skin was obtained from patients after undergoing abdominoplasty surgery and used during skin diffusion studies. Patients signed an informed consent form giving permission for their skin to be used for research purposes and patient information remained confidential. Ethical approval for the use of biological material, i.e. human skin, was obtained from the Research Ethics Committee of the North-West University (reference number NWU-00114-11-A5). After collection, the skin was placed in a freezer at -20°C until used. The skin was firstly checked, by visual examination, for any stretch marks, hairs or lesions, after which it was dermatomed using a Zimmer™ electric dermatome Model 8821 (Zimmer, Ohio, USA). The skin was cut into small pieces with a thickness of 400 µm and placed on Whatman® (Maidstone, UK) filter paper, with the stratum corneum facing upwards. The skin pieces on the filter paper were wrapped in aluminium foil and stored in a freezer at -20°C until needed for the skin diffusion study. The skin on the filter paper was cut into circular shapes to use between the two compartments of the Franz cells.

2.5.5 Skin diffusion studies

For the skin diffusion studies, the same method was used as for the membrane release studies, as described in Section 2.5.3, with a few exceptions. The cut out skin pieces with the stratum corneum facing upwards was used instead of the PVDF membranes, the receptor phase was extracted only once at 12 h and the skin was further used to conduct tape stripping.

2.5.6 Tape stripping

After the 12 h skin diffusion study was completed, the skin was removed from the Franz cell and the filter paper and pinned onto a piece of Parafilm® (Neenah, USA) on a solid surface. A clean paper towel was used to dab the skin gently to remove any excess dispersion. The stratum corneum-epidermis (SCE) was then removed using adhesive tape (3M Scotch® Magic™ tape, Maplewood, USA). The tape strips were cut into small pieces the size of the diffusion area and sixteen strips were needed per Franz cell. The first strip was discarded, as it could possibly contain excess dispersion, the other fifteen strips were used to remove the SCE until the skin had a shiny appearance. The tape strips were placed in a polytop containing 5 ml of absolute ethanol, covering all of the strips. After tape stripping, the remaining skin, known as the epidermis-dermis (ED), was cut into smaller pieces and placed in polytops containing 5 ml of

absolute ethanol. The polytops containing the tape strips and the ED were placed in the refrigerator (2-8°C) for 8 h, after which it was filtered through a 0.45 µm PVDF syringe filter into vials and analysed using an HPLC.

3 Results and discussion

3.1 Preparation of the amorphous forms

The two amorphous forms were prepared using the crystalline raw material and formed the glassy quench cooled and the chloroform desolvated forms. The purity of the amorphous forms was tested through HPLC analysis and found to be good in accordance with **RM**. The purity of **QC** and **CD** was 99.2% and 99.6%, respectively and subsequently compared to the certificate of analysis (CoA) of **RM**, stating a purity of 99.6%.

3.2 Physical characterisation

The three solid-state forms of the API were characterised in terms of XRPD, DSC and FTIR to determine the degree of crystallinity or amorphicity. After the physical characterisation was completed, the lipid films (precursors to niosomes) were prepared and the physical stability was investigated.

3.2.1 Solid-state forms

Crystalline solids have short- and long-range packing of molecules giving them a well-defined structure, compared to amorphous solids, which have no long-range packing of molecules and therefore no distinct structure (Yu; 2001). Clear diffraction peaks are visible on the XRPD patterns for crystalline solids due to long-range ordering of molecules, in contrast to amorphous solids that display a “halo” pattern (Bates *et al.*; 2006). The XRPD pattern for **RM** displayed clear diffraction peaks, whereas **CD** and **QC** had characteristic diffuse patterns. Thermal analysis, by means of DSC, was used to see if a transition temperature (T_g) appeared, which is another indication of an amorphous solid (Bates *et al.*, 2006). The thermograms indicated **RM** had a clear melting point of 121.77°C, whereas **CD** with a T_g of 87.00°C and **QC** with a T_g of 84.98°C was observed. The IR-spectra of amorphous forms vary from that of crystalline forms as they have widespread conformations, which lead to the appearance of broader peaks (Einfalt *et al.*, 2013). The IR-spectra of **CD** and **QC** varied from that of **RM**, as peak broadening was observed in the region of 3600-2000 cm⁻¹. All these solid-state characterisation results compared very well with those previously reported by Aucamp *et al.* (2012) and Milne *et al.* (2016).

3.2.2 Lipid films

From the XRPD patterns obtained for the films containing **RM**, the preparation of lipid films rendered the **RM** samples into an amorphous habit. Diffuse patterns were obtained with no distinct diffraction peaks (Figure 1). This was corroborated by DSC thermograms, as no melting endotherm in the region of $\approx 120^\circ\text{C}$ was visible for roxithromycin. A small endotherm in the region of $\approx 35^\circ\text{C}$ was identified and is proposed to signify a T_g of the amorphous solid dispersion. The IR-spectra for the five **RM** samples also showed peak broadening in the areas of 4000-3200 cm^{-1} , which is an indication of the amorphous nature of the films. It was also seen that the preparation of the lipid films kept **QC** and **CD** amorphous and therefore no crystallisation to the more stable crystalline **RM** occurred. The XRPD, DSC and FTIR were indicative of the amorphous nature of **CD** and **QC**.

Figure 1

3.3 Characterisation results

The dispersions were characterised in terms of zeta-potential, droplet size, Pdl, pH and EE%, as summarised in Table 2.

Table 2

The average zeta-potential measured for all the samples was lower than -45 mV, which is considered to indicate stable niosomes (Silva *et al.*, 2012). Literature states that highly negative zeta-potential values improve skin permeation (Duangjit *et al.*, 2011) and thus the results obtained are favourable for topical delivery of the API. It is said for particles to be delivered topically, a particle size of 3 μm and smaller is required to permeate through the stratum corneum, particles ranging from 3-10 μm can penetrate through the follicular ducts, whereas particles bigger than 10 μm will not permeate through the skin, but stay on the surface (Allec *et al.*, 1997). The measured sizes of the niosomes were not larger than 3 μm and would therefore be able to permeate through the stratum corneum. The Pdl of the samples was closer to 1, indicating the samples were very polydispersed in nature. The pH of the samples varied between 7.93 and 8.61, being slightly basic. A pH between 5.00 and 9.00 is ideal for topical delivery, since it would not harm the skin (Naik *et al.*, 2000), and the results obtained were within these ranges. The EE% of the API within the niosomes was very good, because none of the vesicles entrapped less than 97% of the API. The dispersion of **QC3** had the highest EE%, entrapping $98.66 \pm 0.67\%$ of the API. The higher percentage entrapment could be ascribed to

the higher solubility of **QC** due to the higher degree of free energy. The morphology of the niosomes was investigated using TEM, as observed in Figure 2.

Figure 2

3.4 Diffusion study results

3.4.1 Membrane release results

The membrane release studies indicated that roxithromycin was released from all fifteen niosomes. **QC3** displayed the highest average flux value ($299.81 \pm 72.63 \mu\text{g}/\text{cm}^2 \cdot \text{h}$), which could be due it containing the highest amount of the API encapsulated into the vesicle, as seen during the EE% experiments. Failure of API release from the niosomes was therefore ruled out as a possible cause if no topical delivery occurs. Some Franz cells showed signs of leakage and were therefore removed from the dataset to prevent inaccurate results.

Figure 3

3.4.2 Skin diffusion results

The receptor phase was analysed after 12 h to determine if the API was delivered transdermally. Only four of the fifteen dispersions had quantifiable results above the limit of detection (LOD) and lower limit of quantification (LLOQ) in the receptor phase and were therefore delivered transdermally. These dispersions included **RM1**, **RM2**, **CD2** and **QC2**. The target area for this study is the deep ED layers and the desired outcome was not to obtain transdermal diffusion.

3.4.3 Tape stripping

Tape stripping was performed to determine if any roxithromycin concentrations were obtained in the SCE and ED, resulting in topical delivery.

3.4.3.1 Stratum corneum-epidermis

From the results obtained in the SCE, only six dispersions showed roxithromycin concentrations above the LOD and LLOQ. The six dispersions are ranked from highest to lowest as follows: **QC1**, **RM5**, **QC2**, **RM1**, **CD1** and lastly, **CD5**. It was noticed that dispersions 1 and 5, containing more Span[®] 40 than cholesterol, were favoured in the SCE, which could be ascribed

to Span[®] 40 having a hydrophilic-lipophilic balance (HLB) value of 6.7 making it more oil soluble and would therefore reside in the lipophilic region of the SCE (CRODA, 2015).

Figure 4

3.4.3.2 Epidermis-dermis

The results obtained showed that all the dispersions had concentrations of the API present in the ED, but only six had values above the LOD and LLOQ. These dispersions, from the highest, consisted of **QC2**, **RM4**, **RM3**, **CD3**, **CD2**, and **RM1**. Dispersions 3 and 4 contained a higher concentration of cholesterol than Span[®] 40, and it could be reasoned that cholesterol, due to its amphiphilic properties, favoured the hydrophilic area of the ED. The dispersions of **QC2** and **CD2** contained equal amounts of cholesterol and Span[®] 40 and in this case, it appears the solid-state form may have a larger influence on API diffusion onto the ED than the composition of different excipients used in the formulation of the niosomes.

Figure 5

4 Conclusion

The aim of this study was to investigate the effect the excipient(s) in the niosome formula had on the stabilisation of the amorphous forms and to determine which one of the 15 niosome systems delivered the highest concentration of the API to the target area (ED). The physical characterisation showed the preparation of the lipid films rendered the crystalline **RM** into an amorphous habit and prevent the amorphous forms from crystallising to the more stable crystalline form. Characterisation revealed optimal results for topical delivery of the API. After characterisation was completed, the release of the API from the vesicles was studied. Roxithromycin had been released from all 15 niosome systems, as seen during the membrane release studies, with **QC3** presenting with the highest average flux value. Following the membrane release studies, skin diffusion studies were conducted and it was found only six dispersions delivered API in the SCE, as well as in the ED. It was observed that **QC1** and **QC2** had the highest concentration of the API in the SCE and ED, respectively.

The aims of this study had been reached as quantifiable amounts of roxithromycin were delivered topically to the ED and it was found that the excipients, as well as the solid-state forms, influenced the permeation of the drug into the skin.

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377 **Conflict of Interest**

378 The authors declare that no conflict of interests exists.

379

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457

458 **Tables**

459 **Table 1:** Excipients used to formulate the niosomes

Excipients	Dispersion				
	1	2	3	4	5
API	200 mg	200 mg	200 mg	200 mg	200 mg
Cholesterol	266.66 mg	400 mg	533.33 mg	457.14 mg	355.56 mg
Span [®] 40	533.33 mg	400 mg	266.66 mg	342.86 mg	444.44 mg
Chloroform/methanol (2:1)	5 ml	5 ml	5 ml	5 ml	5 ml
Milli-Q [®] water	10 ml	10 ml	10 ml	10 ml	10 ml

460

461

462 **Table 2:** Characterisation results for the 15 niosomes

Dispersion	Characteristics				
	Zeta-potential (mV)	Droplet size (nm)	Pdl	pH	EE%
RM1	-54.3 ± 1.91	294.9 ± 22.26	0.405 ± 0.09	8.07 ± 0.01	97.86 ± 0.37
RM2	-54.8 ± 1.79	1136.3 ± 24.01	0.616 ± 0.05	7.93 ± 0.01	98.07 ± 0.23
RM3	-52.9 ± 2.60	726.9 ± 14.57	0.645 ± 0.06	8.07 ± 0.03	98.22 ± 0.25
RM4	-46.6 ± 1.13	961.2 ± 12.40	0.712 ± 0.01	7.97 ± 0.02	98.24 ± 0.29
RM5	-52.3 ± 0.55	667.0 ± 21.75	0.588 ± 0.05	7.97 ± 0.01	97.95 ± 0.10
CD1	-48.1 ± 4.63	271.7 ± 23.49	0.351 ± 0.04	8.20 ± 0.03	98.12 ± 0.73
CD2	-56.0 ± 2.32	1065.0 ± 12.12	0.907 ± 0.10	8.51 ± 0.02	98.11 ± 0.83
CD3	-61.8 ± 5.35	983.0 ± 7.89	0.746 ± 0.03	8.37 ± 0.02	98.29 ± 0.63
CD4	-53.4 ± 2.42	460.5 ± 2.89	0.523 ± 0.03	8.40 ± 0.01	98.04 ± 0.80
CD5	-53.8 ± 0.15	553.98 ± 23.16	0.622 ± 0.05	8.19 ± 0.03	98.28 ± 0.55
QC1	-48.6 ± 0.47	289.5 ± 9.70	0.650 ± 0.05	8.18 ± 0.01	97.95 ± 0.33
QC2	-43.5 ± 0.67	1306.3 ± 35.64	0.998 ± 0.003	8.38 ± 0.01	98.15 ± 0.35
QC3	-49.8 ± 2.14	881.1 ± 10.50	0.683 ± 0.05	8.62 ± 0.01	98.66 ± 0.67
QC4	-48.3 ± 1.17	405.8 ± 18.99	0.383 ± 0.02	8.18 ± 0.02	97.90 ± 0.68
QC5	-56.2 ± 0.71	717.8 ± 14.11	0.583 ± 0.02	8.15 ± 0.01	97.77 ± 0.72

463

464

465 **Figure legends**

466 **Figure 1:** XRPD diffraction patterns overlay obtained for (a) **RM**, (b) cholesterol, (c) Span[®] 40,
467 (d) film **RM1**, (e) film **RM2**, (f) film **RM3**, (g) film **RM4** and (h) film **RM5**

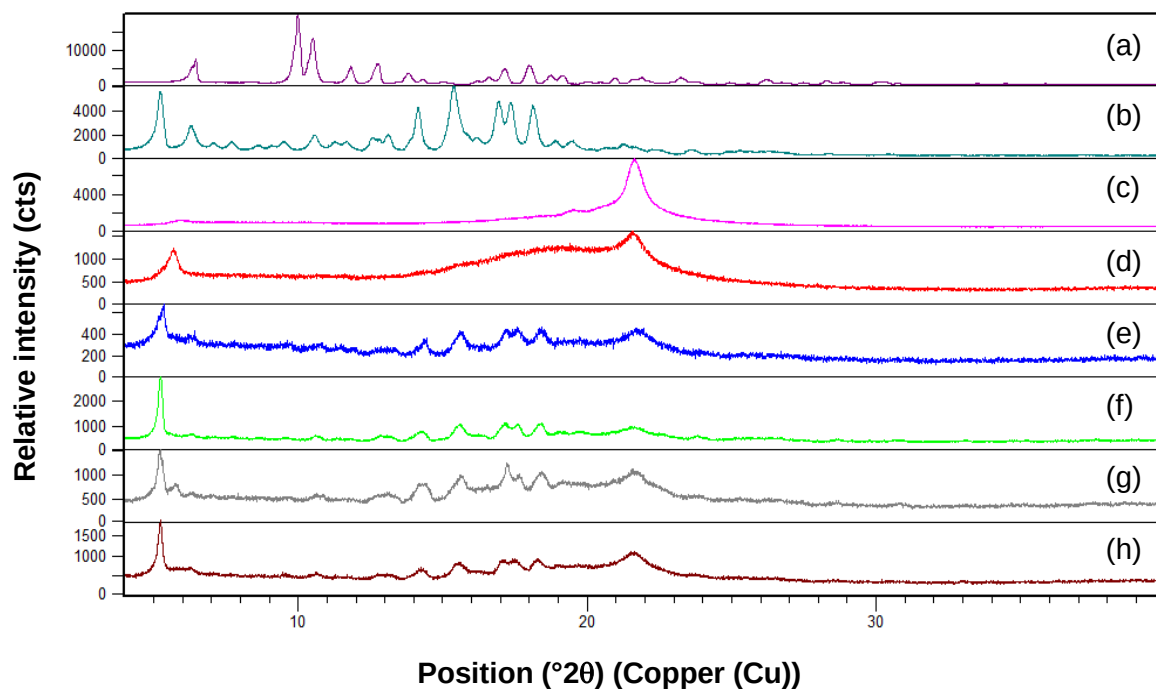
468 **Figure 2:** Appearance of vesicles viewed using TEM: (a) dispersion 1, (b) dispersion 2, (c)
469 dispersion 3, (d) dispersion 4 and (e) dispersion 5

470 **Figure 3:** Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of roxithromycin released after the 6 h membrane release
471 studies for the 15 niosomes

472 **Figure 4:** Average concentration ($\mu\text{g}/\text{ml}$) of roxithromycin in the SCE after tape stripping for the
473 15 niosomes

474 **Figure 5:** Average concentration ($\mu\text{g}/\text{ml}$) of roxithromycin in the ED after tape stripping for the
475 15 niosomes

476



478
 479 **Figure 1:** XRPD diffraction patterns overlay obtained for (a) **RM**, (b) cholesterol, (c) Span® 40,
 480 (d) film **RM1**, (e) film **RM2**, (f) film **RM3**, (g) film **RM4** and (h) film **RM5**

481

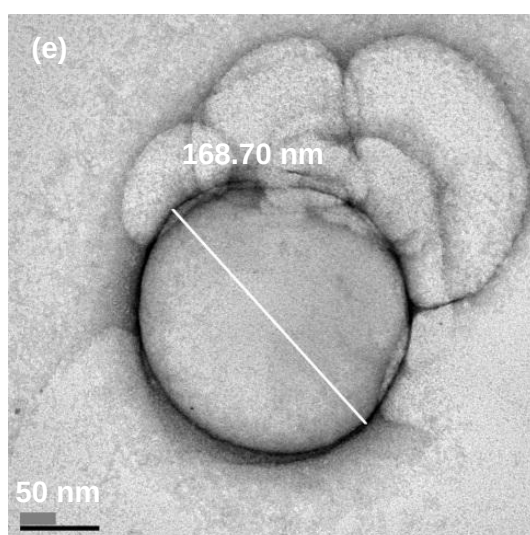
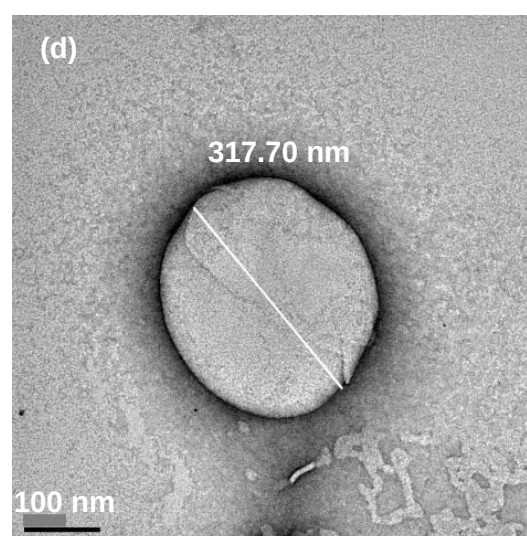
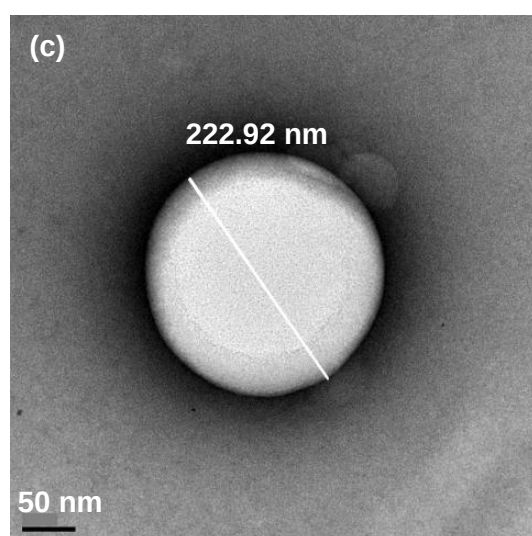
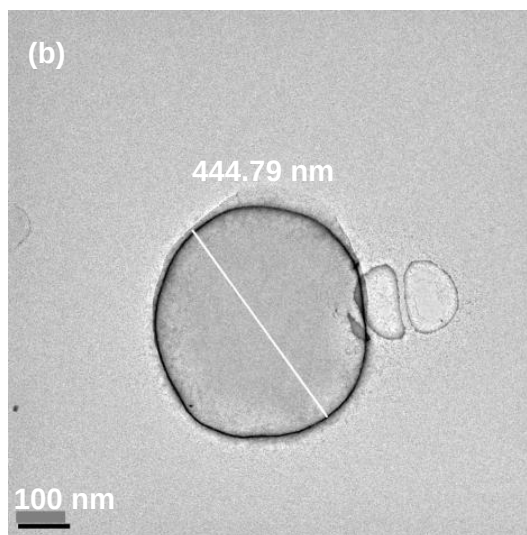
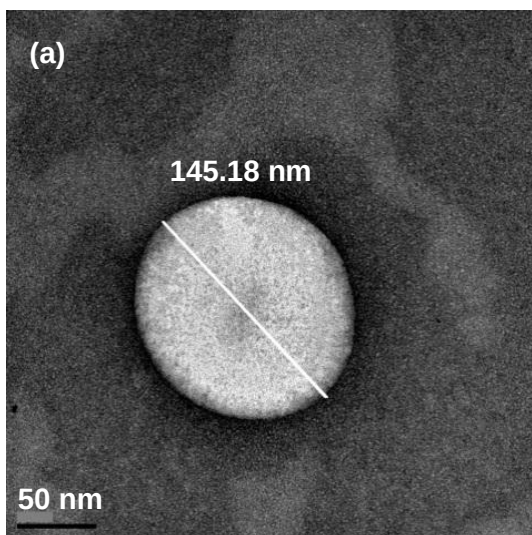


Figure 2: Appearance of vesicles viewed using TEM: (a) dispersion 1, (b) dispersion 2, (c) dispersion 3, (d) dispersion 4 and (e) dispersion 5

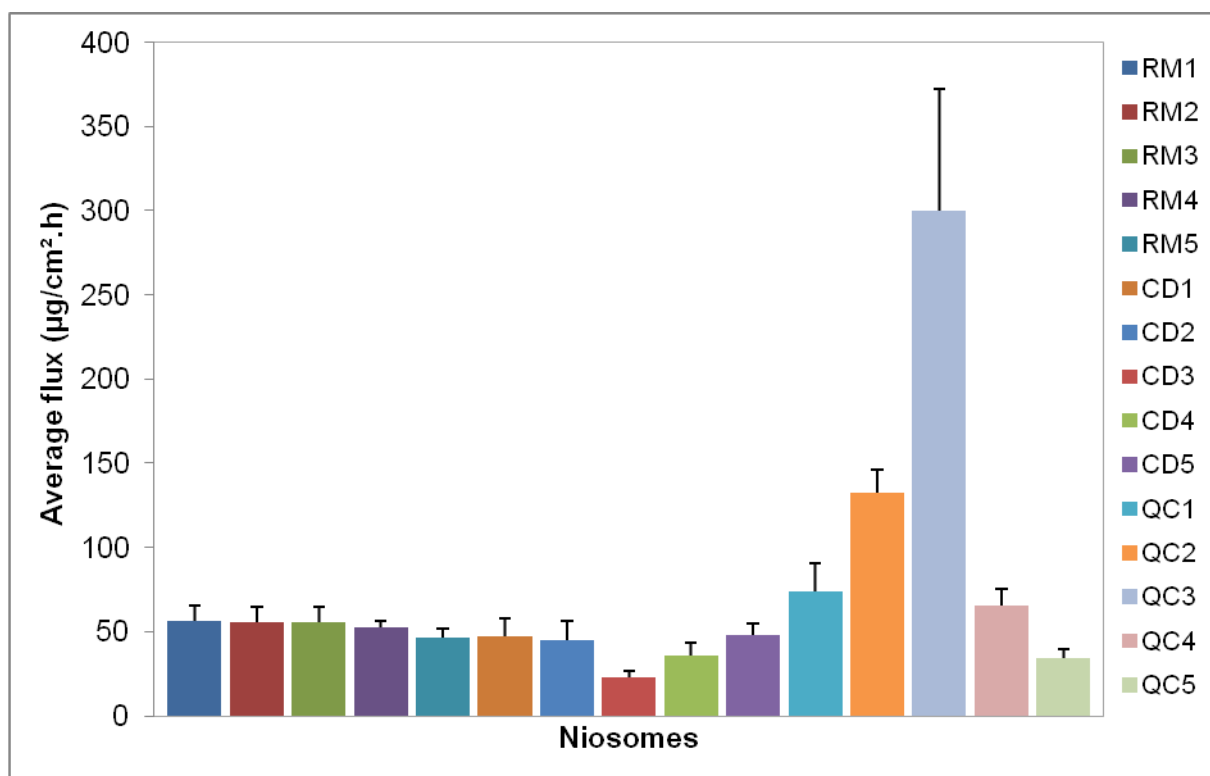


Figure 3: Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of roxithromycin released after the 6 h membrane release studies for the 15 niosomes

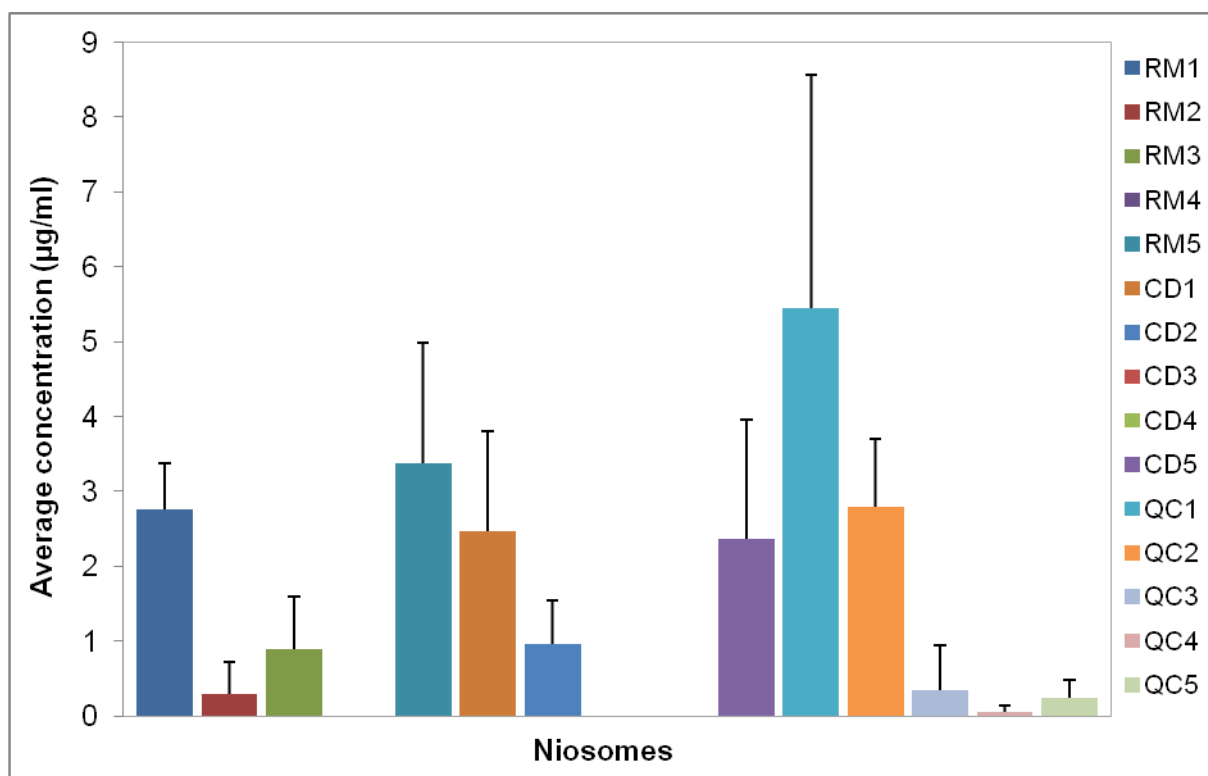


Figure 4: Average concentration (µg/ml) of roxithromycin in the SCE after tape stripping for the 15 niosomes

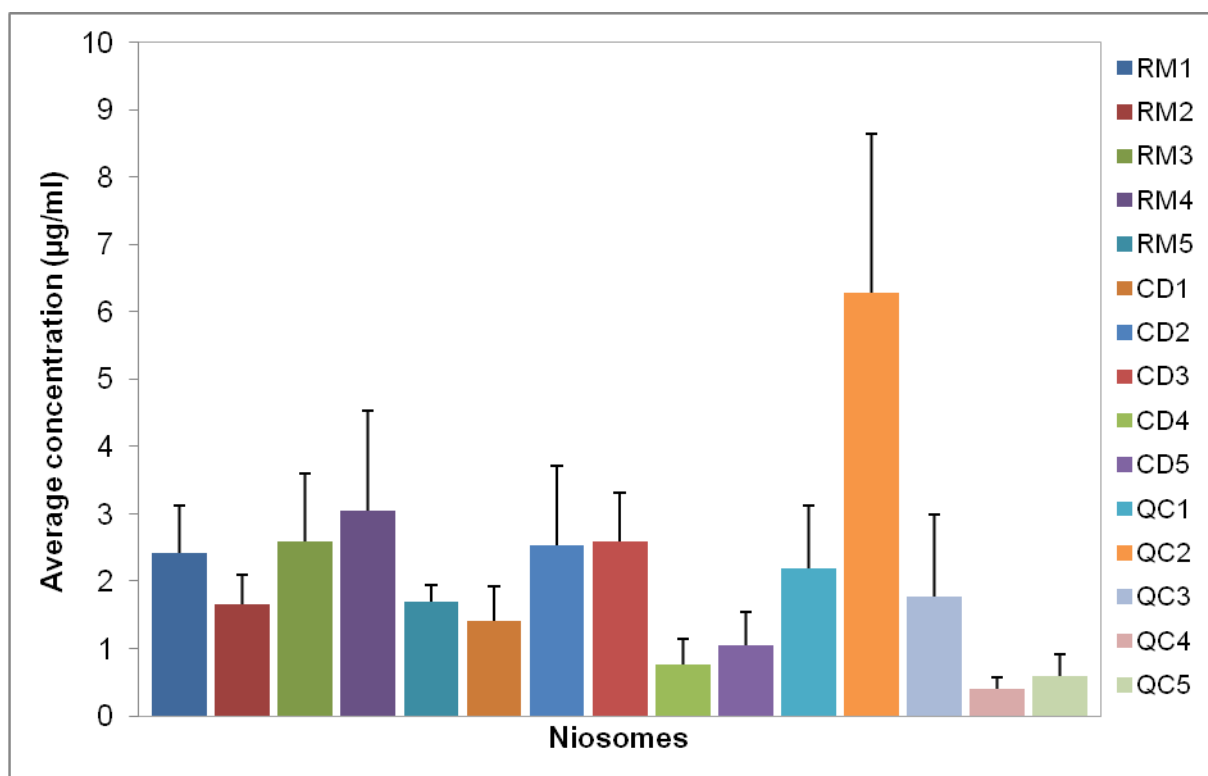


Figure 5: Average concentration (µg/ml) of roxithromycin in the ED after tape stripping for the 15 niosomes

Chapter 4

Conclusion and future recommendations

The stratum corneum, with its unique structure, acts as the core rate-limiting barrier regarding permeation of a drug into the skin (Foldvari, 2000:418). For topical drug delivery to be achieved, certain physicochemical properties are required to help overcome the challenge of drug transport across the skin (Weiss, 2011:471). Roxithromycin is an ideal candidate for topical delivery except for the poor solubility of the API (Aucamp *et al.*, 2013:26) therefore, amorphous forms were investigated, as previous studies showed increased solubility (Aucamp *et al.*, 2013; Csongradi, 2015; Milne *et al.*, 2016). Roxithromycin is also a large molecule, resulting in it being formulated into niosome vesicle systems, since niosomes enhance skin penetration (Kumar & Rajeshwarrao, 2011:214). Roxithromycin could be formulated as a possible topical treatment for acne, as it could reduce the population of *P. acnes* that cause acne, and have a mild anti-inflammatory effect (Krautheim & Gollnick, 2004:401).

The aim of this study was to determine which excipient(s) in the niosome formulas stabilised the amorphous forms of the API and prevented them from converting back to the more stable crystalline form. With the purpose of investigating this occurrence, five niosome dispersions with different ratios of cholesterol and Span[®] 40 were prepared, encapsulating the three solid-state forms of the API. This resulted in the preparation of 15 samples in total (see Table 4.1). The second aim was to investigate which of these 15 niosomes would deliver the highest concentration of the API topically.

Table 4.1: Ratios of excipient concentrations used to prepare the different niosomes containing the **RM**, the **QC** amorphous form and the **CD** amorphous form

Film	Ratio of excipients		Allocated reference numbers
	Cholesterol (%w/w)	Span [®] 40 (%w/w)	
1	0.5	1.0	RM1, QC1 and CD1
2	1.0	1.0	RM2, QC2 and CD2
3	2.0	1.0	RM3, QC3 and CD3
4	2.0	1.5	RM4, QC4 and CD4
5	2.0	2.5	RM5, QC5 and CD5

To achieve the abovementioned aims, the objectives of this study were to:

- Validate the HPLC analytical method to determine the concentration of roxithromycin in each vesicle system.
- Prepare the amorphous **QC** and **CD** forms of roxithromycin.

- Characterise the solid-state forms of roxithromycin using XRPD, DSC and FTIR.
- Prepare the 15 lipid films, which are precursors to niosomes, and establish the crystallisation of the amorphous forms by means of XRPD, DSC and FTIR.
- Prepare the niosome vesicle systems containing the three solid-state forms of the API in the five different ratios of the excipients.
- Characterise the niosomes with and without the APIs in terms of morphology, droplet size and distribution, zeta-potential, pH and EE%.
- Determine the release of roxithromycin from the niosomes through membrane release studies.
- Determine the topical and/or transdermal delivery of roxithromycin by performing Franz cell diffusion studies followed by tape stripping.

An HPLC method was adapted from a previously published method for the quantification of roxithromycin (Aucamp *et al.*, 2016). The method was validated with the assistance and expertise of Prof JL du Preez, in a controlled laboratory environment at the Analytical Technology Laboratory (ATL) of the North-West University (NWU), Potchefstroom Campus, South Africa. This analysis method was proved sensitive, accurate, repeatable and reliable for the quantification of roxithromycin. It was used to determine the concentration of roxithromycin successfully throughout this study in all the prepared experimental samples.

The amorphous forms were prepared using well-known methods including quench cooling of the melt and crystallisation of the **RM** from chloroform. The preparation resulted in two stable amorphous forms, as a glassy **QC** form and a **CD** powder, with a rough appearance.

The three solid-state forms of roxithromycin comprising of **RM**, and the two amorphous forms, **QC** and **CD**, were characterised by means of XRPD, DSC, FTIR and the purity of the amorphous forms were tested through HPLC. Literature states that crystalline solids display clear diffraction peaks on XRPD diffractograms, while amorphous forms have the appearance of a “halo” pattern (Bates *et al.*, 2006:2333). The diffraction patterns presented with these findings, as the **RM** had distinct diffraction peaks and the amorphous forms had the characteristic diffuse pattern indicating the amorphous habit. Further characterisation was performed using thermal analysis by way of DSC, which provides information about phase transitions (Zhang *et al.*, 2004:377). The identification of a T_g is an indication that a solid is amorphous in nature (Bates *et al.*, 2006:2333). The results showed that **RM** had a clear melting point, whereas the amorphous forms displayed only a step change in heat flow known as the T_g . An additional method used to identify the degree of crystallinity was FTIR. IR-spectrums of amorphous solids differ from those of crystalline solids as broader peaks are observed (Einfalt

et al., 2013:322). HPLC analysis was employed to determine the purity of the prepared amorphous forms, which were found to be of high standard correlating with the purchased **RM**.

The study then further progressed towards the investigation of the physical stability of the amorphous forms during the preparation of the lipid films, which are precursors to niosomes. The 15 thin film samples were prepared, where after the degree of crystallinity was confirmed once again by XRPD, DSC and FTIR. Hot stage microscopy (HSM) photos of the thin films were also recorded to provide complimentary information about the phase transition and the temperatures at which it occurred. From the results obtained for the **RM**, it was observed that the preparation of the crystalline form into a lipid film rendered it into an amorphous habit, a fact corroborated by the XRPD patterns showing a diffuse diffraction pattern. The DSC thermograms displayed no clear melting endotherm for roxithromycin in the melting region of $\cong 120^{\circ}\text{C}$, and the FTIR spectra showed peak broadening in the area of $4000 - 3200\text{ cm}^{-1}$. These results were obtained for all five thin films containing **RM** form of roxithromycin. The two amorphous forms revealed the ability to stay amorphous during the preparation of the lipid films and not to convert back to the more stable crystalline form.

The niosome vesicle systems were prepared using an adapted version of the thin film/hand shaking method. The three solid-state forms were encapsulated into niosome vesicles in the five different ratios of the excipients, which resulted in 15 niosome vesicle systems.

The 5 niosome dispersions containing no API were firstly characterised to see whether the vesicles would be able to form in the different ratios. Light microscopy and transmission electron microscopy (TEM) were performed to give a visual representation of the vesicles that formed and the droplet size and size distribution were measured to determine the sizes of the vesicles. It was observed that all the ratios formed vesicles therefore none could be ruled out. The niosomes were then prepared with the API and characterised in terms of light microscopy, zeta-potential, droplet size and distribution, pH and EE%. The zeta-potential of all the samples were highly negative, which would improve permeation of the drug into the skin (Duangjit *et al.*, 2011:6). All the samples displayed sizes that were well within the desired ranges, because all were smaller than $3\text{ }\mu\text{m}$ and would therefore be able to permeate through the stratum corneum into the skin (Allec *et al.*, 1997:119; Barry, 2001:101; Bolzinger *et al.*, 2012:163). The pH values of the samples measured ranged between 7.93 and 8.61 and were within the ideal pH for topical drug delivery, which ranges from 5 to 9 (Sharma *et al.*, 2011:75). All the niosomes displayed had good EE%, entrapping more than 97% of the API, with the dispersion of **QC3** showing the highest entrapment with $98.66 \pm 0.674\%$. Overall, the characterisation of the niosomes showed optimal results for topical drug delivery.

Following the characterisation, membrane release studies were performed to determine if the API was being released from the niosome vesicle system. The results showed that

roxithromycin was released from all fifteen the niosomes. Therefore, failure of release of the API from the vesicle could be eliminated as a possible reason if no topical delivery was achieved. The dispersion containing **QC3** had the highest average flux ($299.81 \pm 72.626 \mu\text{g}/\text{cm}^2.\text{h}$) and the highest average percentage ($4.098 \pm 0.919\%$) of roxithromycin released over the period of 6 h. **QC3** had the highest EE% and could be the reason why it displayed the highest average flux as it had the most API entrapped inside the vesicle. The dispersions that had the second and third best values were **QC2** and **QC1**, respectively. The conclusion that can be made from the membrane release studies is that dispersions containing the amorphous **QC** form of roxithromycin illustrated the best API release from niosome vesicle systems.

After the completion of the membrane release studies, skin diffusion studies followed by tape stripping were performed to determine if the API was delivered topically and/or transdermally. Roxithromycin concentrations were found in the receptor phase of only four of the dispersions. This indicated that minimal transdermal delivery was achieved however, as the aim of this study was topical delivery, finding only a few dispersions that delivered API transdermally was favoured. The four dispersions were **RM1**, **RM2**, **CD2** and **QC2** and as three of these contained an equal amount of cholesterol and Span[®] 40, it could be said that these dispersions had sufficient lipophilic and hydrophilic properties to permeate into and through the lipophilic stratum corneum and the hydrophilic epidermal and dermal layers to reach the systemic circulation (Perrie *et al.*, 2012:393).

Concentrations of roxithromycin were quantified in the stratum corneum-epidermis (SCE) and epidermis-dermis (ED), with six dispersions obtaining results above the limit of detection (LOD) and lower limit of quantification (LLOQ) in both these areas. The dispersion which displayed the highest average concentration in the SCE was **QC1** ($5.442 \pm 3.125 \mu\text{g}/\text{ml}$), followed by **RM5**, **QC2**, **RM1**, **CD1** and **CD5**. It was observed that dispersions residing in SCE contained more Span[®] 40 than cholesterol (dispersions 1 and 5) and could likely be ascribed to Span[®] 40 having an HLB value of 6.7, making it more oil soluble, thus staying in the lipophilic region of the SCE (CRODA, 2015:45). In the ED, the dispersion displaying the highest concentration of the API was **QC2** ($6.277 \pm 2.367 \mu\text{g}/\text{ml}$), followed by **RM4**, **RM3**, **CD3**, **CD2** and **RM1**. Dispersions containing more cholesterol than Span[®] 40 were noticed to be more favoured in the ED. This could be attributed to cholesterol being an amphiphilic molecule and in a hydrophilic environment, it possibly favours the hydrophilic properties more than the lipophilic properties, and therefore resides in the aqueous region of the ED. Dispersions containing an equal ratio of cholesterol to Span[®] 40 were also found in the ED, where it could be deduced that when the ratio of cholesterol to Span[®] 40 is equal, the solid-state forms have a greater influence on penetration than the ratio of the excipients. From the results obtained during the skin diffusion

studies, the dispersions displaying the highest concentration of roxithromycin in the skin were once again those of the amorphous **QC** samples, followed by **RM** and lastly **CD**.

The hypothesis made in Chapter 1, stating that the amorphous forms stayed amorphous in the bilayer of the niosomes, was therefore proved to be true. It was also found that the preparation of the lipid films led to **RM** being rendered into an amorphous habit. It could be figured that because the preparation of the lipid films renders the **RM** form of roxithromycin into an amorphous habit, it does not matter with which solid-state you start with. However, from the results it can clearly be seen that although the raw material was rendered into an amorphous habit, the dispersions containing the **QC** amorphous forms still displayed the best topical results. This can be ascribed to the fact that the amorphous forms could lead to better entrapment within the vesicles due to the inherently higher free energy possessed by these solid-state forms.

The aims of this study were reached as it was established that during the preparation of the lipid films, the amorphous forms of roxithromycin did not crystallise into the more stable crystalline form of roxithromycin. It was found that topical delivery of roxithromycin was successful, as quantifiable concentrations of the API were found in the target area of the ED. Niosomes containing the **QC** amorphous form of roxithromycin had the highest concentration of the API in the skin and it was observed that certain excipients could be used to reach specific target areas.

Future recommendations include:

- Sonicate the niosomes for a longer period to achieve smaller droplet sizes.
- Incorporate a higher concentration of roxithromycin into the niosomes to improve skin permeation results.
- Formulation of vesicles encapsulating roxithromycin into optimised topical preparations, for example gels, creams, nano-emulsions and nano-emulgels.

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Appendix A:

Method validation for high-performance liquid chromatographic analysis of roxithromycin

A.1 Introduction

Validation means to assess validity or to demonstrate effectiveness. Method validation can therefore be described as the process that provides documented evidence, which confirms, to a high degree of certainty, that the product and the equipment used will meet the particular requirements for its intended purpose (Lavanya *et al.*, 2013:1280). This method was developed and validated primarily to determine the concentration of roxithromycin encapsulated into niosome vesicle systems during membrane release and skin diffusion studies.

Table A.1: A summary of the results obtained from the validation tests of roxithromycin

Test	Results
Specificity	Complies
Range	1.70 - 526.00 µg/ml
Linearity	$R^2 = 0.9999$
Accuracy	%Recovery = 98.07%
Precision	%RSD* = 0.90%

*Relative standard deviation

A.2 Chromatographic conditions

The analytical method was adapted from a previously published HPLC method for the identification and quantification of roxithromycin (Aucamp *et al.*, 2016). The adapted method was validated with the assistance of Prof JL du Preez from the NWU, Potchefstroom Campus. All analyses pertaining to the validation of this method were performed in a controlled laboratory environment, at a room temperature of 25 °C (ambient temperature). This method validation was done in conjunction with Me. M Swart, as both our studies included roxithromycin as an API therefore a single set of data was recorded, but processed separately by each student. The parameters used during method development were:

Analytical instrument: An Agilent® 1200 series HPLC system (Agilent Technologies, United States of America) was used for concentration analysis. A Shimadzu (Kyoto, Japan) UFLC (LC-20AD) chromatographic system was also used. The system consisted of a SIL-20AC auto-sampler fitted with a sample temperature controller, a UV/VIS Photodiode Array detector (SPD-M20A) and a LC-20AD solvent delivery module. Data analysis

was conducted using LabSolutions, LCsolution, Release 1.21 SP1 (Shimadzu, Kyoto, Japan).

Column: A Venusil XBP C₁₈ (2) 150 x 4.6 mm column with a 5 µm particle size was used.

Mobile phase: The mobile phase consisted of 0.01 M ammonium di-hydrogen phosphate (NH₄H₂PO₄) buffer solution with the pH adjusted to 7.0 with diluted ammonia (7% w/v) solution. The buffer was mixed with methanol in the ratio 15:85. The mobile phase was filtered through a 0.22 µm nylon membrane filter (Membrane Solutions®, Kent, USA) before use.

Flow rate: 1.0 ml/min

Injection volume: 50 µl

Detection wavelength: UV at 205 nm

Retention time: ± 5.0 min

Stop time: 10.0 min

Solvent: The solvent used in this study was methanol.

A.3 Standard preparation

Standard solutions were prepared by weighing 20 mg of roxithromycin reference standard powder into a 100 ml volumetric flask, methanol was added to dissolve the powder and the flask was placed in an ultrasonic bath for 1 min to ensure all the roxithromycin had dissolved. The volumetric flask was made up to volume with methanol, after which a stopper was fitted, and then shaken by hand. Subsequently the solution was filtered into an HPLC vial and this solution was then injected using different injection volumes. Each injection volume was injected in duplicate.

A.4 Validation parameters

The parameters used to validate this analytical method included linearity, limit of detection (LOD) and lower limit of quantification (LLOQ), accuracy, precision (inter- and intra-day variation), ruggedness (repeatability and stability) and specificity.

A.4.1 Linearity

The linearity of an analytical method is the ability to acquire results, which are directly proportional to the amount (concentration) of API present in the sample within the range of a standard curve (ICH, 2005:5). Visual inspection of a plot of signals as a function of the analyte

concentration can be used to determine linearity and if a linear relationship exists, the results should be evaluated through statistical methods by means of calculating the regression coefficient (Lavanya *et al.*, 2013:1283). A correlation coefficient (R^2) close to one indicates a method has high linearity (Araujo, 2009:2229). According to Shabir (2006:7), the acceptance criteria for linearity of an analytical method requires a regression coefficient yield of $R^2 \geq 0.997$. The linear equation adapted from Araujo (2009:2229) can be used to interpret the data yielded by the regression:

$$y = mx + c$$

Equation A.1

Where:

y: peak area

m: slope

x: concentration of roxithromycin ($\mu\text{g/ml}$)

c: y-intercept

Table A.2: Linearity results of roxithromycin standard solution

Standard ($\mu\text{g/ml}$)	Peak area		
	1	2	Average
1.05	14.81	13.51	14.16
2.63	38.89	37.63	38.26
5.26	76.52	69.18	72.85
10.52	146.40	150.04	148.22
21.04	336.70	304.80	320.75
31.56	487.20	443.40	465.30
42.08	598.90	624.30	611.60
52.60	765.50	782.40	773.95
105.20	1769.60	1734.30	1751.95
210.40	3547.40	3378.90	3463.15
315.60	5271.40	5031.70	5151.55
420.80	7009.40	6880.50	6944.95
526.00	8641.20	8598.20	8619.70

From Table A.2 and Figure A.1, it can be seen the R^2 obtained for roxithromycin for this analytical method was 0.9999, therefore the method proves linear over the concentration range 1.70 – 526.00 $\mu\text{g/ml}$ and should be suitable for single point calibration.

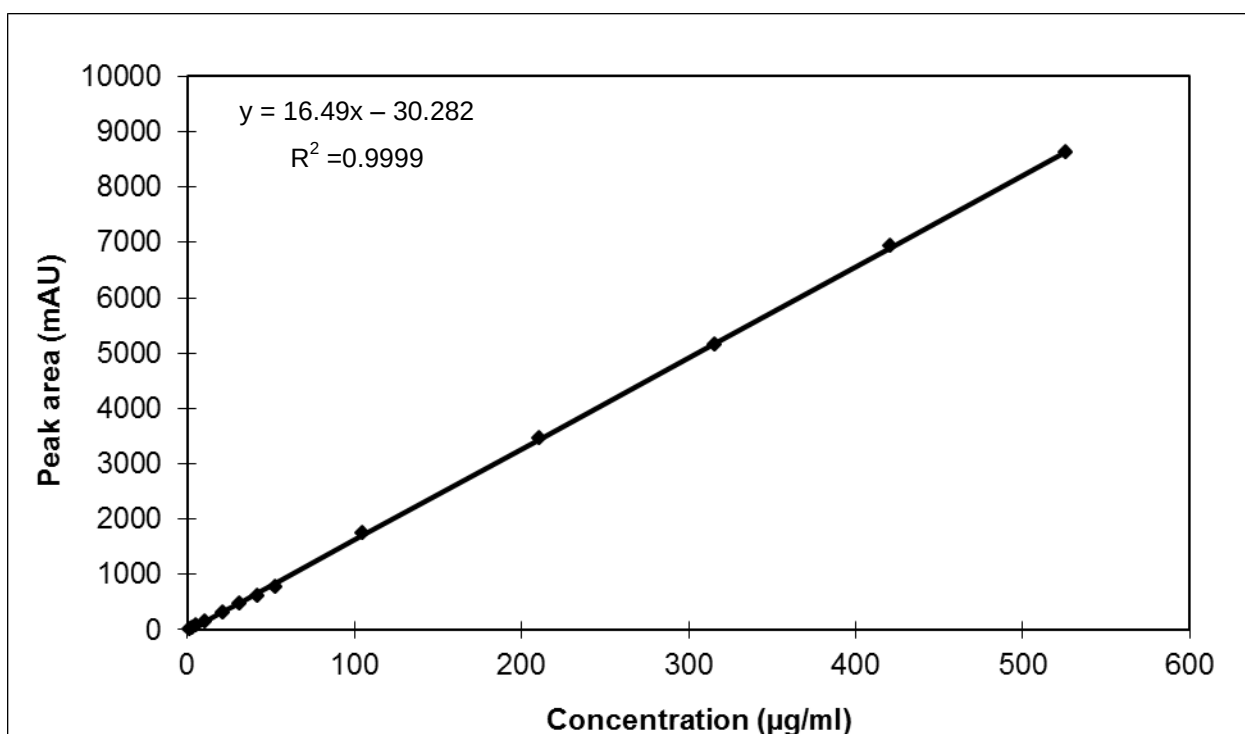


Figure A.1: Linear regression curve of roxithromycin

A.4.2 Limit of detection and lower limit of quantification

The LOD of an analytical method is the lowest amount of analyte identified in the sample, but cannot be quantified by analytical procedures as a numerical value (Araujo, 2009:2231). The LLOQ of an analytical method is the lowest amount of analyte identified in the sample, which can be quantitatively measured with sufficient accuracy and precision (FDA, 2001:20).

Six standards with low concentrations were prepared and injected six consecutive times. For the purpose of this study, the accepted percentage relative standard deviation (%RSD) for LOD should not exceed 20% and the LLOQ 15% (FDA, 2001:6). The standard deviation (SD), as well as the %RSD, is given in Table A.3.

Table A.3: Results for the LOD and LLOQ of roxithromycin

STD (µg/ml)	Peak area							SD	%RSD
	1	2	3	4	5	6	Ave		
0.851	11.30	8.30	13.20	8.90	14.50	12.10	11.38	2.21	19.38
1.703	22.00	20.00	24.90	24.90	32.20	24.50	24.75	3.78	15.27
2.554	44.20	45.90	35.00	39.20	39.40	36.00	39.95	3.97	9.93
3.405	55.80	49.00	55.20	49.30	46.50	45.60	50.23	3.95	7.86
6.810	111.60	106.70	101.60	106.90	144.40	105.60	107.54	3.89	3.62
10.215	156.30	165.40	155.90	155.60	163.30	168.00	160.01	4.98	3.11

Table A.3 illustrates the LOD for roxithromycin was 0.85 µg/ml, with a %RSD of 19.38%, and the LLOQ was 1.70 µg/ml with an RSD of 15.27%. Although the %RSD of the LLOQ is slightly higher than 15%, it still falls within the acceptable range, hence both LOD and LLOQ proved to be satisfactory.

A.4.3 Accuracy

Accuracy indicates the closeness between experimental results obtained and the accepted reference value, often referred to as trueness (ICH, 2005:4). A minimum of nine standards over three concentrations covering the specific range should be used to determine accuracy and expressed as percentage recovery (Lavanya *et al.*, 2013:1283). The mean recovery should be between 98.00 – 102.00% (APVMA, 2004:5). In Table A.4, it is clear the mean recovery for roxithromycin is 98.07%, which indicates the analytical method was accurate.

Table A.4: Results for accuracy of roxithromycin

Concentration (µg/ml)	Peak area			Recovery	
	1	2	Average	µg/ml	%
20.20	296.00	313.40	304.70	19.56	96.83
21.50	312.50	301.60	307.05	19.72	91.68
21.70	320.00	312.00	316.55	20.33	93.71
50.50	761.40	772.50	766.95	50.91	100.80
53.80	797.20	784.40	790.80	52.52	97.67
54.20	791.90	796.80	794.35	52.76	97.31
202.00	3003.20	2997.60	3000.40	202.36	100.18
215.10	3144.40	3130.70	3137.55	211.66	104.78
216.90	3177.20	3175.50	3176.35	214.29	99.62
				Mean	98.07
				SD	3.67
				%RSD	3.75

A.4.4 Precision

Precision of an analytical procedure describes how closely a set of measurements are, obtained from applying the method repeatedly to the same sample under the prescribed conditions (ICH, 2005:4). Precision can be divided into intra-day precision (repeatability), inter-day precision (reproducibility) and intermediate precision. During this study, only the repeatability and reproducibility were determined.

A.4.4.1 Intra-day precision

The intra-day variation refers to the repeatability of an analytical method during a specific day under the same set of conditions. Three standards of three different roxithromycin concentrations, nine standards in total, were prepared and injected in duplicate. According to Snyder *et al.* (1997:691), the acceptance criteria for intra-day precision is a %RSD of less than 2%.

Table A.5: Results for intra-day precision of roxithromycin

Concentration (µg/ml)	Peak area			Recovery	
	1	2	Average	µg/ml	%
26.58	373.10	386.10	379.60	26.03	97.92
27.78	412.30	421.80	417.05	28.60	102.93
28.55	421.30	423.40	422.35	28.96	101.43
53.15	779.70	762.40	771.05	52.87	99.47
55.55	815.70	821.20	818.45	56.12	101.02
57.10	859.00	846.30	852.65	58.46	102.39
106.30	1567.20	1550.20	1558.70	106.87	100.54
111.10	1610.90	1617.70	1614.30	110.69	99.63
114.20	1699.20	1674.30	1686.75	115.65	101.27
				Mean	100.73
				SD	1.46
				%RSD	1.45

Intra-day precision was satisfactory with a %RSD of 1.45%, as shown in Table A.5.

A.4.4.2 Inter-day precision

According to the ICH (2005:5), inter-day precision (reproducibility) indicates the variations that can occur in the same laboratory when repeating the experiments on the second, third or more days. A standard of 100 µg/ml was prepared, as well as three samples of the same concentration as the standard. The standard and the samples were prepared freshly on three consecutive days and injected in duplicate. The percentage recovery (%) results from the repeatability results were used as “Day 1” (Section A.4.4.1). The acceptance criteria for inter-day precision are a %RSD value of 3% or less (Rafael *et al.*, 2007:100). Table A.6 shows the results for the inter-day precision of the three samples on the three consecutive days.

Table A.6: Results for inter-day precision of roxithromycin

	Day 1 (%)	Day 2 (%)	Day 3 (%)	Mean between days
	99.47	99.80	101.10	
	101.02	100.56	102.35	
	102.39	100.18	102.28	
Mean (%)	100.96	100.17	101.91	101.46
SD	1.19	0.32	0.57	0.91
%RSD	1.18	0.32	0.56	0.90

The inter-day precision, as shown in Table A.6, has a %RSD of 0.90% therefore reproducibility was within acceptable limits.

A.4.5 Ruggedness

Ruggedness can be described as the extent of reproducibility of analytical results obtained by analysing the same samples under various conditions (Lavanya *et al.*, 2013:1285) and consists of system repeatability and stability.

A.4.5.1 System repeatability

Repeatability determines the preciseness of the method under the same conditions over a short period of time (ICH, 2005:5). Repeatability was tested by injecting a standard six successive times and measuring the peak areas and retention times, which should have a %RSD of 2% or less for a system to be repeatable (Snyder *et al.*, 1997:691). System performance proved well within the acceptable range, with %RSD values of 1.95% for peak area and 0.80% for retention time, as seen in Table A.7.

Table A.7: Results for system repeatability of roxithromycin

Injection number	Peak area	Retention time (min)
1	1538.60	5.184
2	1538.20	5.184
3	1595.70	5.193
4	1536.40	5.202
5	1595.70	5.191
6	1518.60	5.081
Mean (%)	1553.87	5.173
SD	30.35	0.04
%RSD	1.95	0.80

A.4.5.2 Stability

Chemical stability of an analyte is tested in a particular matrix for a predetermined period under specific conditions (FDA, 2001:21). It is known that samples should not be used after the time it takes for them to degrade by 2%. The standard was injected hourly over a period of 24 h, where the peak area for every hour was compared to the peak area of 0 h. Table A.8 illustrates that roxithromycin is stable over a period of 24 h with a %RSD of 1.57% and therefore should remain stable during diffusion studies, which are performed over a 12 h time period.

Table A.8: Results for stability of roxithromycin

Time (h)	Peak area	%Remaining
0	1564.70	100.00
1	1636.00	104.56
2	1583.90	101.23
3	1583.80	101.22
4	1574.40	100.62
5	1568.50	100.24
6	1583.60	101.21
7	1600.60	102.29
8	1595.80	101.99
9	1588.10	101.50
10	1585.40	101.32
11	1583.30	101.19
12	1547.00	98.87
13	1573.60	100.57
14	1536.30	98.18
15	1593.70	101.85
16	1547.60	98.91
17	1575.00	100.66
18	1568.10	100.21
19	1536.30	98.18
20	1528.60	97.69
21	1569.70	100.32
22	1554.80	99.37
23	1557.80	99.56
24	1526.90	97.58
Mean (%)	1570.50	100.37
SD	24.63	1.57
%RSD	1.57	1.57

A.4.6 Specificity

Specificity is used to determine the analyte in the presence of other expected components and to ensure no interference from these components occurs (ICH, 2005:4). Specificity was tested by preparing a standard and weighing approximately 50 mg of roxithromycin in a 100 ml volumetric flask then making it up to volume with methanol. The niosome formulation consists of roxithromycin, cholesterol and Span[®] 40, therefore Span[®] 40 and cholesterol were separately prepared and dissolved in methanol. A mixture of cholesterol and roxithromycin, Span[®] 40 and roxithromycin and a mixture with all three components were also prepared. A pipette was used to transfer 2 ml of the standard solution into four different test tubes, after which 500 µl of water (H₂O), sodium hydroxide (NaOH) and 100 µl of hydrogen peroxide (H₂O₂) or hydrochloric acid (HCl) were added to the four tubes, respectively. These four components were added to a standard solution to see what degradation products formed and whether this interfered with the roxithromycin peak. All the samples were injected in duplicate for 10.0 min. For specificity to be acceptable, none of the peaks should interfere with the roxithromycin peak.

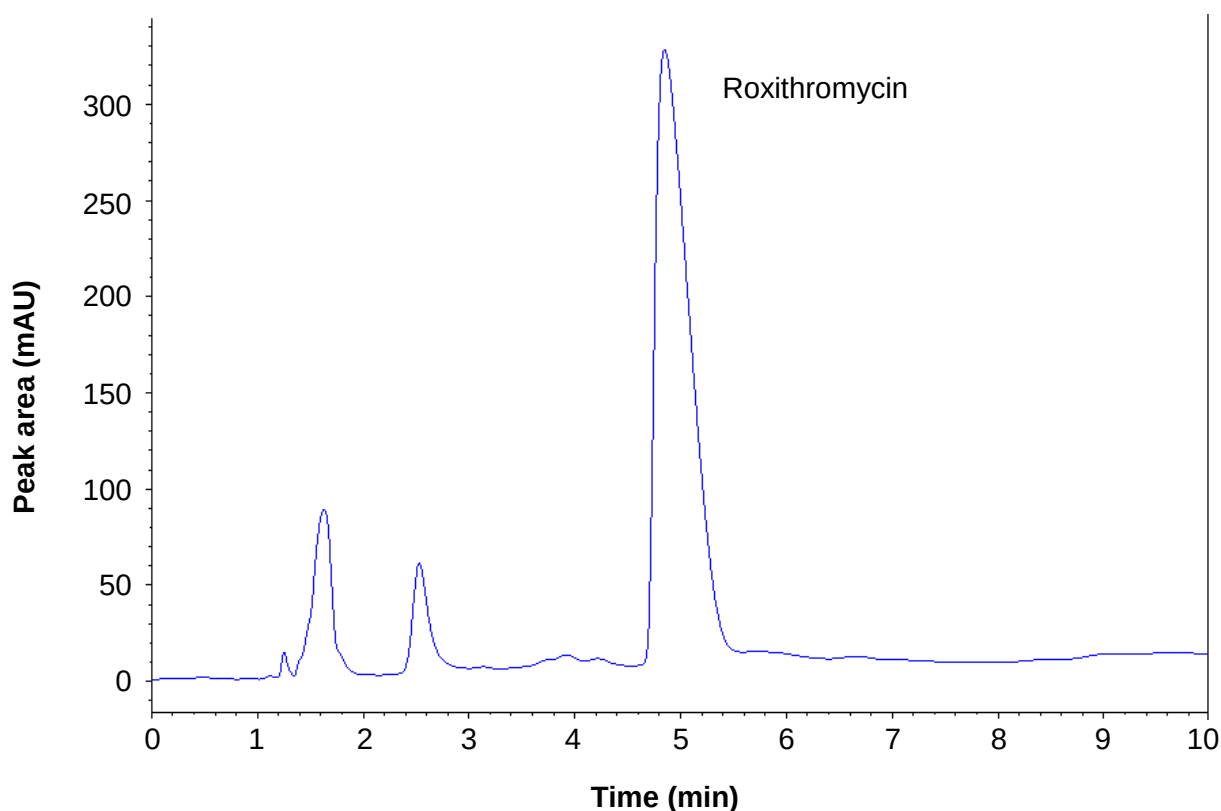


Figure A.2: Roxithromycin standard obtained during specificity testing

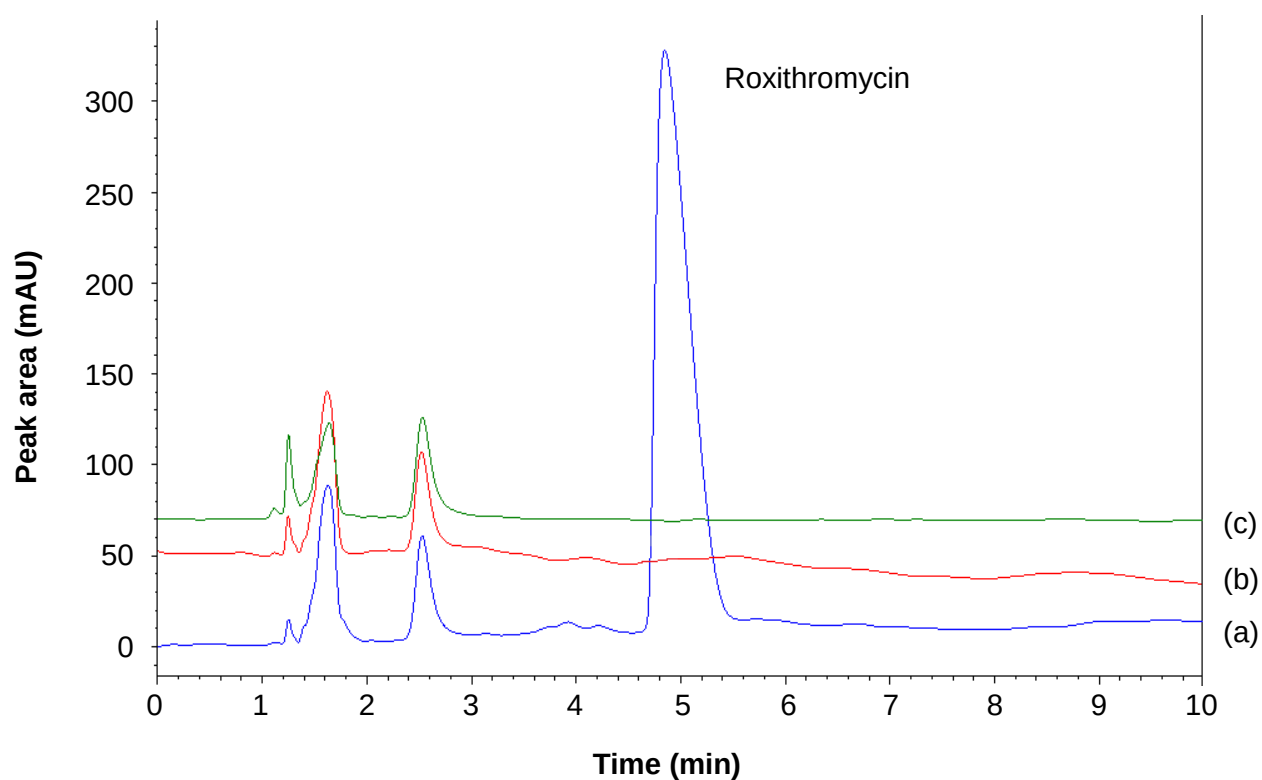


Figure A.3: Specificity results for (a) roxithromycin, (b) cholesterol and (c) Span® 40

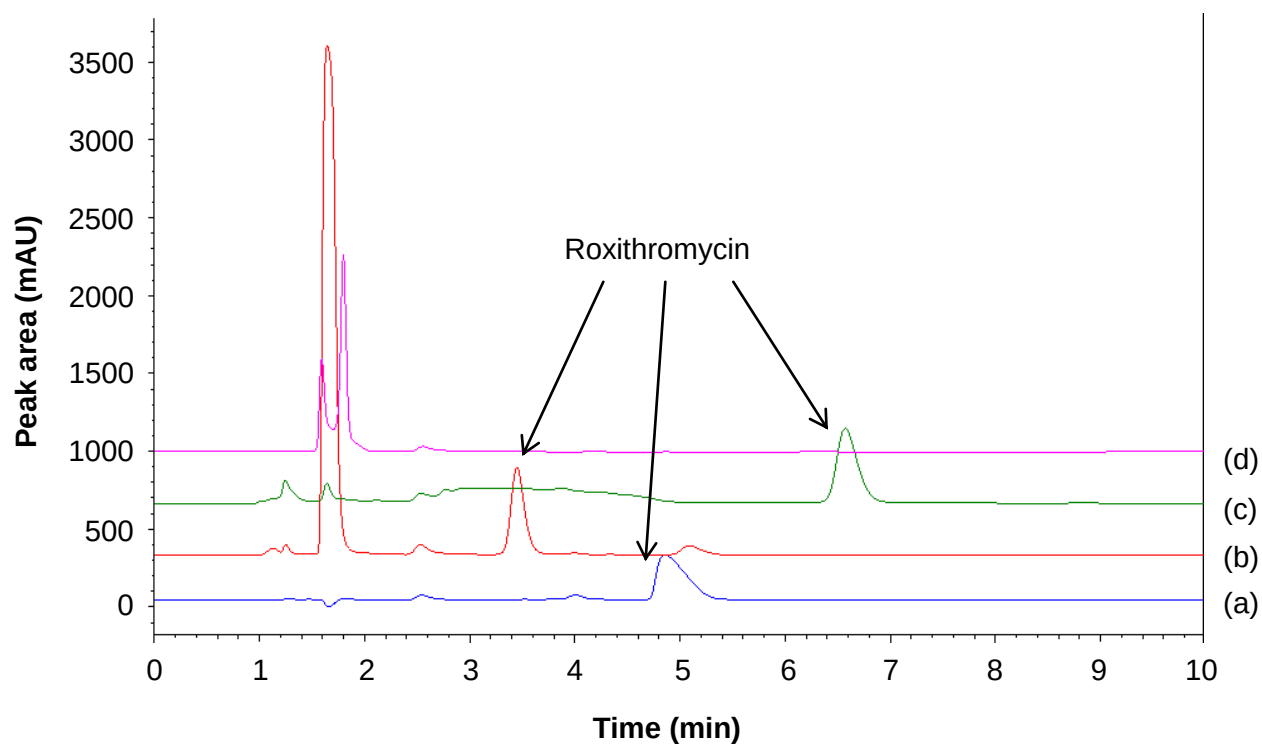


Figure A.4: Specificity results for the specificity of roxithromycin in relation to (a) H₂O, (b) H₂O₂, (c) NaOH and (d) HCl

Figure A.3 shows the chromatogram for the three components the niosome formulation consists of, namely roxithromycin, cholesterol and Span[®] 40. It is clear no peaks interfered with the roxithromycin peak and so the method was specific.

In Figure A.4, the effect of the four added components on the roxithromycin peak can be seen. Water does not have a big impact, because it is not known to cause chemical degradation of roxithromycin. With the H₂O₂ solution, a peak appears early, but the peak due to roxithromycin is visible, with the NaOH-solution, roxithromycin elutes later and lastly, the HCl-solution showed complete degradation of roxithromycin due to the absence of the API peak. From this it can be concluded that the degradation products would have no impact on any experimental results should they occur and therefore specificity complies.

A.5 Conclusion

The validated HPLC method had acceptable results for linearity, limit of detection (LOD) and lower limit of quantification (LLOQ), accuracy, precision (inter- and intra-day variation), ruggedness (repeatability and stability) and specificity of roxithromycin. This method was found to be valid and suitable for determining the concentration of roxithromycin in the niosome vesicle system, which will be used during the membrane release and skin diffusion studies.

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APVMA **see** Australian Pesticides and Veterinary Medicines Authority.

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Appendix B:

Preparation and physical characterisation of roxithromycin solid-state forms

B.1 Introduction

It is a known fact that different solid-state forms of roxithromycin exist. It has also been reported that different amorphous forms of roxithromycin exist and that these differ in terms of morphology, thermodynamics and kinetics (Milne, 2016). The rationale of this study was to determine if three different solid-state forms of roxithromycin could be successfully included into thin films, which would be precursors to the preparation of drug delivery vesicles such as niosomes, and to determine if the two amorphous forms would remain stable during the thin film formation process, during handling, agitation and exposure to high relative humidity. Roxithromycin raw material, also known as the monohydrate form, was purchased from DB Fine Chemicals (Johannesburg, RSA).

B.2 Preparation of the amorphous forms of roxithromycin

B.2.1 Preparation of the quench cooled amorphous form

The glassy amorphous form was prepared using the well-known quench cooling of the melt method. This method involved melting a thin layer of the crystalline roxithromycin in a glass Petri dish in a laboratory oven (Binder, Germany) at a temperature of up to $\cong 130$ °C. Once all the crystalline roxithromycin reached the molten stage, the sample was removed from the oven and quenched on a cold granite surface. The quench cooling rendered the sample into a glass form (Aucamp *et al.*, 2012:48).

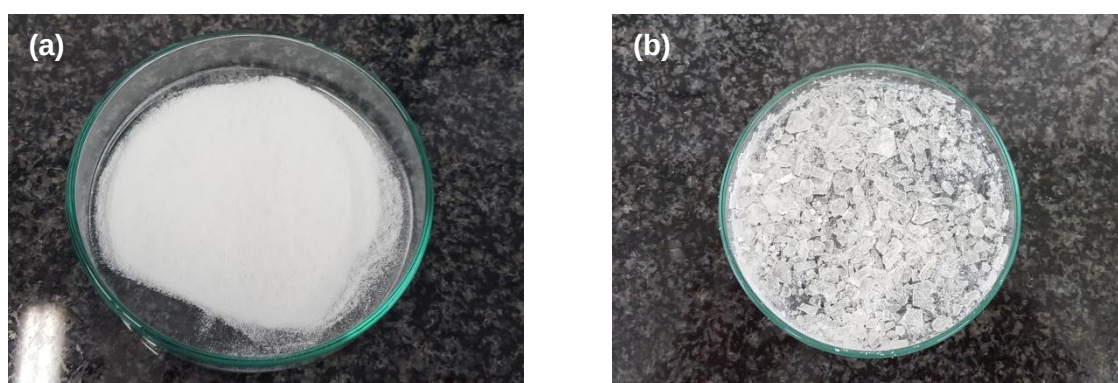


Figure B.1: Preparation of the **QC** amorphous form: (a) crystalline roxithromycin in a Petri dish and (b) glassy amorphous form after crushing the molten product into smaller pieces

B.2.2 Preparation of the chloroform desolvated amorphous form

The method involved recrystallisation of the crystalline roxithromycin from chloroform. Roxithromycin was added to the chloroform while continuously stirring and heating the solution to approximately 60 ± 3 °C. A sufficient amount of roxithromycin was added to the chloroform in order to form a saturated solution. The solution was left to cool to ambient temperature and subsequently covered with Parafilm® (Beemis NA, Neenah, USA). After slow evaporation of the chloroform, a dense mass was formed and the desolvated form was obtained by desolvating the chloroform solvated form in an oven at 60 ± 2 °C, for 24 h (Aucamp *et al.*, 2012:48). Chloroform is not considered as an organic solvent of choice during typical API processing techniques due to its toxic nature (Merck, 2017), however, a previous study found a drying period of 24 h at 60 °C is sufficient so that no residual solvent is left and therefore not harmful to the skin or when absorbed systemically.

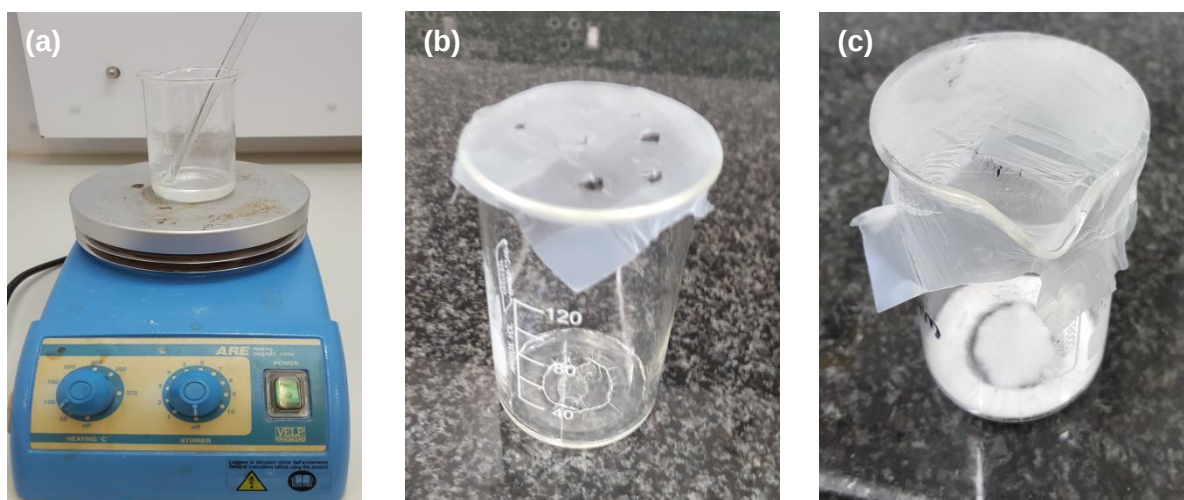


Figure B.2: Preparation of the **CD** amorphous form: (a) saturated roxithromycin solution in chloroform on a hot plate, (b) solution left to cool down and (c) chloroform desolvated amorphous form

B.3 Physical characterisation of roxithromycin and the amorphous forms

Through the physical characterisation of amorphous forms, certain types of information are revealed. This information includes: 1) structure, which includes the molecular packing as well as the degree of crystallinity; 2) changes, such as crystallisation and structural relaxation; 3) thermodynamic properties including enthalpy, entropy and free energy; 4) multi-component systems (Yu, 2001:30).

Several analytical techniques are used to characterise the crystal form of the drug during the different stages of drug development, because pharmaceutical processes can have an impact

on the final crystalline form and therefore change the bioavailability of the product (Vippagunta *et al.*, 2001:5).

Although various literature reports already explain the physical and chemical characterisations of both roxithromycin amorphous forms, these two forms were still compared with data obtained from crystalline roxithromycin, to form a sound basis for this study. These forms were characterised in terms of XRPD, DSC, FTIR and the purity was tested through HPLC analysis.

B.3.1 Methods used during physical characterisation

B.3.1.1 X-ray powder diffraction

According to Chauhan and Chauhan (2014:1-2), XRPD forms the primary means of solid-state characterisation and are a basic requirement to distinguish between crystalline and amorphous solids. XRPD patterns were obtained using a PANalytical Empyrean diffractometer equipped with a PIXcel^{3D} detector (PANalytical, Almelo, Netherlands). The measurement conditions were: target, Cu; voltage, 40 kV; current, 30 mA; divergence slit, 2 mm; anti-scatter slit, 0.6 mm; detector slit, 0.2 mm; monochromator and scanning speed, 2°/min (step size, 0.025°; step time, 1.0 sec). The diffraction pattern obtained from the XRPD analysis is shown in a diffractogram and plots the intensity in counts against the angle of diffraction, degrees two Theta (2θ).

B.3.1.2 Differential scanning calorimetry

In this study, a Shimadzu (Kyoto, Japan) DSC-60 instrument was used to record the DSC thermograms, which shows the difference in heat flow (mW) against temperature (°C). Samples (3 – 5 mg) were accurately weighed and sealed in aluminium crimp cells with unpierced lids. The samples were heated from 25 to 200 °C with a heating rate of 10 °C/min and a nitrogen gas purge of 35 ml/min. The onset temperatures of the thermal events were reported so that reporting will be standard throughout the study. Since amorphous forms, as well as possible amorphous excipients, were tested it was considered more significant to report the onset of glass transition temperatures (T_g), rather than peak or end temperatures of thermal events.

B.3.1.3 Fourier-transform infrared spectroscopy

Irradiation with infrared light triggers molecular vibrations, with certain bonds vibrating faster than others. This reaction can be detected and translated into a visual representation called a spectrum (Derrick *et al.*, 1999:4). Infrared mostly provides information about the presence or absence of certain functional groups and consequently the identification of organic compounds (Stuart, 2004:71). IR-spectra were recorded using a Shimadzu IR Prestige-21 spectrophotometer (Kyoto, Japan) over a range of 400 – 4000 cm^{-1} , potassium bromide (KBr) was used as a background. The diffuse reflectance method was implemented and involved

grinding the sample with KBr in a mortar, with a pestle, and measuring the IR-spectrum in a reflectance cell.

B.3.2 Results and discussion for the physical characterisation

Crystalline solids are characterised by both short-range and long-range ordering of molecules, giving it a well-defined molecular structure. Non-crystalline or amorphous solids do not possess these long-range packing and therefore do not have a distinct molecular structure (Yu; 2001:30). Due to this long-range ordering, crystalline solids show distinct diffraction peak intensities during XRPD analysis, while amorphous solids show the appearance of a “halo” pattern (Bates *et al.*; 2006:2333). The XRPD patterns of amorphous solids can have noise signals, small order bumps or smeared peaks (Chauhan & Chauhan 2014:4).

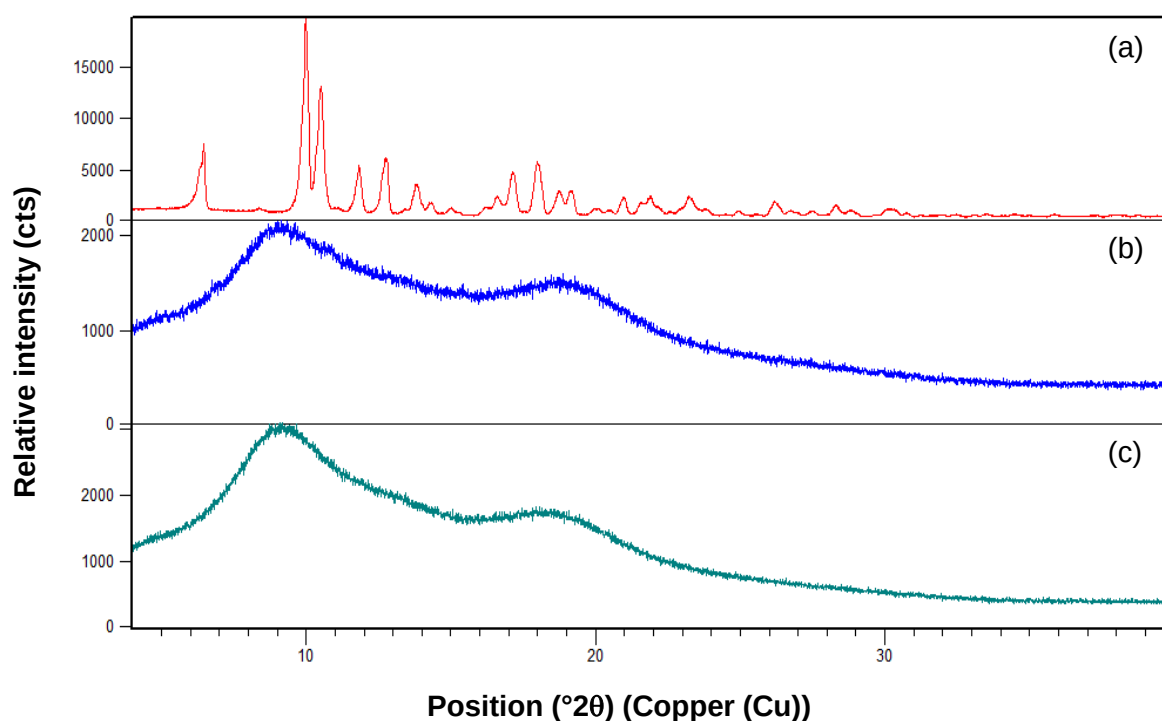


Figure B.3: An overlay of the XRPD patterns of (a) **RM**, (b) **CD** amorphous form and (c) **QC** amorphous form

Figure B.3 depicts the XRPD diffractograms for the three forms of roxithromycin that were used during this study. It shows (a) roxithromycin raw material (commercially obtained), (b) roxithromycin amorphous form obtained through the desolvation method of roxithromycin chloroform solvate and (c) the amorphous form obtained through quench cooling of the melt. The distinct diffraction peaks visible in Figure B.3 (a) are indicative of the crystalline nature of **RM**, while the characteristic diffuse diffraction patterns showing the amorphous habit of **CD** and **QC** samples are illustrated in Figures B.3 (b) and (c), respectively.

The solids are further characterised by thermal analysis by means of DSC. The appearance of a glass transition temperature (T_g) is also an indication that the solid is amorphous (Bates *et al.*, 2006:2333). The T_g is the point where there is a step change in heat flow, unlike crystals that have sharp melting points (Florence & Attwood, 2015:25). Figure B.4 shows the DSC thermograms for the three roxithromycin forms, where it depicts the thermal analysis of **RM** with a melting point of 121.77 °C (a), **CD** with a T_g of 87.00 °C (b) and **QC** with a T_g of 84.98 °C (c).

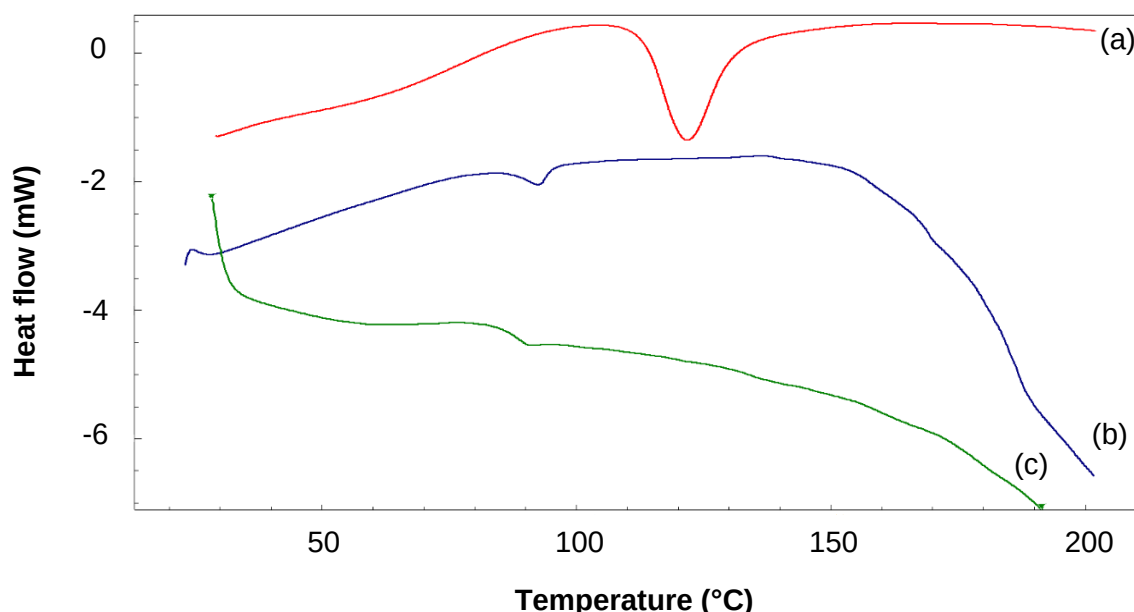


Figure B.4: An overlay of the DSC thermograms obtained for (a) **RM**, (b) amorphous **CD** and (c) amorphous **QC**

FTIR was also used to characterise the solid-state forms, as the IR-spectrums of the amorphous form differs from the crystal form due to the widespread conformations that exists with amorphous solids. Due to these conformations, broader peaks are observed relative to the peaks found with crystalline forms (Einfalt *et al.*, 2013:322). FTIR analysis and comparison of roxithromycin raw material and the two amorphous samples indicated that the purity of roxithromycin remained intact. This can be concluded because the definitive functional groups were still detected in the region of 3600 – 2000 cm^{-1} as seen in Table B.1. The functional groups and their corresponding wavenumbers in Table B.1 were found in Stuart (2004:46-47). The wavenumbers of the roxithromycin solid-state forms were recorded from their FTIR spectrums in Figures B.5 and B.6 and noted in Table B.1.

Table B.1: Roxithromycin functional groups and IR-spectrum wavenumbers ((a), (b) and (c) indicate the wavenumbers of **RM** found also in Figure B.5)

Wavenumbers of roxithromycin solid-state forms				
Regions	Functional groups	RM	CD	QC
4000 – 3000 cm ⁻¹	O-H (3700 – 3600) (strong & broad) N-H (3500 – 3300)	(a) 3631.96 cm ⁻¹ (b) 3564.45 cm ⁻¹ (c) 3466.08 cm ⁻¹	3439.08 cm ⁻¹	3466.08 cm ⁻¹
3000 – 2500 cm ⁻¹	O-H (3300 – 2500) C-H (3000 – 2800)	2976.16 cm ⁻¹ 2939.52 cm ⁻¹ 2883.58 cm ⁻¹ 2837.29 cm ⁻¹ 2791.00 cm ⁻¹	2972.31 cm ⁻¹ 2937.59 cm ⁻¹ 2877.79 cm ⁻¹ 2829.57 cm ⁻¹ 2787.14 cm ⁻¹	2970.38 cm ⁻¹ 2937.59 cm ⁻¹ 2877.79 cm ⁻¹ 2829.57 cm ⁻¹ 2785.21 cm ⁻¹
2500 – 2000 cm ⁻¹	C≡C (2050 – 2300) C≡N (2200 – 2300)	2358.94 cm ⁻¹ 2335.80 cm ⁻¹ 2117.84 cm ⁻¹ 2050.33 cm ⁻¹		2360.87 cm ⁻¹ 2337.72 cm ⁻¹

The most characteristic difference between the IR-spectrum of **RM** in Figure B.5 and the IR-spectrum of the amorphous forms in Figure B.6 lies in the 4000 – 3200 cm⁻¹ region. **RM** displays three distinct peaks at (a) 3631.96, (b) 3564.45 and (c) 3466.08 cm⁻¹ in contrast to the one broad peak shown at 3439.08 and 3466.08 cm⁻¹ for **CD** and **QC**, respectively. This one broad peak is because no water molecules are bound. The amorphous habit of the prepared amorphous forms is therefore identified through peak broadening. This correlates with previous reports for amorphous roxithromycin forms (Aucamp *et al.*, 2012:469; Aucamp *et al.*, 2013:21; Milne, 2016:110).

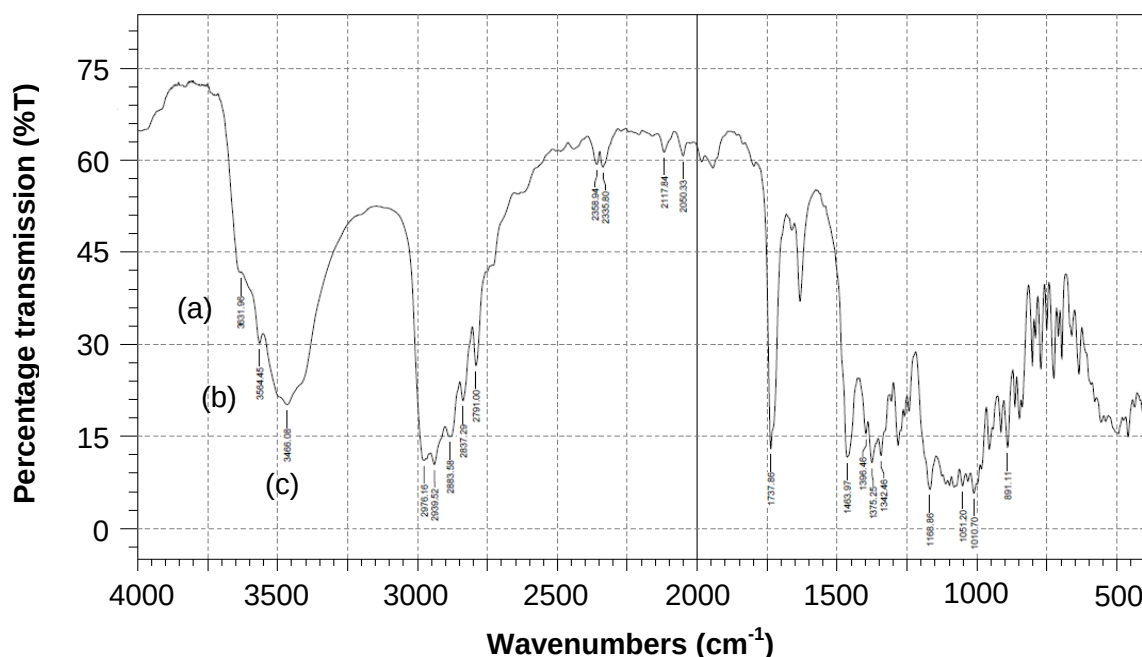


Figure B.5: An FTIR spectrum obtained for **RM**

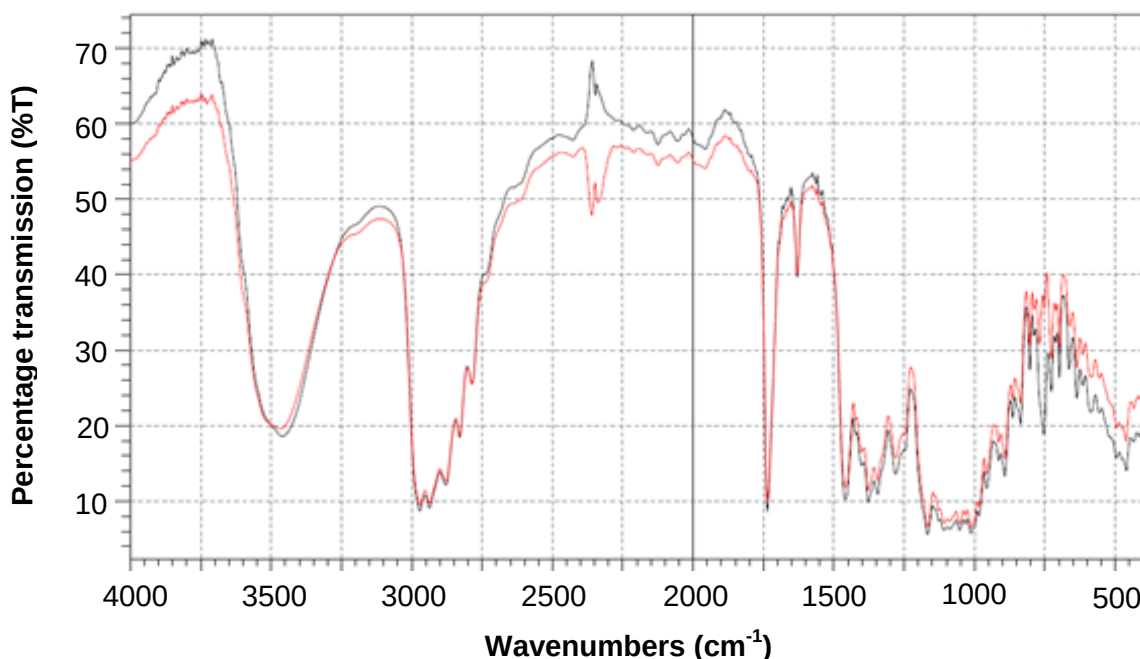


Figure B.6: An overlay of the FTIR spectra obtained for the amorphous **QC** form (red spectrum) and the amorphous **CD** (black spectrum)

The purity of the prepared **QC** and **CD** samples was further confirmed through HPLC analysis and found to be in good correlation with **RM**. According to the raw material certificate of analysis (CoA), the purity of **RM** was 99.6% and the percentage purity of the amorphous **QC** form and the amorphous **CD** was 99.2% and 99.6% respectively, which indicates the quench cooling and desolvation processes did not detrimentally affect the purity of the API.

B.4 Preparation of the lipid films (precursor to niosomes)

B.4.1 Components used in the preparation of the lipid films

Table B.2: Excipients, supplier, batch number and function as used during the formulation of the niosomes

Excipient	Supplier	Batch number	Function
Roxithromycin	DB Fine Chemicals	IF-RO-081116	API
Span[®] 40	Fluka	85545	Surfactant
Cholesterol	Sigma	421393	Additive
Chloroform	Merck	102245	Organic solvent
Methanol	Associated chemical enterprises (ace)	28958	Organic solvent
Milli-Q[®] water	In house	Merck Millipore	Hydration agent

B.4.1.1 Span[®] 40

Span[®] 40, also known as sorbitan monopalmitate, is classified as a non-ionic surfactant used to prepare niosome vesicle systems, where it acts as a wetting agent, emulsifier, solubiliser and a permeability enhancer (Kumar & Rasjeshwarrao, 2001:209). Other properties including stability, compatibility and lack of toxicity, making it the preferred choice of surface active agent when formulating vesicles (Mahale *et al.*, 2012:47). The non-ionic surfactant forms the lipid bilayer of the niosome by orientating their hydrophilic heads to the outside and the hydrophobic tails to the inside. This unique structure enables the vesicle to encapsulate both lipophilic and hydrophilic substances (Moghassemi & Hadjizadeh, 2014:23-24). Generally, when preparing niosomes, the maximum amount of surfactant used is 1.0 – 2.5% w/w (Mahale *et al.*, 2012:49).

B.4.1.2 Cholesterol

Cholesterol is a steroid derivative, and the incorporation thereof in niosomes affects properties including rigidity and membrane permeability, as well as entrapment efficiency (Rajera *et al.*, 2011:945-946). Cholesterol prevents the aggregation of the surfactants and therefore enables the formation of the vesicles (Kumar & Rasjeshwarrao, 2001:211). It has a membrane stabilising action, which helps to stabilise the surfactant bilayer of the niosomes and it reduces the leakiness of the membrane (Biswal *et al.*, 2008:3).

B.4.1.3 Organic solvents (chloroform and methanol)

Chloroform (CHCl₃) is a volatile, colourless and sweet-smelling liquid commonly used as a solvent (OED, 1989). Methanol (CH₃OH), also abbreviated as MeOH, is a volatile, colourless, flammable and poisonous liquid used in chemical synthesis, in antifreeze and as a solvent (NCBI, 2004). Chloroform and methanol are miscible and used as an organic solvent in this study as all compounds used are easily dissolved in it (Merck, 2017).

B.4.1.4 Roxithromycin

Roxithromycin was the API used during this study and was entrapped into the lipid layer of the niosomes. The monohydrate form has an aqueous solubility of 0.0335 mg/ml at 25 °C (Aucamp *et al.*, 2013:26) and due to this poor solubility, the amorphous forms of the API were explored. The **CD** and **QC** amorphous forms were also encapsulated into the vesicle systems.

B.4.2 Method of preparation

The preparation of the lipid films was performed using an adaption of the thin-film hydration method (Chandu *et al.*, 2012:27). The lipid mixture consisting of Span[®] 40 and cholesterol, as well as roxithromycin, were weighed and dissolved in 5 ml of a 2:1 volume ratio of chloroform

and methanol in a small beaker. The beaker was placed on a hot plate at 50 °C, above the transition temperature of the lipids (Kumar & Rasjeshwarrao, 2001:210), heated and stirred until all the organic solvents had evaporated, leaving a thin film at the bottom of the beaker.

B.5 Investigation of the physical characterisation of amorphous roxithromycin solid-state forms during thin film formation

Once the physical characterisation and confirmation of drug purity of the amorphous forms were complete, the study progressed towards the investigation of the physical stability thereof during the preparation of the thin films. These thin films were the precursors for niosome vesicle system dispersions. The first step of this stability study was to confirm the amorphous or crystalline nature of the thin films after preparation. A total of 15 films were prepared containing varying concentrations of cholesterol and Span[®] 40 and each concentration level of these two excipients were used to prepare films containing **RM**, **QC** amorphous form or the **CD** amorphous form. The concentration of roxithromycin stayed constant at 2% (w/v) during the preparation of all the dispersions. The XRPD patterns, DSC thermograms and IR-spectra were once again measured, as well as HSM photos of the thin films.

B.5.1 Roxithromycin raw material film 1 – 5 (RM1 – RM5)

The XRPD data obtained for the thin films of **RM1 – RM5** in comparison with **RM**, cholesterol and Span[®] 40 are illustrated in Figure B.7 and the peak angles (°2θ) and relative intensities (%) are shown in Table B.3.

From Figure B.7 (d) it is apparent the prepared film rendered **RM** into an amorphous habit. Only the sharp single diffraction peak of Span[®] 40 at 21.65°2θ and a single diffraction peak of cholesterol at 5.25°2θ was visible with the XRPD pattern of thin film **RM1**. Although these diffraction peaks were visible, it should also be noted that the intensity of these peaks were significantly reduced within the thin film sample.

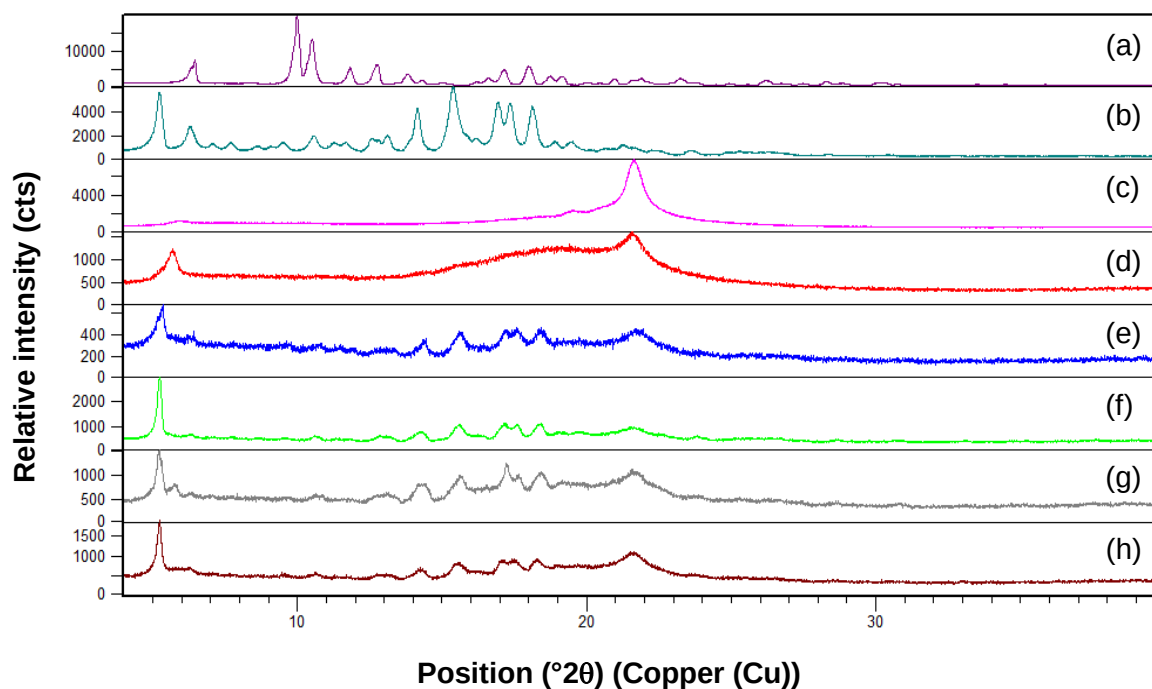


Figure B.7: An overlay of the XRPD diffraction patterns obtained for (a) **RM**, (b) cholesterol, (c) Span[®] 40, (d) film **RM1**, (e) film **RM2**, (f) film **RM3**, (g) film **RM4** and (h) film **RM5**

From the DSC thermograms (Figure B.8), it became apparent that film **RM1** rendered **RM** into the amorphous state or the melting of cholesterol and Span[®] 40 in the region of $\cong 41 - 44\text{ }^{\circ}\text{C}$ resulted in the solubilisation of roxithromycin during the heating process. This is evident from the fact that a melting endotherm for roxithromycin cannot be identified in the melting region of $\cong 120\text{ }^{\circ}\text{C}$ (Figure B.8 (d)). However, from the XRPD data depicted in Figure B.7 (d), it can be concluded that the film resulted in an amorphous solid dispersion of **RM**.

Figure B.9 depicts an overlay of the DSC thermograms obtained with the powder mixture and the thin film consisting of the **RM** in combination with cholesterol and Span[®] 40. It is apparent from this data that Span[®] 40 (melting at $\cong 41.00\text{ }^{\circ}\text{C}$) melts first thereby solubilising the rest of the powder mixture. The DSC thermogram obtained with the thin film showed a small endotherm in the region of $\cong 35\text{ }^{\circ}\text{C}$. It is proposed that this signifies a T_g of the amorphous solid dispersion or that it could be a small amount of either cholesterol or Span[®] 40, which did not convert to the amorphous solid dispersion.

Table B.3: Comparison of the XRPD diffraction peaks for **RM**, cholesterol, Span[®] 40, and thin films of **RM1 – RM5**

RM		Cholesterol		Span [®] 40		Film RM1		Film RM2		Film RM3		Film RM4		Film RM5	
°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%
-	-	5.25	91.07	-	-	-	-	5.33	100.00	5.26	100.00	5.24	100.00	5.27	100.00
-	-	-	-	-	-	5.69	100.00	-	-	-	-	-	-	-	-
6.34	23.79	6.32	38.01	-	-	-	-	-	-	-	-	-	-	-	-
6.48	36.19	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.85	42.21	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.01	100.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.12	45.43	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.34	27.88	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.48	61.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.57	60.07	10.58	25.12	-	-	-	-	-	-	-	-	-	-	-	-
-	-	11.71	15.10	-	-	-	-	-	-	-	-	-	-	-	-
11.84	25.31	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.62	17.72	12.57	20.39	-	-	-	-	-	-	-	-	-	-	-	-
12.73	28.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.82	26.51	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	13.14	27.06	-	-	-	-	-	-	-	-	-	-	-	-
-	-	14.17	69.29	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	14.41	32.04	-	-	14.49	25.77	-	-
-	-	15.36	100.00	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	15.52	21.97
-	-	-	-	-	-	-	-	15.65	50.23	15.62	22.47	15.69	42.06	-	-
-	-	16.23	23.07	-	-	-	-	-	-	-	-	-	-	-	-
-	-	16.97	81.24	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	17.16	20.01	-	-	17.07	21.14
17.23	19.59	-	-	-	-	-	-	17.18	42.52	-	-	17.25	59.18	-	-
-	-	17.44	71.19	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	17.64	18.30	-	-	-	-
18.01	27.52	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18.09	24.93	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	18.14	74.90	-	-	-	-	-	-	-	-	-	-	-	-
-	-	18.20	69.94	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	18.45	39.37	18.45	17.55	18.47	31.98	18.30	17.45
-	-	18.95	18.17	-	-	-	-	-	-	-	-	-	-	-	-
-	-	19.50	18.17	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	21.65	100.00	21.59	98.51	21.78	43.84	-	-	21.85	34.99	21.59	31.31

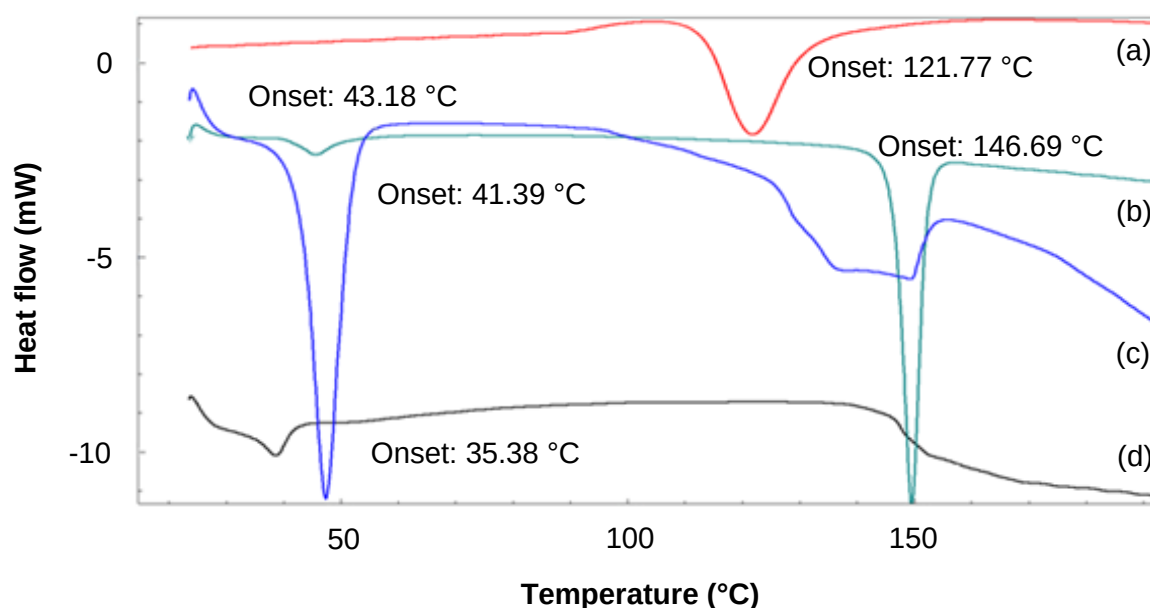


Figure B.8: An overlay of the thermograms obtained by DSC analysis for (a) **RM**, (b) cholesterol, (c) Span[®] 40 and (d) film **RM1**

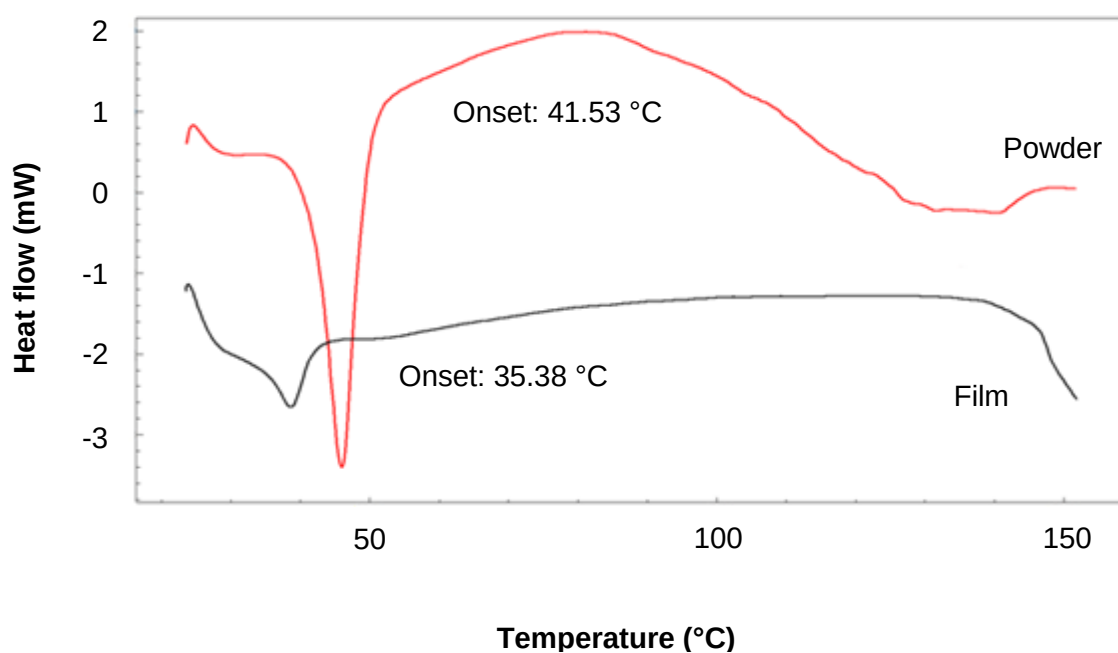


Figure B.9: An overlay of the thermograms: red is the dry powder mixture and black is the thin film obtained with **RM1**

According to Zhang *et al.* (2004:377), HSM is a means to provide complementary information on phase transitions. A Nikon Eclipse microscope (E4000), fitted with a Nikon DS-Fi1 camera (Linkam THMS600, Japan) and equipped with a T95 LinkPad temperature controller, was used to view the samples. A very small piece of the thin film

was transferred into a quartz sample holder and viewed through 10 x magnification. The heating stage was set to a heating rate of 10 °C/min and the sample was examined to observe the melting temperature or any other solid-state transformations. The temperature at which the sample first started to show any signs of melting was recorded. This temperature might slightly differ from that of the DSC data recorded, as the DSC instrument is more sensitive to change in heat flow and could record the precise point of melting.

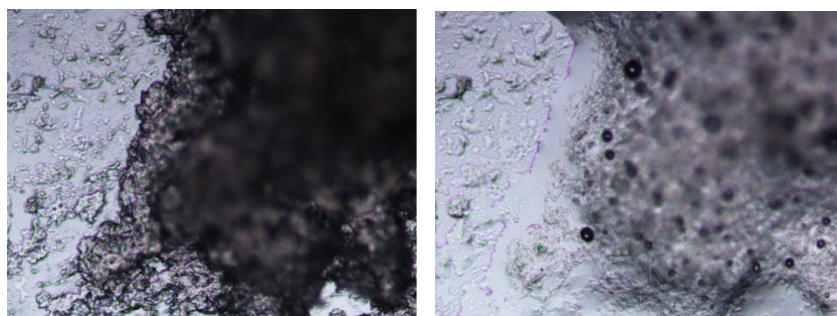


Figure B.10: HSM results for thin film **RM1** which started to melt at 43.6 °C

Just as with thin film **RM1**, the film preparation of the **RM2** combination resulted in an amorphous solid dispersion of the **RM** in combination with cholesterol and Span[®] 40. This is presumed from the fact that although small diffraction peaks were visible within the film sample (Figure B.7 (e)), these diffraction peaks correlate with those of cholesterol and Span[®] 40 (Table B.3). Only one diffraction peak could possibly correlate with that of the **RM** ($17.18^{\circ}2\theta$), however this diffraction peak is very close to the diffraction peak at $16.97^{\circ}2\theta$ observed with cholesterol and therefore it is difficult to conclude that it can be ascribed to the **RM**. It would be more likely to detect the **RM** at $10.01^{\circ}2\theta$, since this is the diffraction position, which shows 100.00% relative intensity for the **RM**.

From Figure B.11, it can be concluded that with the melting of cholesterol and Span[®] 40 at relative low temperatures, **RM** dissolved into the molten or partially molten excipients and therefore no melting endotherm for **RM** was visible. The thermogram of the film does not show a clear melting endotherm for cholesterol, Span[®] 40 or **RM**. The endotherm with an onset of 37.35 °C could be a glass transition of the amorphous solid dispersion, or it could be melting due to a small fraction of either cholesterol or Span[®] 40 that did not convert to an amorphous matrix during the film preparation. This conclusion can be substantiated by the fact that XRPD diffraction peaks for mainly cholesterol were identified (Figure B.7 (e)).

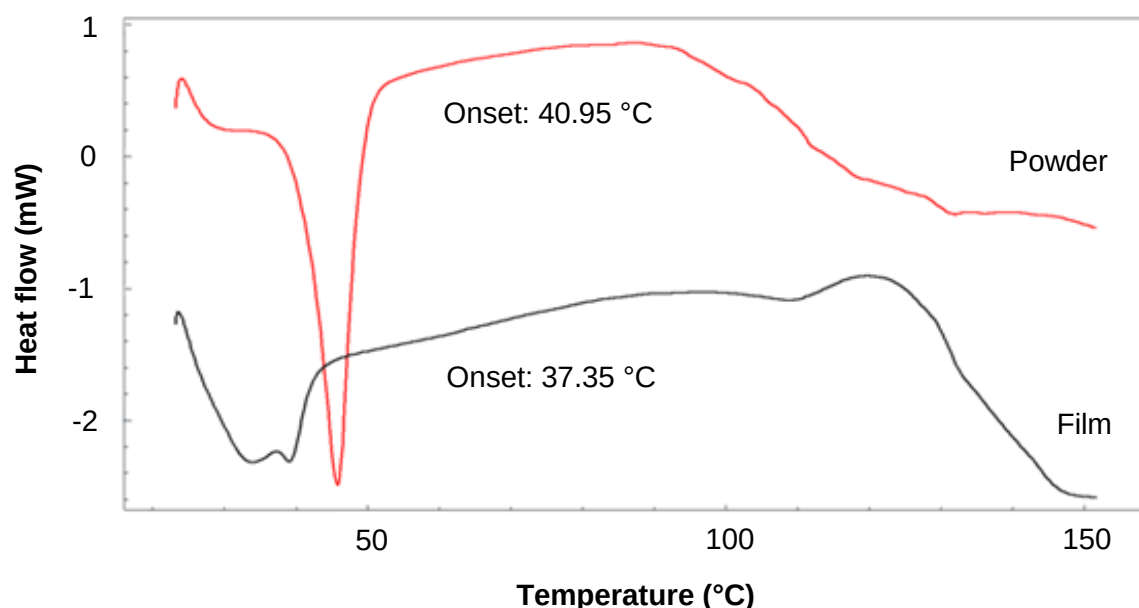


Figure B.11: An overlay of the thermograms: red is the dry powder mixture and black is the thin film obtained with **RM2**

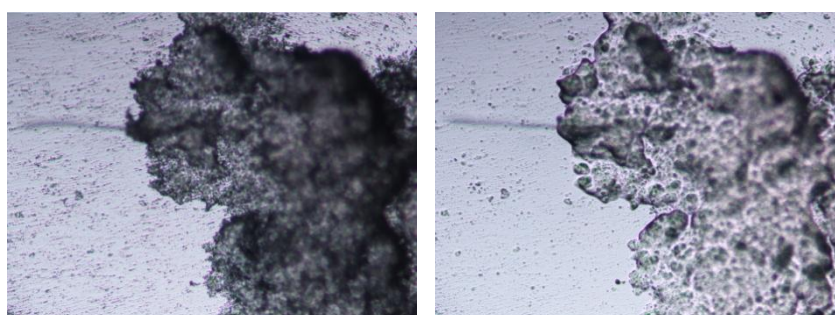


Figure B.12: HSM results for thin film **RM2** which started to melt at 45.1 °C

Compared to the previous data (**RM1** and **RM2**), the film obtained with **RM3** shows to contain more crystalline content, which is concluded from the higher intensity peak at $5.26^{\circ}2\theta$ (Table B.3 and Figure B.7 (f)). It is further presumed this crystalline content is due to cholesterol and not **RM**, indicating that although **RM** was used in this formulation, the thin film preparation still rendered the API into an amorphous state.

The results from Figure B.13 confirm the higher degree of crystallinity of the film compared to that of **RM1** and **RM2**, since a clear melting endotherm was observed with an onset at 34.44 °C.

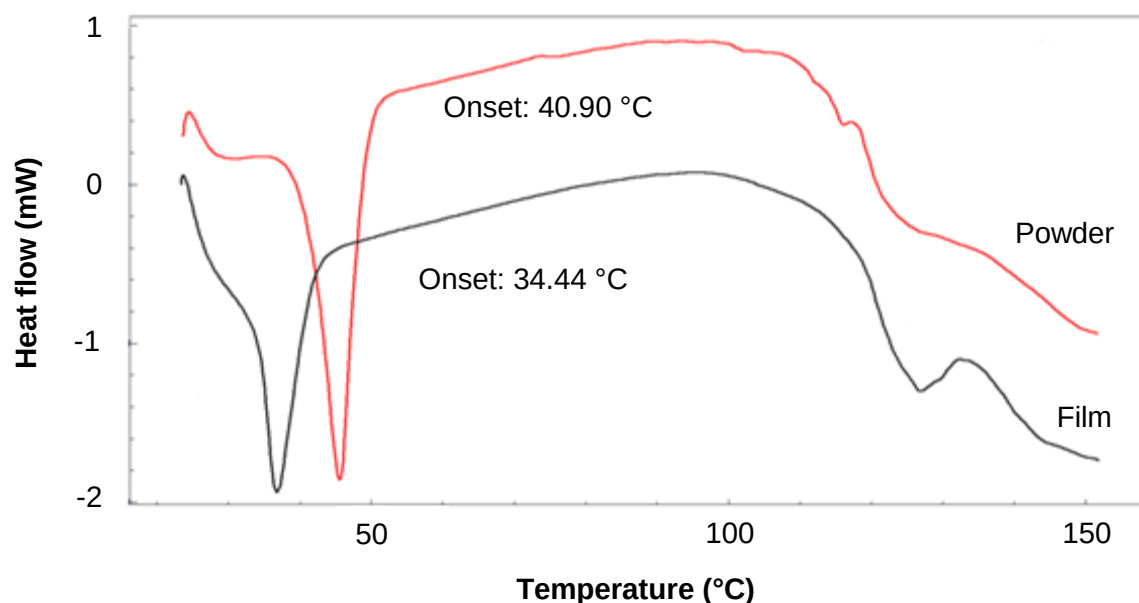


Figure B.13: An overlay of the thermograms: red is the dry powder mixture and black is the thin film obtained with **RM3**



Figure B.14: HSM results for thin film **RM3** which started to melt at 42.6 °C

Just as with the thin film obtained with **RM3**, the film of **RM4** also shows some degree of crystallinity and by comparing the diffraction peaks as listed in Table B.3, it can be concluded that the diffraction peaks were due to cholesterol that was not rendered into a complete amorphous habit. The same conclusion can be made from the DSC data depicted in Figure B.15.

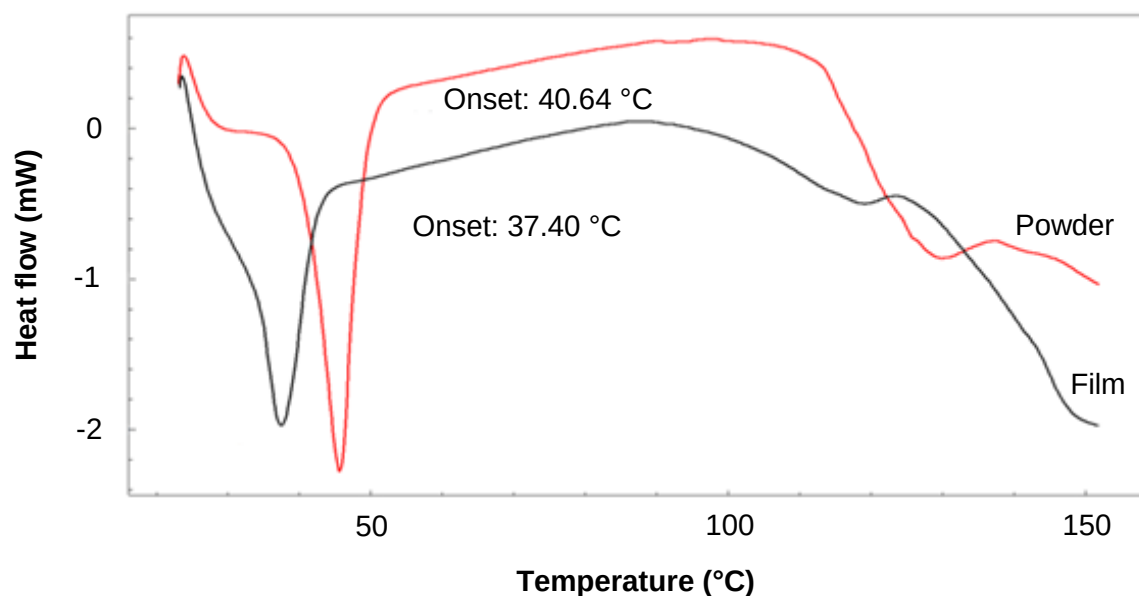


Figure B.15: An overlay of the thermograms: red is the dry powder mixture and black is the thin film obtained with **RM4**

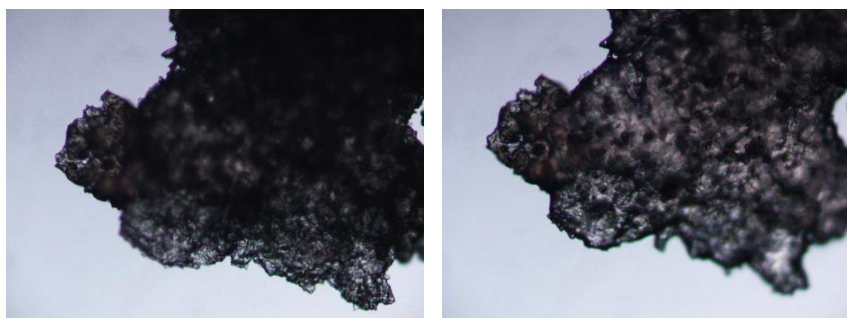


Figure B.16: HSM results for thin film **RM4**, which started to melt at 42.6 °C

Figure B.7 (h) depicts the film of **RM5** and shows some degree of crystalline cholesterol within the thin film, which is corroborated by the DSC thermograms as depicted in Figure B.17.

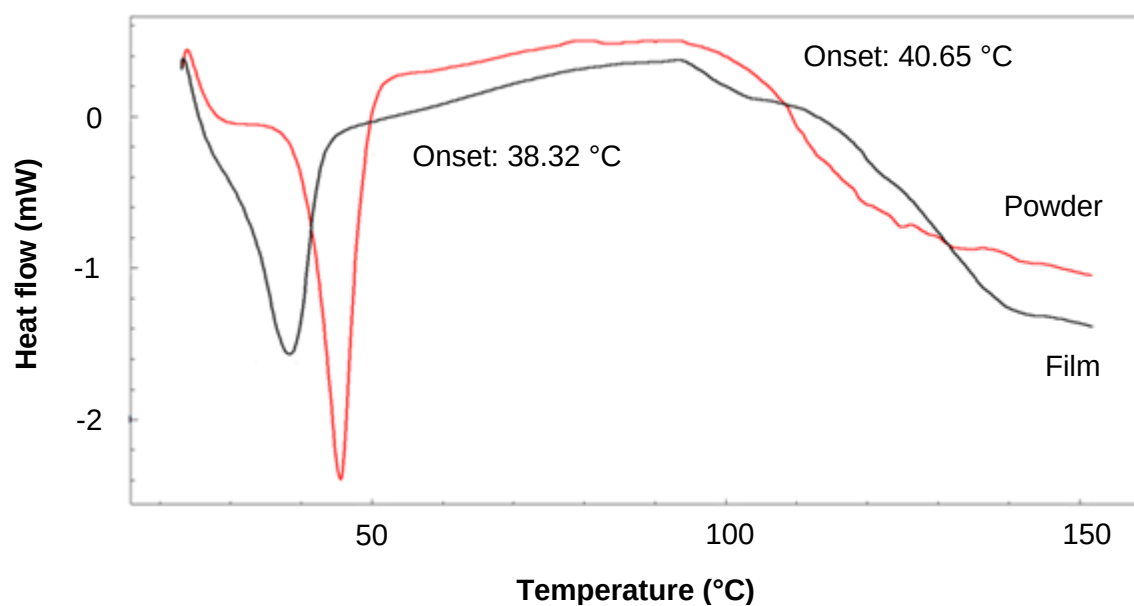


Figure B.17: An overlay of the thermograms: red is the dry powder mixture and black is the thin film obtained with **RM5**

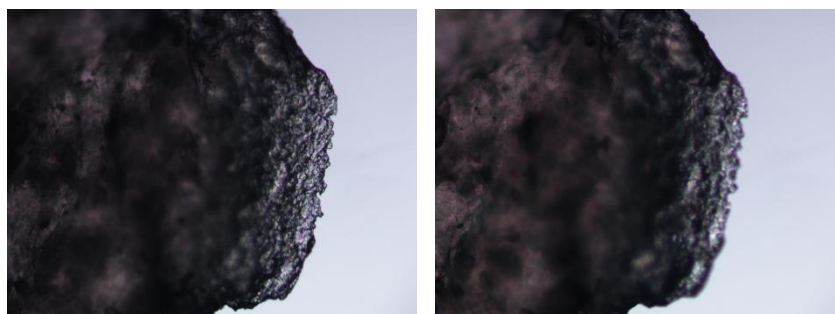


Figure B.18: HSM results for thin film **RM5** which started to melt at 43.0 °C

The FTIR spectra of the five **RM** thin films (**RM1 – RM5**) are illustrated in Figure B.19, where very broad peaks in the areas of $4000 - 3200 \text{ cm}^{-1}$ are visible, an indication that the films are amorphous in nature.

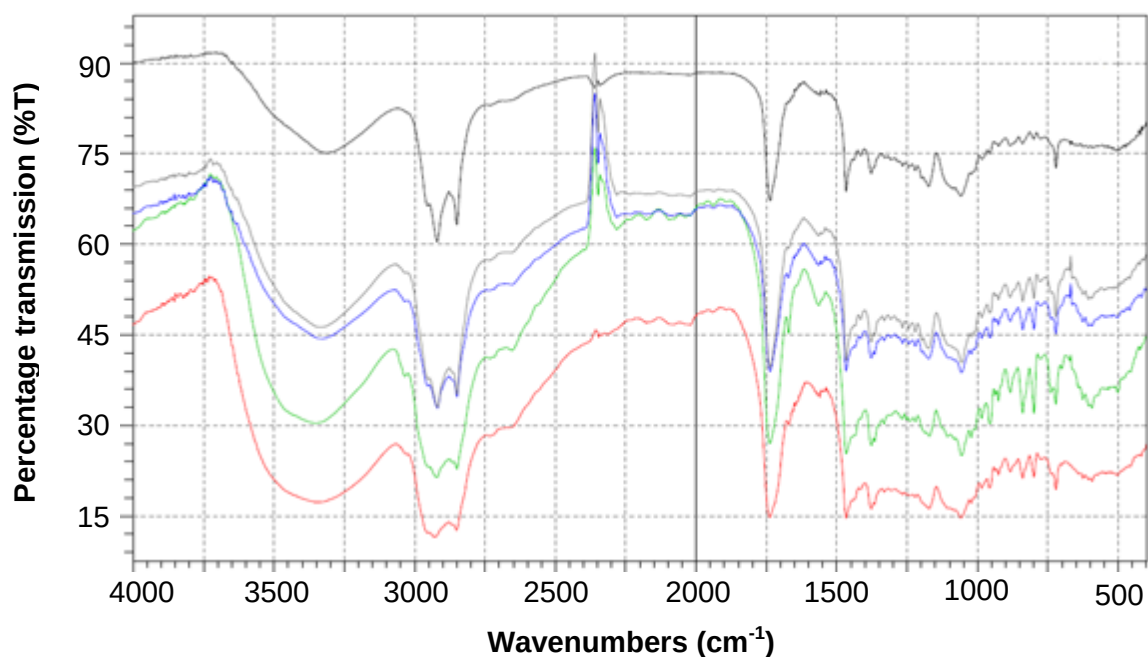


Figure B.19: An overlay of the FTIR spectra obtained for film **RM1** (black), film **RM2** (red), film **RM3** (green), film **RM4** (blue) and film **RM5** (grey)

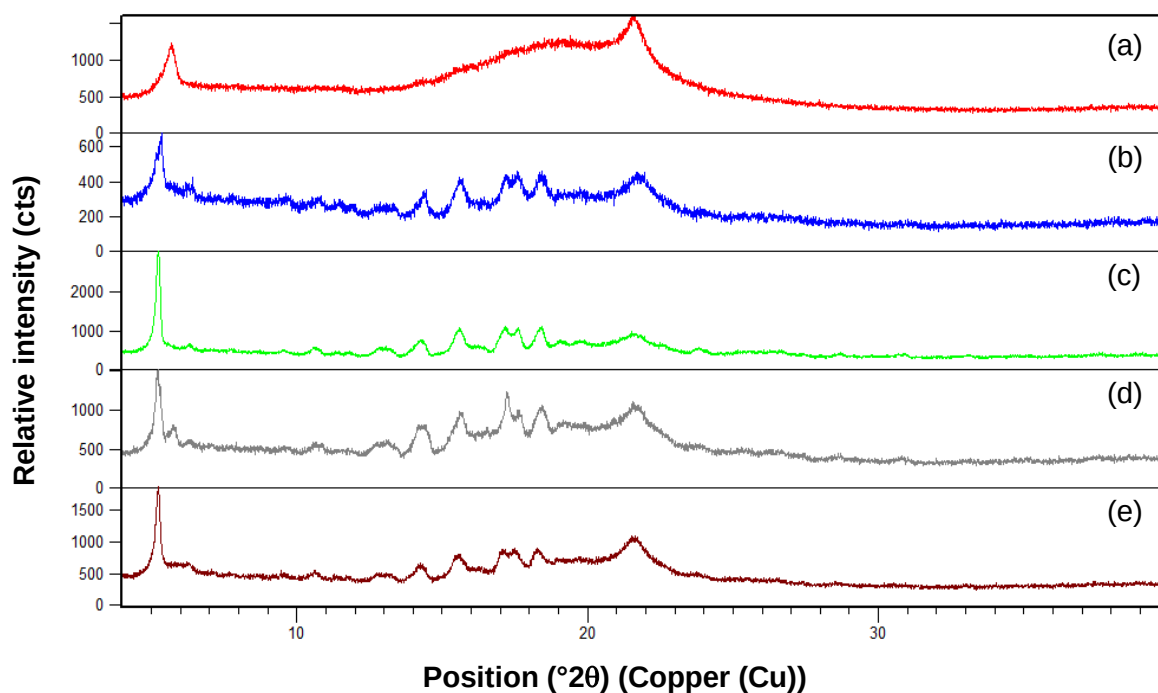


Figure B.20: An overlay of the XRPD diffractograms of the thin films containing **RM** with (a) being film **RM1**, (b) film **RM2**, (c) film **RM3**, (d) film **RM4** and (e) film **RM5**

In summary, Figure B.20 presents an overlay of all the XRPD patterns obtained with the thin films (**RM1 – RM5**). It can clearly be seen that all five films containing the **RM** show an amorphous habit, as no clear diffraction peaks are visible, therefore the preparation of the

thin films (precursor to niosomes) rendered **RM** into an amorphous habit. From Table B.3, it can be seen that the films of **RM2** and **RM4** showed peaks that could be associated with the crystalline form of roxithromycin. Figure B.20 (c) shows the highest intensity diffraction peak at position $5.26^{\circ}2\theta$, compared to all the other diffractograms. From this data it can be concluded that in terms of amorphicity of the resultant thin films/amorphous solid dispersions, the films can be ranked as follows: **RM3** > **RM5** > **RM4** > **RM1** > **RM2**.

B.5.2 Amorphous chloroform desolvate film 1 – 5 (CD1 – CD5)

The XRPD data obtained for the thin films of **CD1** – **CD5**, compared to the roxithromycin amorphous form obtained through the desolvation method of roxithromycin chloroform solvate, cholesterol and Span[®] 40, are illustrated in Figure B.21 and the peak angles ($^{\circ}2\theta$) and relative intensities (%) are shown in Table B.4. The amorphous **CD** form does not show any distinct diffraction peaks on the XRPD diffractogram, therefore the diffraction peaks of the **RM** are shown in Table B.4. The films are compared to the peaks of the **RM** to ensure it stayed amorphous during the thin film formation and did not convert back to the more stable crystalline form.

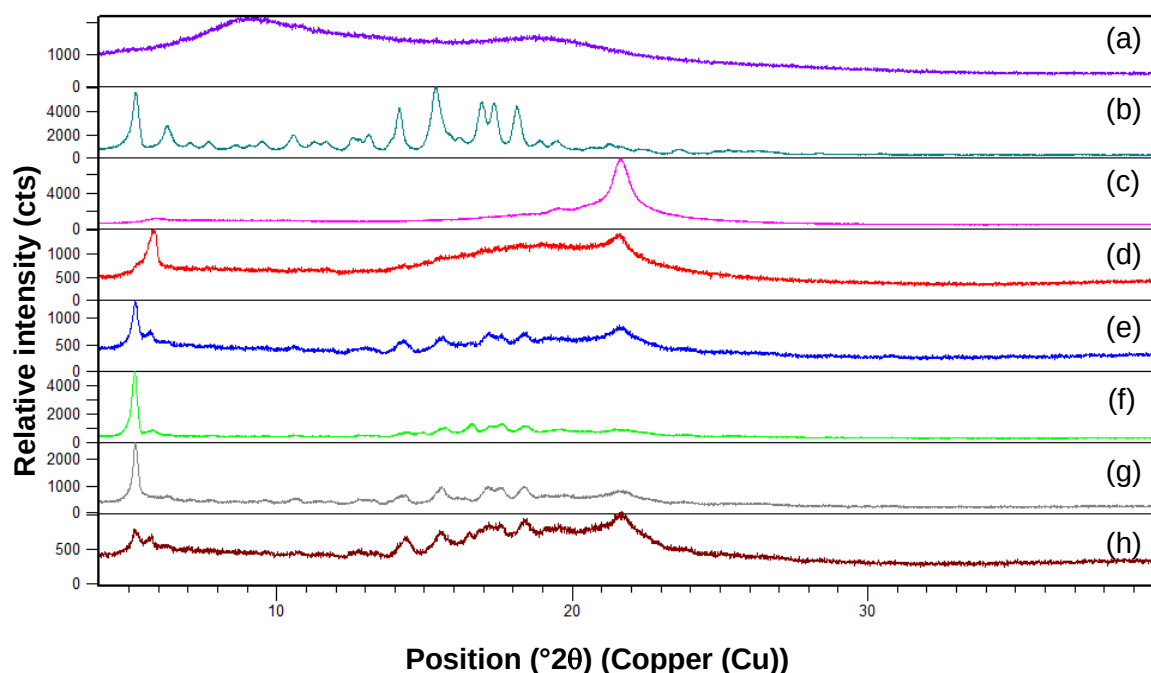


Figure B.21: An overlay of the XRPD diffraction patterns obtained for (a) amorphous **CD**, (b) cholesterol, (c) Span[®] 40, (d) film **CD1**, (e) film **CD2**, (f) film **CD3**, (g) film **CD4** and (h) film **CD5**

Table B.4: Comparison of the XRPD diffraction peaks for **RM**, cholesterol, Span[®] 40, and thin films of **CD1 – CD5**

RM		Cholesterol		Span [®] 40		Film CD1		Film CD2		Film CD3		Film CD4		Film CD5	
°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%
-	-	5.25	91.07	-	-	-	-	5.24	100.00	5.23	100.00	5.23	100.00	5.25	100.00
-	-	-	-	-	-	5.92	100.00	-	-	-	-	-	-	-	-
6.34	23.79	6.32	38.01	-	-	-	-	-	-	-	-	-	-	-	-
6.48	36.19	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.85	42.21	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.01	100.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.12	45.43	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.34	27.88	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.48	61.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.57	60.07	10.58	25.12	-	-	-	-	-	-	-	-	-	-	-	-
-	-	11.71	15.10	-	-	-	-	-	-	-	-	-	-	-	-
11.84	25.31	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.62	17.72	12.57	20.39	-	-	-	-	-	-	-	-	-	-	-	-
12.73	28.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.82	27.60	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	13.14	27.06	-	-	-	-	-	-	-	-	-	-	-	-
-	-	14.17	69.29	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	14.35	19.73	-	-	-	-	14.39	65.18
-	-	15.36	100.00	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	15.60	23.41	-	-	15.60	25.40	15.56	76.56
-	-	16.23	23.07	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	16.65	17.64	-	-	-	-
-	-	16.97	81.24	-	-	-	-	-	-	-	-	-	-	-	-
17.23	19.59	-	-	-	-	-	-	17.17	23.33	-	-	17.16	21.22	17.23	68.19
-	-	17.44	71.19	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	17.70	16.98	17.64	18.92	-	-
18.01	27.52	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18.09	24.93	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	18.14	74.90	-	-	-	-	-	-	-	-	-	-	-	-
-	-	18.20	69.94	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	18.41	19.30	-	-	18.46	18.18	18.41	72.54
-	-	18.95	18.17	-	-	-	-	-	-	-	-	-	-	-	-
-	-	19.50	18.17	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	21.65	100.00	21.68	47.52	21.66	33.86	-	-	-	-	21.54	95.15

The XRPD diffraction patterns in Figure B.21 (d) – (h) illustrate the amorphous habit of the thin films, as there are no distinct peak intensities visible. Two peaks that can be recognised throughout are those of cholesterol and Span[®] 40, with cholesterol clearly distinguishable. Film **CD3**, shown in Figure B.21 (f), once again displays the highest peak intensity of the five films, due to the highest concentration of cholesterol present in the film. From Table B.4 it can be seen that film **CD1** showed a diffraction peak at $5.92^{\circ}2\theta$, which is at a higher diffraction angle than that identified in the diffractogram of pure cholesterol ($5.25^{\circ}2\theta$); it also shows a peak correlating with that of Span[®] 40 ($21.65^{\circ}2\theta$). The film of **CD2** shows three peaks, where two of them can be ascribed to cholesterol and Span[®] 40, while the third at $17.17^{\circ}2\theta$ is very near to **RM**; it has a very low relative intensity and could not be definitely ascribed to the **RM**. The film of **CD3** only shows one diffraction peak, clearly correlating with that of cholesterol. Two peaks are seen in the film of **CD4**, where one is associated with cholesterol and the other with the **RM**. Film **CD5** shows three peaks, two of which can again be presumed to be from cholesterol and Span[®] 40, while the third exactly corresponds with the peak of $17.23^{\circ}2\theta$ visible for the crystalline form (**RM**).

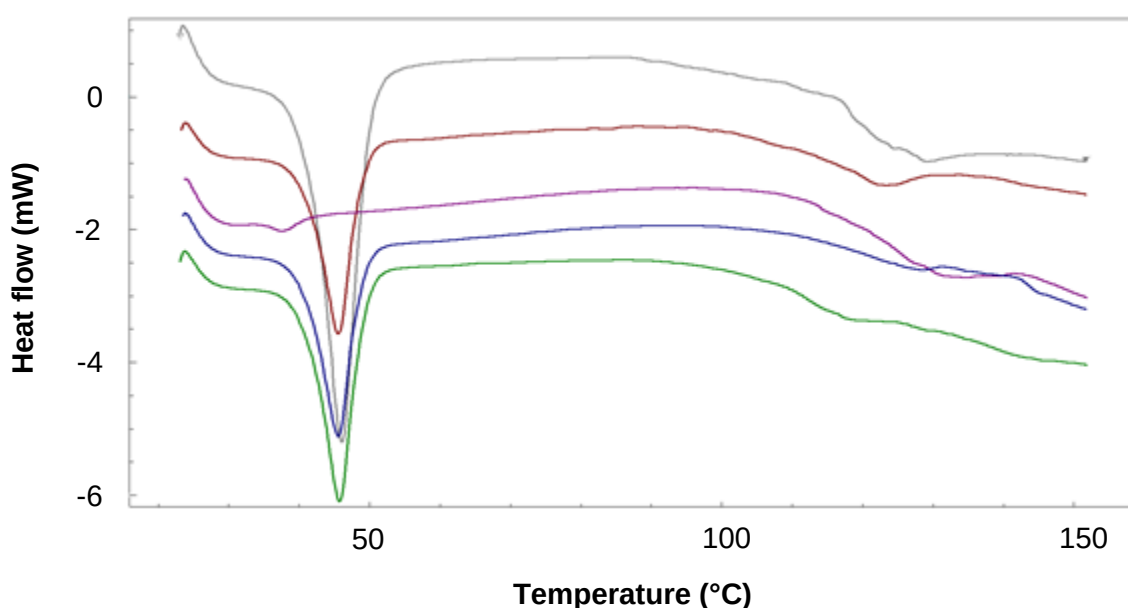


Figure B.22: An overlay of the thermograms obtained by DSC analysis for the powder mixtures used to prepare the thin films of **CD1** (grey), **CD2** (brown), **CD3** (purple), **CD4** (blue) and **CD5** (green)

In Figure B.22, the powder mixtures for the five films (**CD1** – **CD5**) showed a big endotherm in the region of $\cong 41 - 44^{\circ}\text{C}$ except for **CD3** (purple), which only had a small endotherm. The reason for this small endotherm is unclear, but can be a result of an instrument malfunction or a problem with the sample itself.

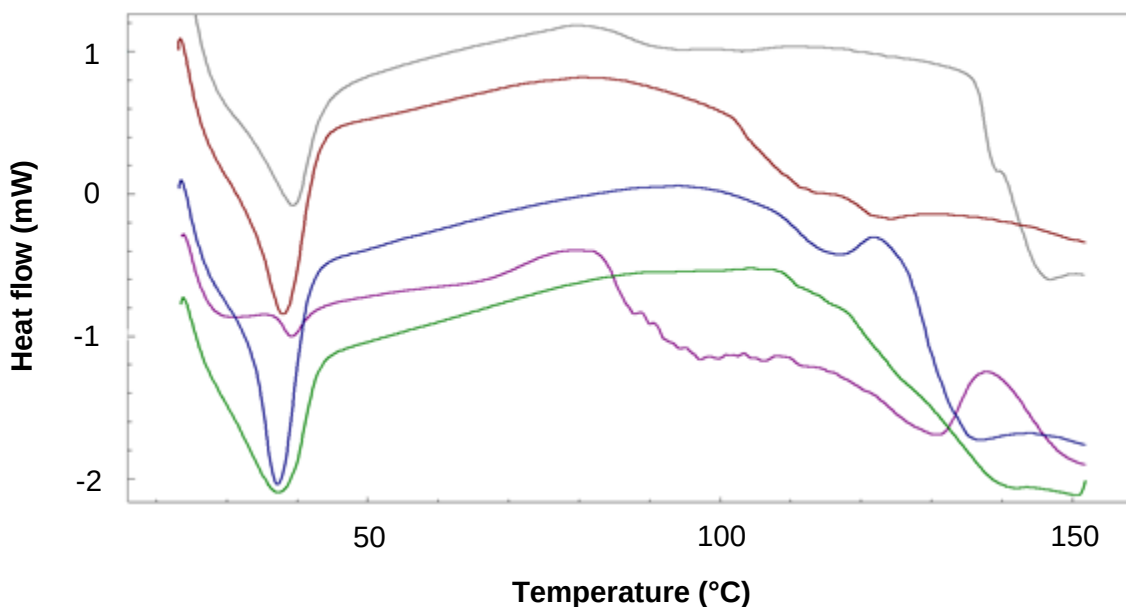


Figure B.23: An overlay of the thermograms obtained by DSC analysis for the prepared films of **CD1** (grey), **CD2** (brown), **CD3** (purple), **CD4** (blue) and **CD5** (green)

The thermograms displayed in Figure B.23 show smaller endotherms in the region of $\cong 35 - 40$ °C compared to their powder counter parts. It is proposed these smaller peaks signify the T_g and for that reason, the films are considered to be amorphous in nature.

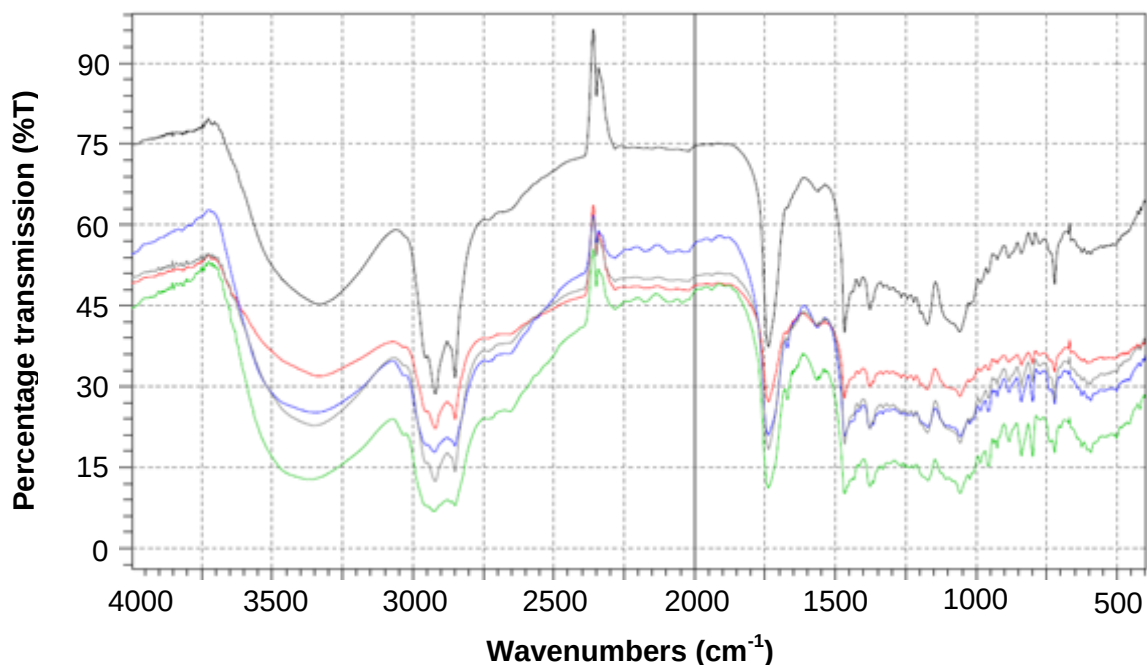
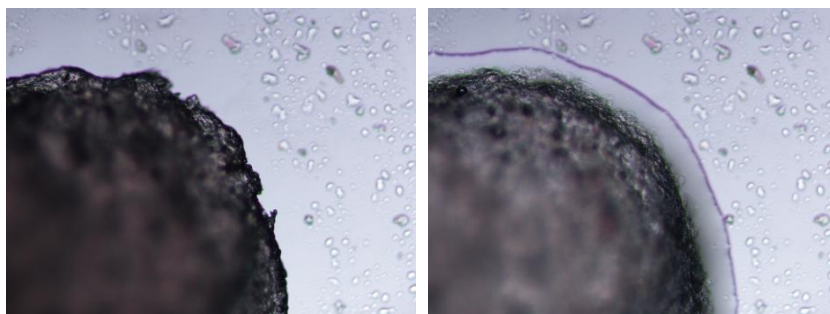


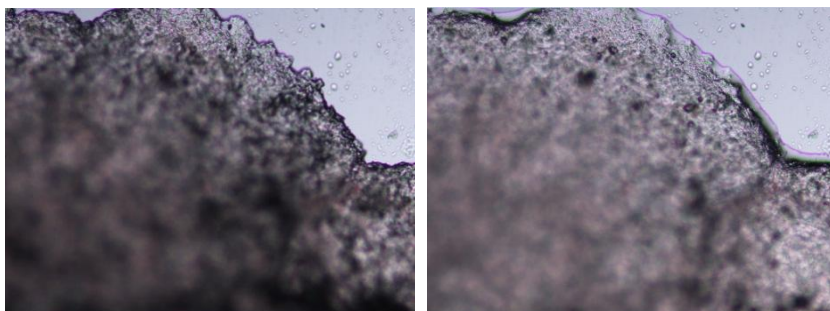
Figure B.24: An overlay of the FTIR spectra obtained for film **CD1** (black), film **CD2** (red), film **CD3** (green), film **CD4** (blue) and film **CD5** (grey)

An overlay of the FTIR spectrums of the five chloroform desolvate thin films are illustrated in Figure B.24, which shows a very broad peak in the areas of $3800 - 3200 \text{ cm}^{-1}$ and therefore an indication the films are amorphous in nature.

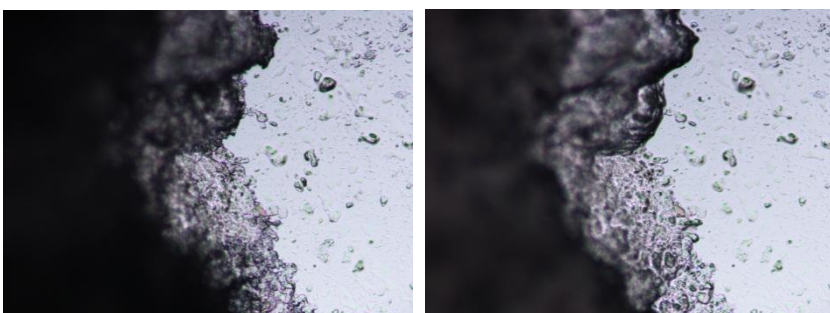
Film **CD1** started to melt at 43.5°C



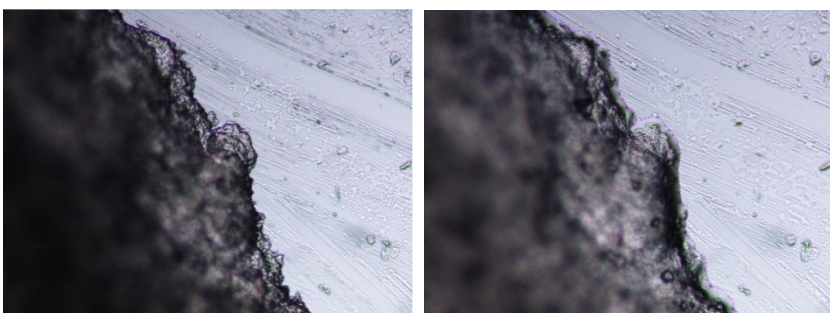
Film **CD2** started to melt at 41.0°C



Film **CD3** started to melt at 43.1°C



Film **CD4** started to melt at 41.6°C



Film **CD5** started to melt at 43.0°C

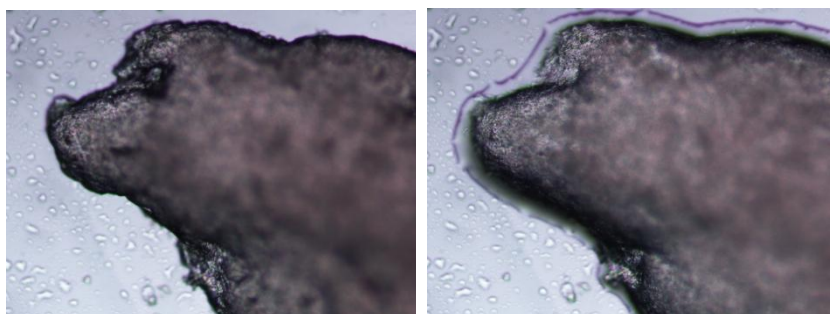


Figure B.25: HSM results for thin films of **CD1 – CD5**

In summary, from the XRPD overlay shown in Figure B.21, it can be seen that the thin film of **CD3** (Figure B.21 (f)) showed the highest intensity at position $5.26^{\circ}2\theta$ compared to the other diffractograms. In Table B.4, the films of **CD2**, **CD4** and **CD5** had peaks that can be ascribed to crystalline roxithromycin (**RM**). The amorphousness of the films can be concluded from this data and as a result, the thin film/amorphous solid dispersions can be ordered as follow: **CD3** > **CD4** > **CD1** > **CD2** > **CD5**.

B.5.3 Amorphous quench cooled film 1 – 5 (QC1 – QC5)

The XRPD data obtained for the thin films of **QC1** – **QC5** in comparison with the roxithromycin amorphous form obtained through quench cooling of the melt method, cholesterol and Span[®] 40 are illustrated in Figure B.26 and the peak angles ($^{\circ}2\theta$) and relative intensities (%) are shown in Table B.5. The amorphous **QC** form does not show any distinct diffraction peaks on the XRPD diffractogram and therefore the diffraction peaks of **RM** were once again used to compare the peaks of films to ensure it stayed amorphous during the thin film formation.

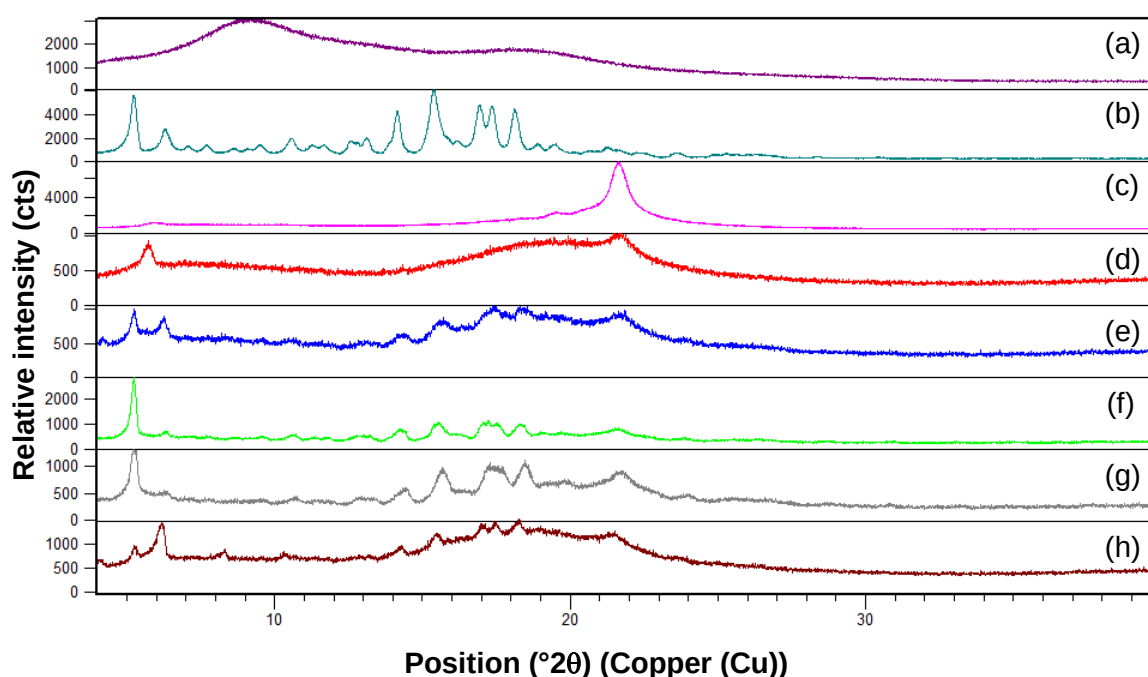


Figure B.26: An overlay of the XRPD diffraction patterns obtained for (a) amorphous **QC**, (b) cholesterol, (c) Span[®] 40, (d) film **QC1**, (e) film **QC2**, (f) film **QC3**, (g) film **QC4** and (h) film **QC5**

Table B.5: Comparison of the XRPD diffraction peaks for **RM**, cholesterol, Span[®] 40, and thin films of **QC1 – QC5**

RM		Cholesterol		Span [®] 40		Film QC1		Film QC2		Film QC3		Film QC4		Film QC5	
°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%	°2θ	%
-	-	5.25	91.07	-	-	-	-	5.27	100.00	5.25	100.00	-	-	5.26	41.50
-	-	-	-	-	-	-	-	-	-	-	-	5.34	100.00	-	-
-	-	-	-	-	-	5.75	100.00	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	6.27	73.16	-	-	-	-	6.25	100.00
6.34	23.79	6.32	38.01	-	-	-	-	-	-	-	-	-	-	-	-
6.48	36.19	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.85	42.21	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.01	100.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.12	45.43	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.34	27.88	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.48	61.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.57	60.07	10.58	25.12	-	-	-	-	-	-	-	-	-	-	-	-
-	-	11.71	15.10	-	-	-	-	-	-	-	-	-	-	-	-
11.84	25.31	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.62	17.72	12.57	20.39	-	-	-	-	-	-	-	-	-	-	-	-
12.73	28.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.82	27.60	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	13.14	27.06	-	-	-	-	-	-	-	-	-	-	-	-
-	-	14.17	69.29	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	14.34	19.61	-	-	14.48	25.61	-	-
-	-	15.36	100.00	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	15.49	21.91	-	-	15.51	44.01
-	-	-	-	-	-	-	-	15.62	50.04	-	-	15.68	60.32	-	-
-	-	16.23	23.07	-	-	-	-	-	-	-	-	-	-	-	-
-	-	16.97	81.24	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	17.11	21.67	-	-	-	-
17.23	19.59	-	-	-	-	-	-	-	-	-	-	17.20	51.93	-	-
-	-	17.44	71.19	-	-	-	-	17.36	66.67	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	17.61	17.83	-	-	17.50	56.88
18.01	27.52	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18.09	24.93	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	18.14	74.90	-	-	-	-	-	-	-	-	-	-	-	-
-	-	18.20	69.94	-	-	-	-	-	-	-	-	-	-	18.27	55.83
-	-	-	-	-	-	-	-	18.44	52.69	18.38	17.06	18.50	52.95	-	-
-	-	18.95	18.17	-	-	-	-	-	-	-	-	-	-	-	-
-	-	19.50	18.17	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	21.65	100.00	21.73	69.27	21.74	47.45	-	-	21.63	36.15	21.47	32.32

Figure B.26 illustrates the five thin film samples of **QC** forms stayed amorphous, as no distinct diffraction peaks, due to the **RM**, were visible from the XRPD patterns in Figure B.26 (d) – (h). Film **QC3** presented with the highest diffraction peak intensity of all the films, since it contained the highest concentration of cholesterol. In Table B.5, the film of **QC1** showed a diffraction peak at $5.75^{\circ}2\theta$, which can be the result of cholesterol and a peak belonging to Span[®] 40. Two peaks correlating to cholesterol and Span[®] 40 are visible in film **QC2**. The film of **QC3** once again only had one peak resembling that of cholesterol. Three peaks in the film of **QC4** are observed, with two belonging to cholesterol and Span[®] 40 and the third being close to crystalline roxithromycin (**RM**). The film of **QC5** had peaks correlating with those of cholesterol and Span[®] 40.

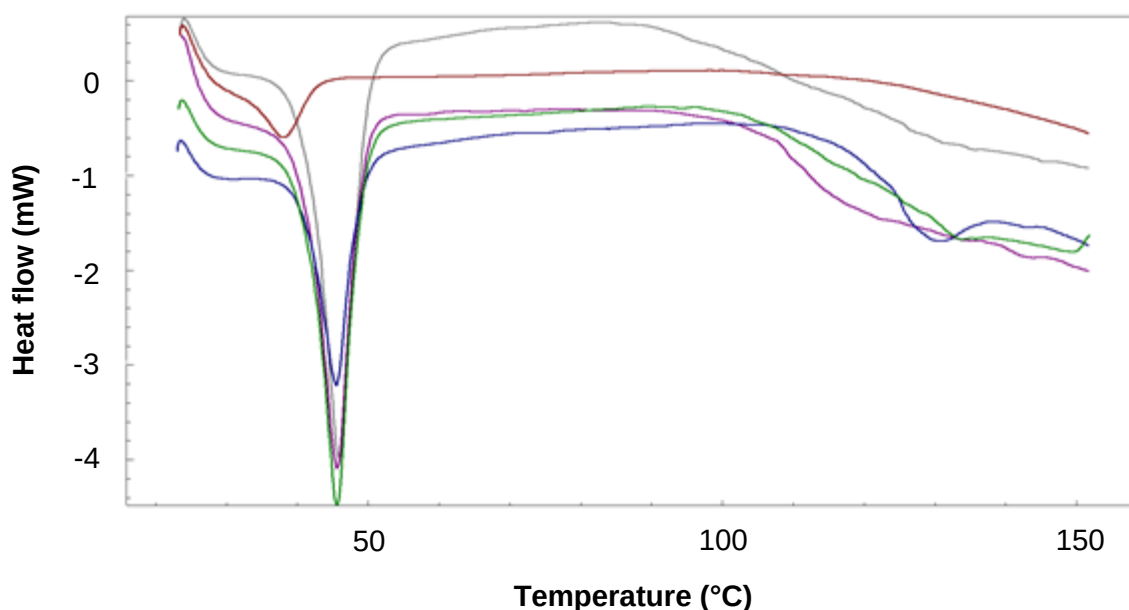


Figure B.27: An overlay of the thermograms obtained by DSC analysis for the powder mixtures used to prepare the thin films of **QC1** (grey), **QC2** (brown), **QC3** (purple), **QC4** (blue) and **QC5** (green)

The overlay of the five powder mixtures for the **QC** forms is shown in Figure B.27, where a big melting endotherm is visible in the region of $\cong 41 - 44^{\circ}\text{C}$ for all the samples except for **QC2** (brown).

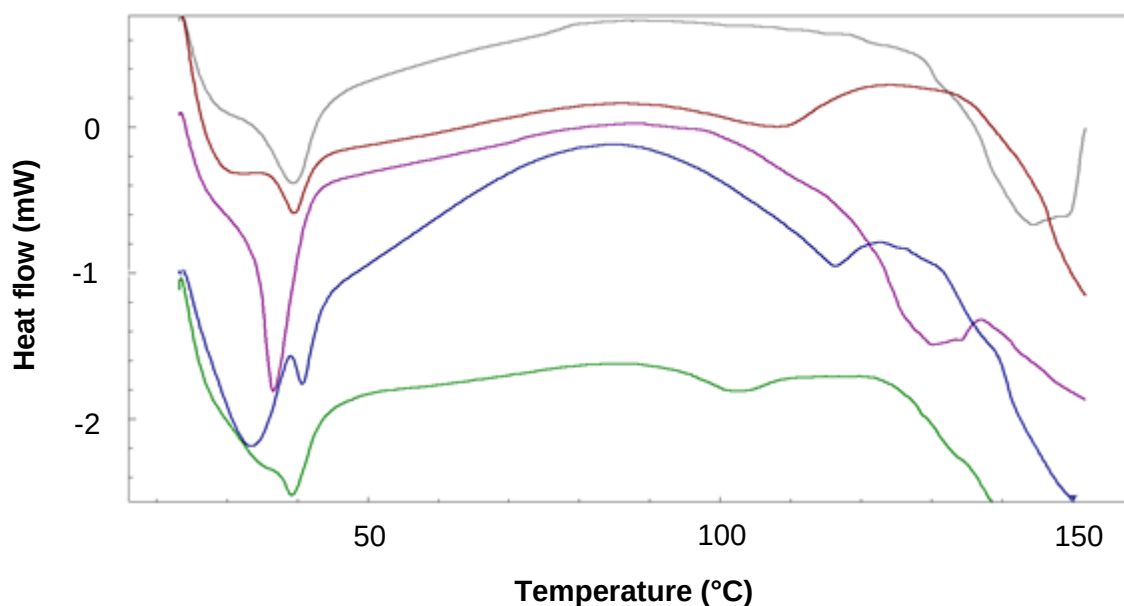


Figure B.28: An overlay of the thermograms obtained by DSC analysis for the prepared films of **QC1** (grey), **QC2** (brown), **QC3** (purple), **QC4** (blue) and **QC5** (green)

Figure B.28 displays the thermograms for the **QC** films, where smaller endotherms in the region of $\approx 35 - 40$ °C can be seen compared to their corresponding powder mixtures. It is assumed that these smaller peaks signify the T_g and consequently the films in the amorphous state.

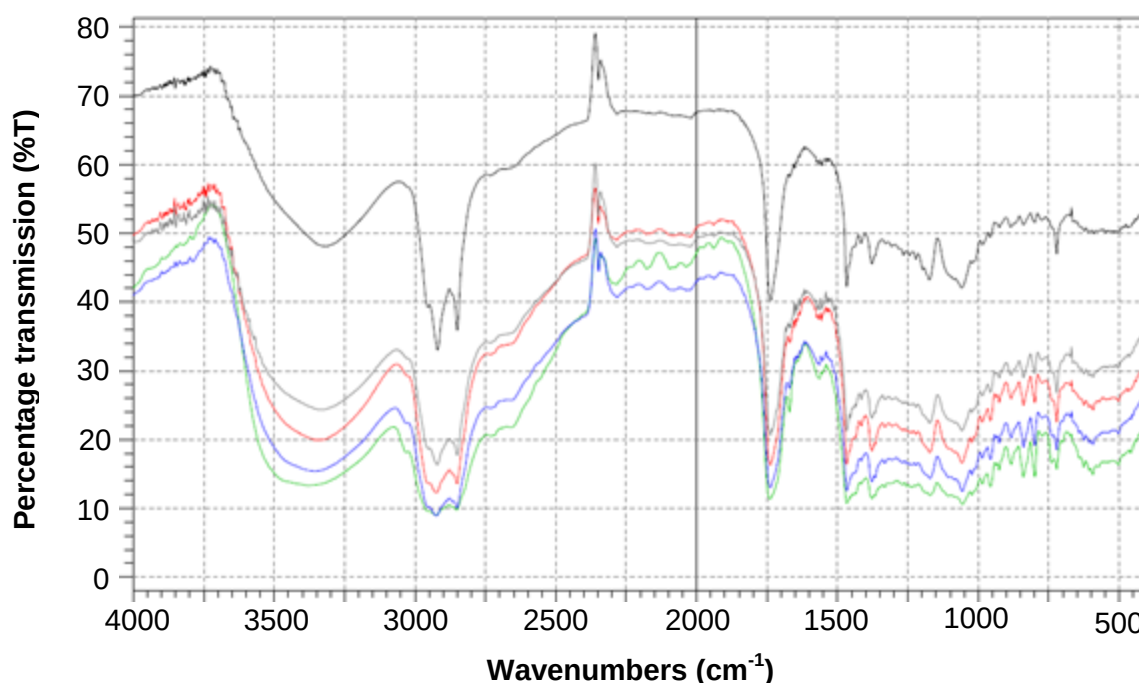
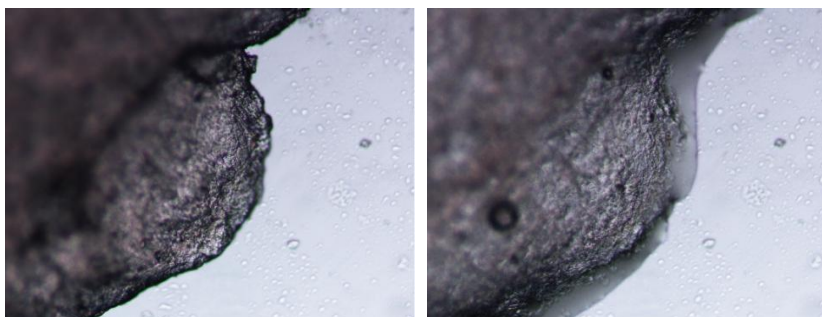


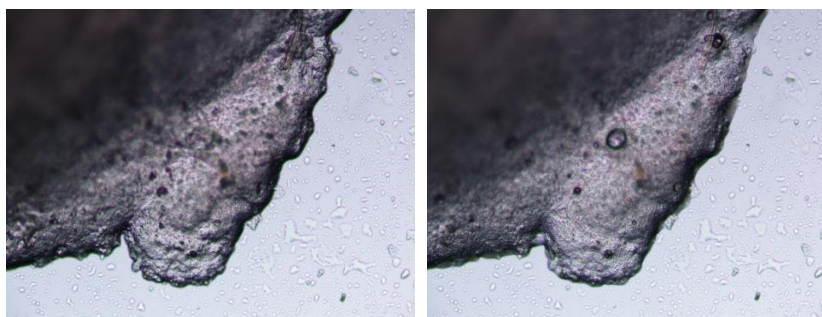
Figure B.29: An overlay of the FTIR spectra obtained for film **QC1** (black), film **QC2** (red), film **QC3** (green), film **QC4** (blue) and film **QC5** (grey)

Figure B.29 shows an overlay of the five thin films of the quench cooled samples, where once more it can be seen that a very broad peak is visible in the area between $3800 - 3200 \text{ cm}^{-1}$, representative of the amorphous nature of the films.

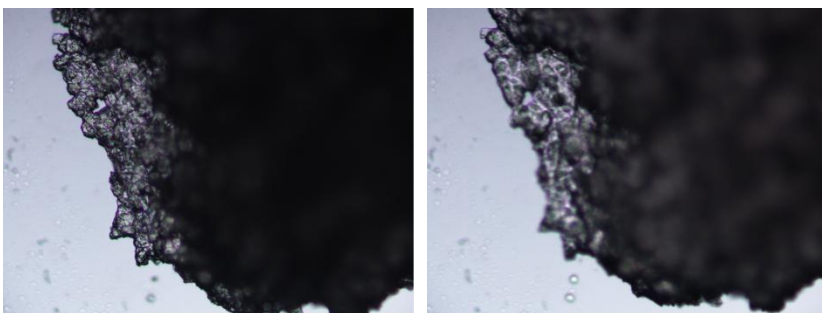
Film **QC1** started to melt at 43.7°C



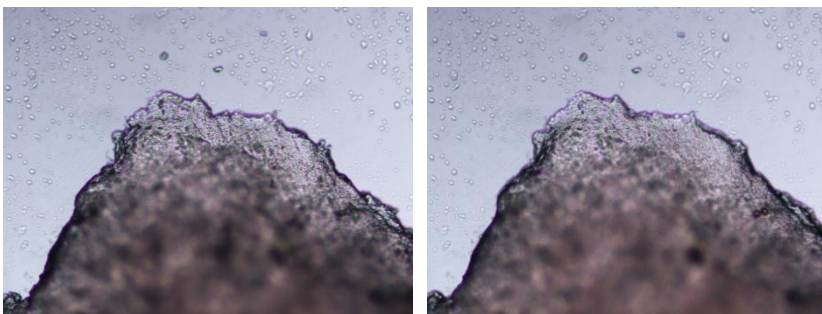
Film **QC2** started to melt at 43.1°C



Film **QC3** started to melt at 43.0°C



Film **QC4** started to melt at 43.3°C



Film **QC5** started to melt at 43.8°C

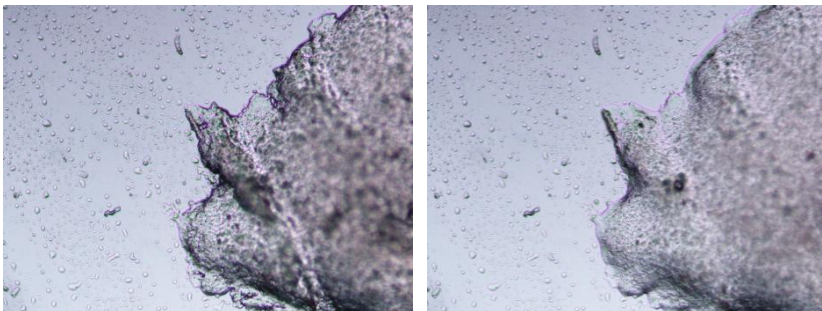


Figure B.30: HSM results for thin films of **QC1 – QC5**

In summary, the thin film of **QC3** again showed the highest intensity at the peak position of $5.26^{\circ}2\theta$, compared to the other films (Figure B.26). Only the film of **QC1** had a peak that could correspond with the peak of crystalline roxithromycin (**RM**). The amorphicity of the films can therefore be ranked as follow: **QC3** > **QC4** > **QC5** > **QC2** > **QC1**.

B.6 Conclusion

The physical characteristics and stability of the films was investigated by interpretation of the obtained XRPD patterns, DSC thermograms, FTIR spectra and hot stage photomicrographs (HSM). It was observed that the formation of the lipid films rendered **RM** samples into an amorphous state. It was also seen that the preparation method retained the **CD** and **QC** samples amorphous. During formation of the thin film samples, no recrystallisation occurred, therefore it was concluded that no crystallisation of the amorphous forms to the more stable **RM** occurred. It is further assumed that the film samples containing no peaks associated with that of **RM** have better stability and as a result, are more suitable to use. From the data collected, the **QC** form only had one film containing a small fraction of **RM**, whereas the **RM** films and the **CD** films showed more crystalline content. From this the expectation is that the **QC** samples will perform better than the other two forms during drug entrapment and subsequent membrane release and skin diffusion studies, as it is likely to show better drug entrapment due to complete amorphicity and to remain stable during the thin film production phase. Another anticipation is that film 3 would perform better than the rest, as in all three films (**RM3**, **CD3** and **QC3**) it had the highest degree of amorphicity.

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OED **see** Oxford English Dictionary

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Appendix C:

Formulation and characterisation of niosome vesicle systems for topical delivery

C.1 Introduction

In recent years, the topical route has competed with the oral route for the most successful novel research area in drug delivery (Barry, 2001:101). Even though topical delivery has many advantages, it presents formulators with one enormous obstacle - the stratum corneum. The stratum corneum serves as an excellent barrier of the skin, therefore protecting the body from outside influences and as a result, it is not so easy to overcome (Naik *et al.*, 2000:318). Vesicle systems designed to overcome this barrier and enhance drug delivery, need to have certain characteristics to improve delivery of the drug through the skin (Foldvari, 2000:417). Not only does the vesicle system need to be compatible with the API and non-irritating, the physicochemical properties of the API can influence the entire formulation, which in turn may affect the release of the drug and absorption into the skin (Weiss, 2011:471).

C.2 Formulation of niosome vesicle systems

For a product to be formulated, it needs to go through a series of stages with different tests, before the final formulation can be made and further studies can be conducted. There are three stages, namely pre-formulation, initial formulation and final vesicle formulation. The pre-formulation consists of a literature review to determine what all the components in the vesicle system will be and what effect they may have on the final formulation (this was done and discussed in the literature study in Chapter 2). The initial formulation was formulated and discussed in Appendix B to see whether the three solid-state forms of roxithromycin could be successfully included into thin films (precursors to niosomes) and if they remained stable. It was discovered during the initial pre-formulation that these thin films form amorphous solid dispersions, regardless of the solid-state form used. After this was done and confirmed, the study progressed and the five niosome dispersions without any encapsulated API were made to determine whether all five ratios would be able to form vesicles. The characterisation of each vesicle system is necessary to determine which concentration of the lipid ratios are the optimal formulation for topical delivery. Morphology (light microscopy and TEM) and droplet size were used to characterise these dispersions and to see if any could be ruled out. The final formulation would then be selected, depending on how well and if the vesicles in the different ratios formed during the initial formulation.

The tests used to characterise the final formulations included:

- Light microscopy
- Zeta-potential
- Droplet size and distribution
- pH determination
- Entrapment efficiency

C.2.1 Preparation of niosomes without the API

The lipid mixture containing cholesterol and Span[®] 40 were dissolved in 5 ml chloroform:methanol (2:1, v/v), in a small glass beaker. This was placed on a hot plate at 50 ± 2 °C above the transition temperature of the lipids (Kumar & Rasjeshwarrao, 2001:210) and stirred until a thin film formed at the bottom of the beaker and all the organic solvents had evaporated, 10 ml of Milli-Q[®] water was then added to the beaker to hydrate the film along with a magnet. The mixture was stirred and heated at a temperature of 50 ± 2 °C, for 30 min to form a milky suspension without any big droplets visible to the eye. Thereafter, the mixture was left to cool to room temperature (25 ± 2 °C) and sonicated on ice with a sonicator probe for 2 min to form multilamellar niosomes with good droplet size distribution (Csongradi, 2015:141). An ultrasonicator (Model UP200St, Hielscher Ultrasonics, Teltow, DE) was used. To allow swelling of the vesicles, the niosomes was left for 24 h at room temperature (Muzzalupo *et al.*, 2011:29).



Figure C.1: Preparation process of niosomes: (a) powder lipid mixture after weighing, (b) thin films that formed after evaporation of the organic solvents, (c) hydrated solution heated and stirred, (d) sonication of the dispersion with a sonicator probe and (e) final niosome dispersions

C.2.2 Characterisation tests performed on niosomes without the API

C.2.2.1 Morphology

Morphology is studied to determine the shape and size of the vesicles and was done using some form of microscope. The size, which is a very important characteristic, and shape of the vesicles are greatly affected by the temperature of the hydration medium and therefore it should always be above the gel to liquid phase transition temperature of the lipids (Mahale *et al.*, 2012:47). In this study, a light microscope was used to view the samples and to determine whether vesicles had formed, subsequently TEM was used to study the vesicles further by verifying that they had formed and by measuring their size.

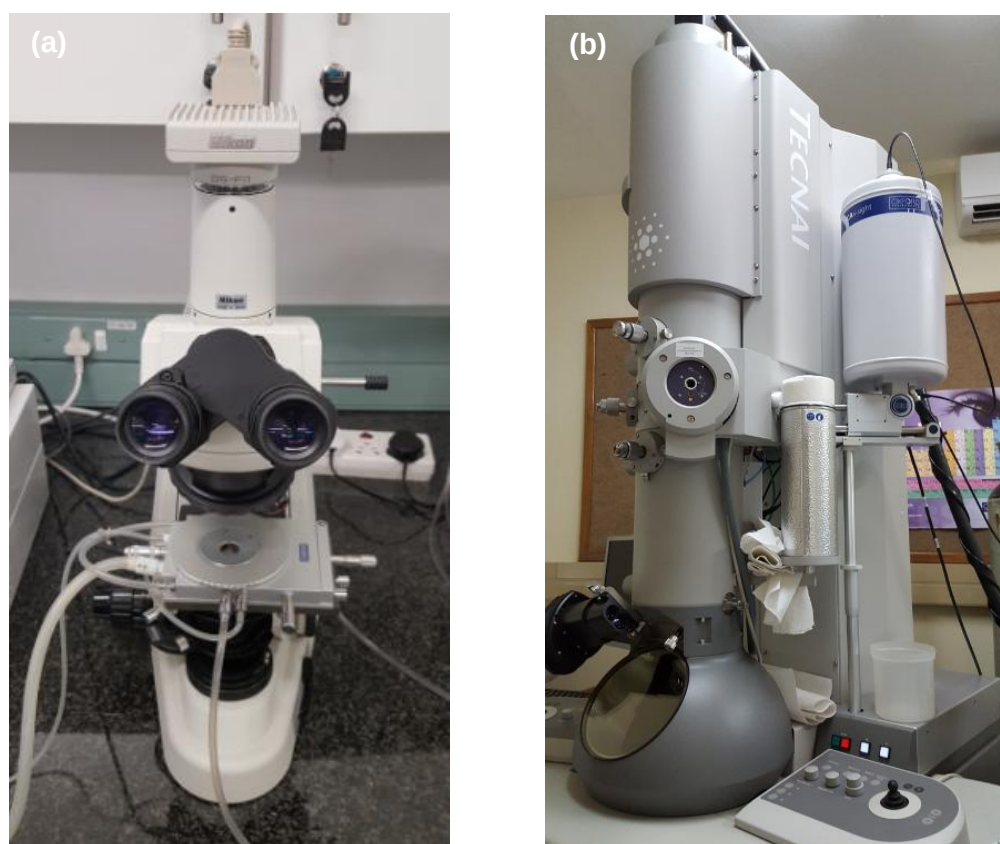


Figure C.2: Microscopy instruments used during morphology: (a) Nikon Eclipse E4000 microscope and (b) FEI Tecnai G2 high resolution transmission electron microscope

C.2.2.1.1 Light microscopy

Light microscopy was firstly used to see if the niosome vesicles had formed, since it is a quick and easy way to determine this. The light microscopy was performed on a Nikon Eclipse E4000 microscope, fitted with a Nikon DSFi1 camera (Nikon, Japan Linkam THMS600) (Figure C.2.(a)). The microscope was equipped with a T95 LinkPad temperature controller

(Surrey, England). A small drop of sample was placed on a transparent quartz sample holder, as well as two drops of Azure blue dye (Sigma, Johannesburg) and viewed under the microscope. The lipid layer of the vesicles reacted with the colorant and turned green, making them more visible. The samples were studied using a variety of magnifications, with a magnification of 50x finally used to view the samples ($n = 5$), since the best visual results were obtained.

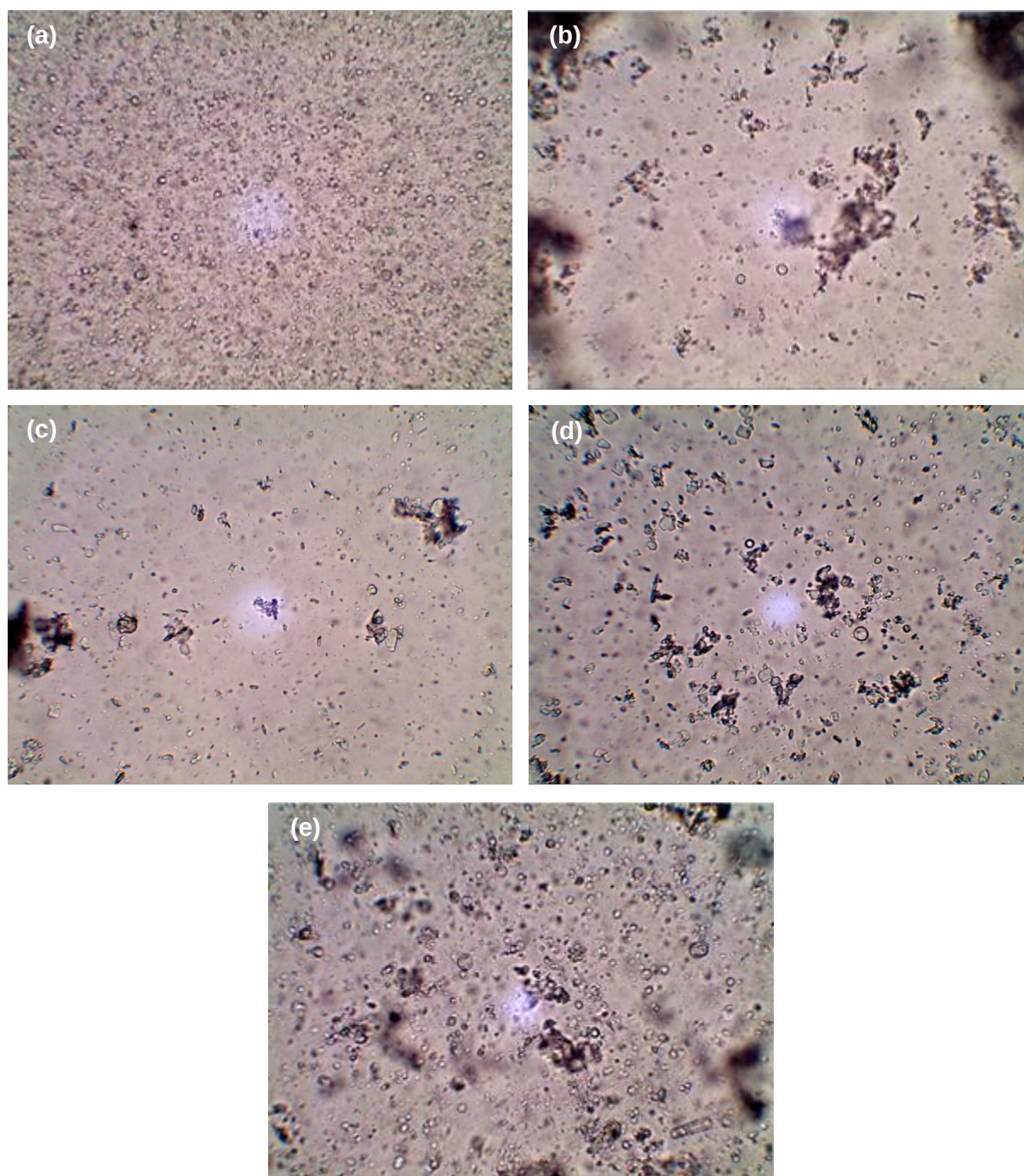


Figure C.3: Micrographs of the formed niosomes: (a) dispersion 1, (b) dispersion 2, (c) dispersion 3, (d) dispersion 4 and (e) dispersion 5

A few micrographs of each sample were taken, but only one micrograph of each was reported for each of the five dispersions. Figure C.3 displays the micrographs for the five niosomes obtained. The results from the light microscopy showed that all five ratios formed vesicles and some dispersions resulted in the formation of more vesicles than did others. It should be noted that this microscope only had low magnification ability and therefore only the larger vesicles were visible, whereas the smaller vesicles might not be observed.

C.2.2.1.2 Transmission electron microscopy

TEM was used to examine the morphology of the niosomes and to establish if all five samples did indeed form vesicles. An FEI Tecnai G2 20S-Twin 200 kV high resolution transmission electron microscope (HRTEM) (Czech Republic, Europe) (Figure C.2.(b)), fitted with an Oxford INCA X-Sight EDS System was used and operated by Dr A Jordaan (Laboratory for Electron Microscopy, NWU, Potchefstroom Campus). Each sample was diluted with distilled water roughly 10x due to the opaque appearance of the samples. A drop of sample was stratified onto a 300 mesh copper carbon-coated grid and left for 5 min to dry. Excess sample was removed by a piece of filter paper. The lipid layers of the vesicles were stained using a drop of osmium tetroxide and the sample left to dry for 15 min, before being inserted and viewed through the microscope. TEM can only be performed on vesicles containing no API, as precipitation of the API can cause damage to the microscope. Samples were viewed at magnifications of 5000 – 10000 x.

The micrograph results from the TEM are shown in Figure C.4. Visible in the results is the fact that all five ratios formed vesicles. The vesicles are surrounded by a membrane that is darker in colour due to the osmium reacting with the lipid layers of the vesicle. All the vesicles had a spherical shape, but the membrane thickness differed due to the different concentrations of cholesterol and Span[®] 40 used in each dispersion. However, from this it can be concluded that the vesicles consisted of the hydrophobic outer layer as described in Chapter 2.

When the samples were viewed through the microscope, dispersion 3 had the most vesicles visible compared to dispersion 5, which had the least. Dispersion 3 also displayed the best vesicle shape, as seen in Figure C.4.(c), compared to dispersion 5, which showed some degree of vesicle aggregation.

Reportedly the size of vesicles can range from 20 nm to about 50 000 nm (Moghassemi & Hadjizadeh, 2014:30). For topical drug delivery, particles with a particle size smaller than 3 µm could permeate through the stratum corneum, particles ranging from 3 – 10 µm can selectively penetrate through the follicular ducts; and those larger than 10 µm will remain on the surface of the skin and not penetrate through (Allec *et al.*, 1997:119; Barry, 2001:101; Bolzinger *et al.*,

2012:163;). All the dispersions resulted in vesicles smaller than 3 μm , therefore acceptable for topical drug delivery.

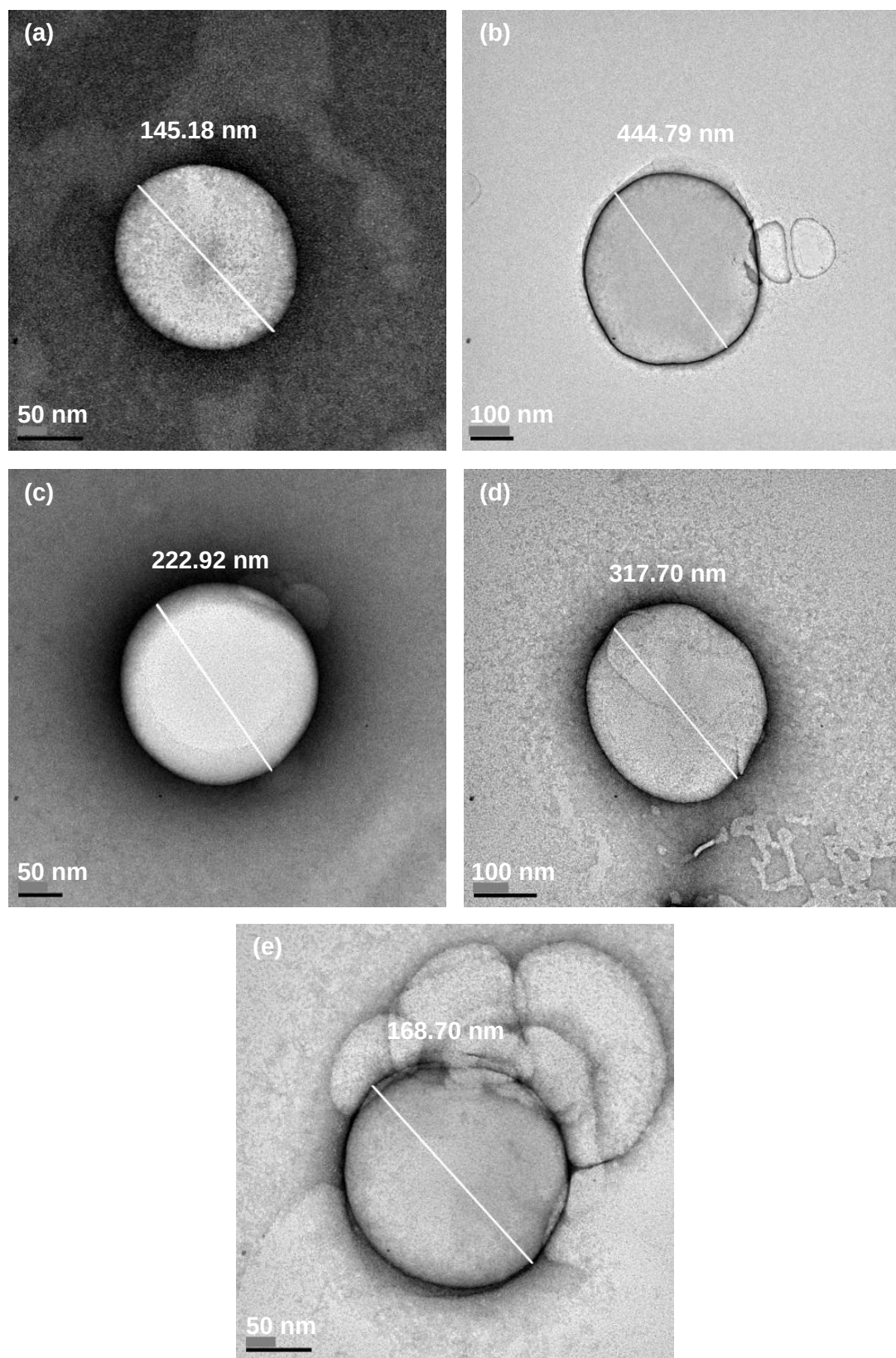


Figure C.4: TEM micrographs showing the appearance of vesicles: (a) dispersion 1, (b) dispersion 2, (c) dispersion 3, (d) dispersion 4 and (e) dispersion 5

C.2.2.2 Droplet size and distribution

The size of the niosome vesicles was determined by Dynamic Light Scattering (DLS), using a Malvern Zetasizer Nano ZS 2000 (Malvern Instruments Ltd, Worcestershire, UK) (Figure C.5.(b)). A drop of each sample was transferred to a small glass beaker then diluted 25 x with Milli-Q® water. Approximately 2 ml of this solution was injected into a clear disposable zeta cell (DTS1070 folded capillary cell) (Figure C.5.(a)) with a syringe and placed into the instrument. Each sample was measured in triplicate at a temperature of 25 ± 2 °C. The particle size (Z-average) measured in d.nm and the polydispersity index (Pdl) results were recorded, the Malvern Zetasizer also specified the quality of the results by saying “good” or “refer to quality report”. The Pdl are defined as the measure of the broadness of the size distribution calculated, and ranges on a scale of 0 to 10 (Shaw, 2017). A Pdl value closer to 0 indicates the sample is monodispersed, while values closer to 1 are an indication of a very polydispersed sample. All samples recorded had a “good” result quality report, thereby indicating the results were acceptable. The size of the vesicles, were well within the desired range.

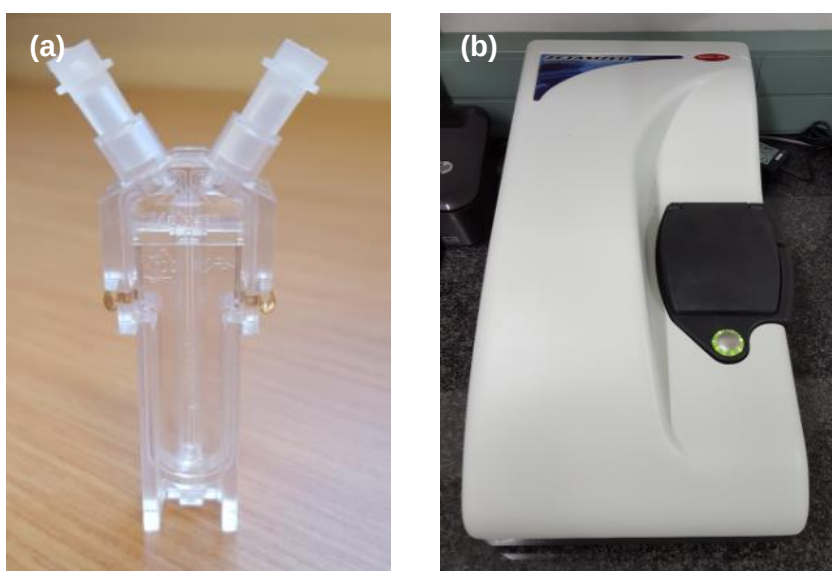


Figure C.5: (a) A clear disposable zeta cell and (b) a Malvern Zetasizer Nano ZS 2000

Table C.1: Size and the average Pdl for the five preparations of niosomes containing no API

Placebo	Measurement			Average droplet size (d.nm)	Average Pdl
	1	2	3		
1	267.4	285.0	288.8	280.4 ± 11.418	0.612 ± 0.034
2	294.0	270.0	276.0	280.0 ± 12.490	0.415 ± 0.049
3	230.7	210.6	191.7	211.0 ± 19.503	0.652 ± 0.043
4	312.3	299.2	294.0	301.8 ± 9.430	0.358 ± 0.005
5	347.6	319.1	311.9	326.2 ± 18.879	0.385 ± 0.021

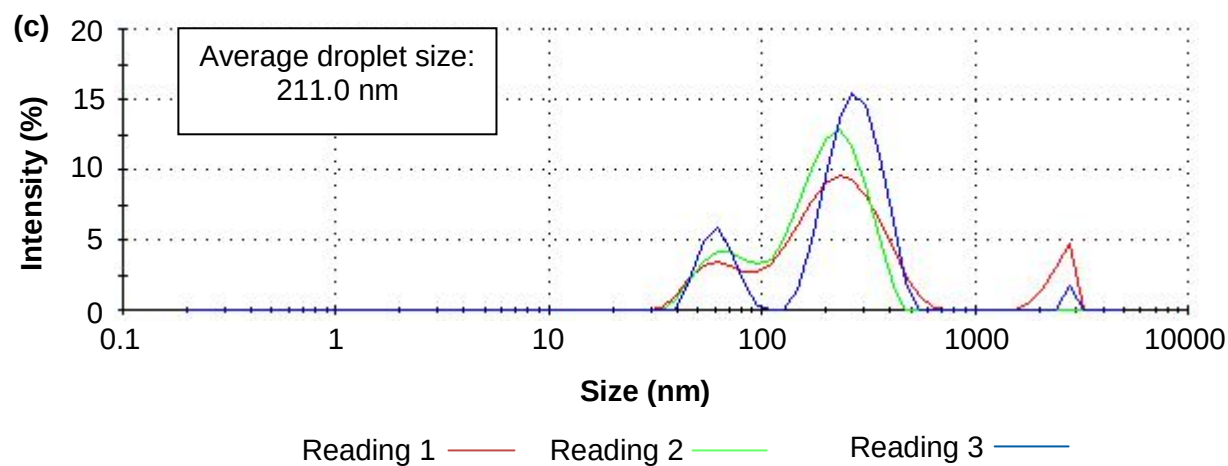
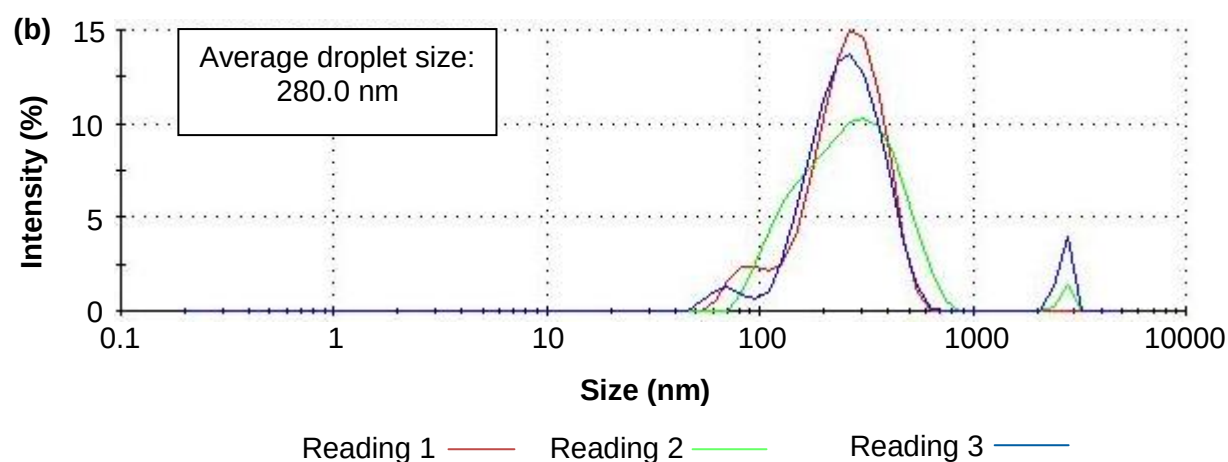
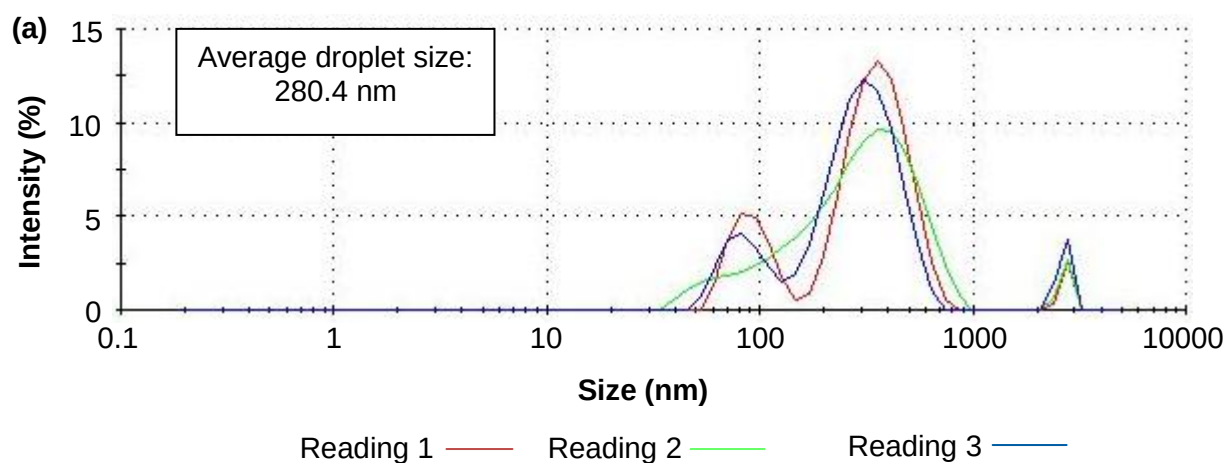


Figure C.6: Size distribution results of niosomes: (a) dispersion 1, (b) dispersion 2 and (c) dispersion 3

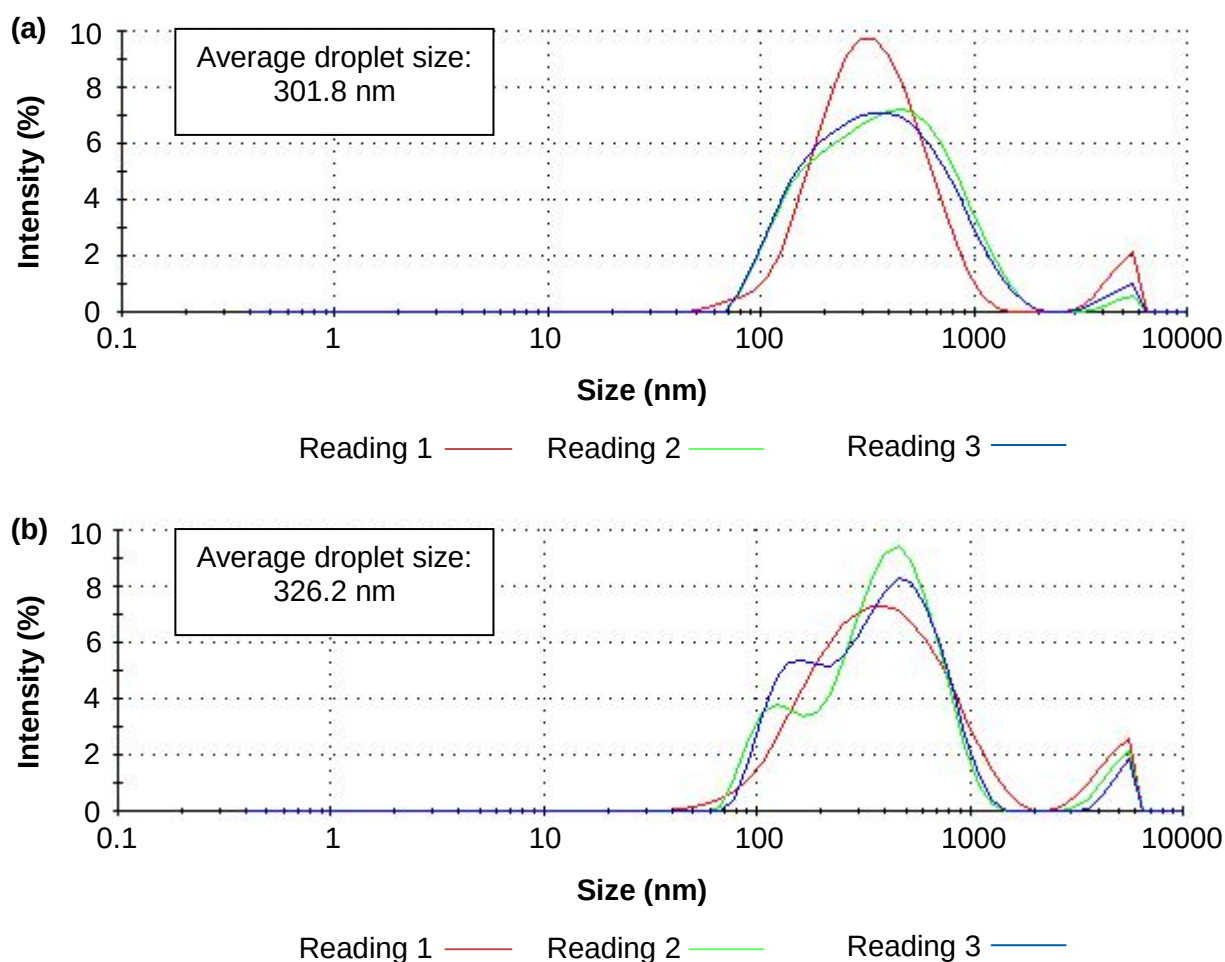


Figure C.7: Size distribution results of niosomes: (a) dispersion 4 and (b) dispersion 5

C.2.3 Conclusion on the vesicle systems containing no API

The morphology results in Figures C.3 and C.4 clearly indicates all five ratios formed niosomes. The size distribution results were favourable for topical delivery, since all niosomes had average droplet sizes below 3 μm and therefore all five dispersions ratios were chosen for the final formulation stage, which would also be tested topically. Further characterisation tests were performed on these dispersions as seen in this Appendix.

C.2.4 Preparation of niosomes encapsulating roxithromycin

The same method as mentioned in Section C.2.1 was used, except that roxithromycin (crystalline or amorphous) was weighed (2% w/v) and mixed with the lipid mixture and dissolved in the organic solvent. The API was entrapped by means of a passive loading method, which entails dissolving the drug in the organic solvents because the drug is lipophilic (Moghassemi & Hadjizadeh, 2014:30). The rest of the method stayed the same.

C.2.5 Characterisation tests performed on niosomes encapsulating roxithromycin

C.2.5.1 Light microscopy

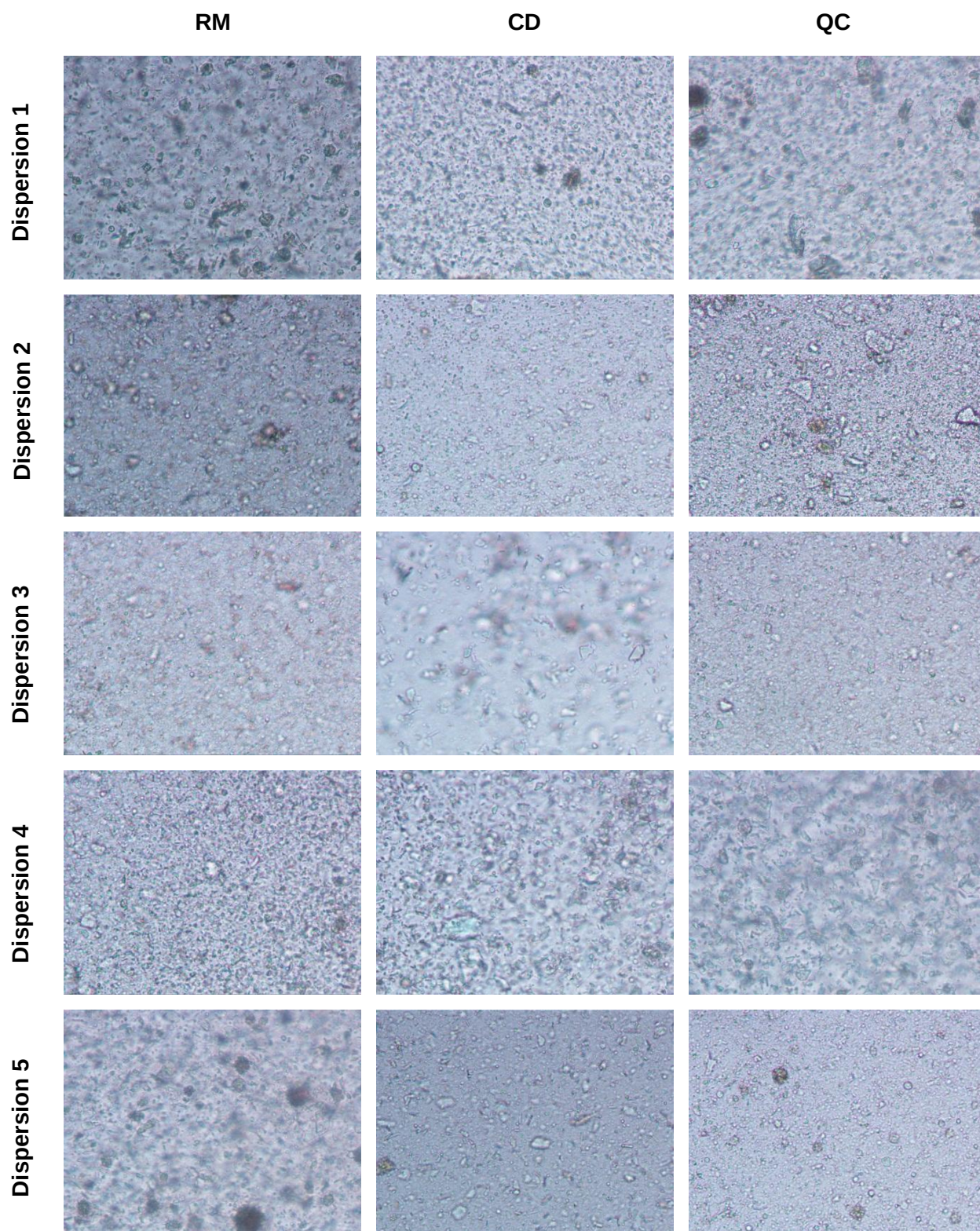


Figure C.8: Micrographs of niosomes encapsulating roxithromycin using light microscopy

Light microscopy was performed on all the samples. Figure C.8 shows the samples of **RM**, **CD** and **QC** encapsulated into the five niosomes, resulting in fifteen final dispersions. Although the vesicles could clearly be seen through the microscope, it may be difficult to see them from the resulting micrographs.

Some of the dispersions showed more vesicles than did others, with a few having particles that did not dissolve so well. From the images, it is apparent that all the dispersions formed vesicles of different sizes and therefore the exact sizes of the formed niosomes had to be determined next.

C.2.5.2 Zeta-potential and droplet size

The charge and size of the vesicles have a substantial effect on the encapsulation of the drug, as well as the stability of the niosomes (Kumar & Rajeshwarrao, 2011:213). A Malvern Zetasizer Nano ZS 2000 (Malvern Instruments Ltd, Worcestershire, UK) was used to determine the zeta-potential and droplet size of the niosomes. A drop of each sample was diluted 25 x with distilled water, mixed thoroughly and placed in a clear disposable zeta cell with a syringe and the reading taken in triplicate at $25 \pm 2^\circ\text{C}$. For sufficient electrostatic stabilisation, the zeta-potential values should range between -41.7 and -58.4 mV (Moghassemi & Hadjizadeh, 2014:30). Literature states that negative zeta-potential values strongly improve skin permeation of a drug during transdermal delivery (Duangjit *et al.*, 2011:6).

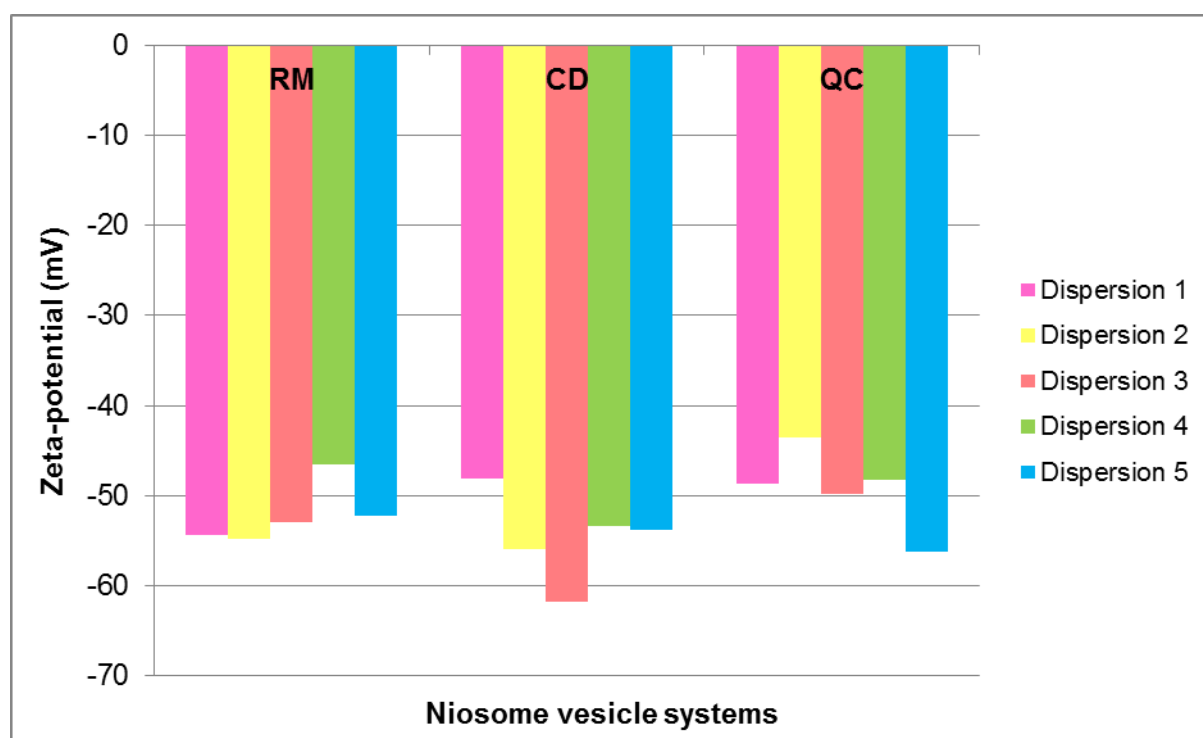


Figure C.9: Zeta-potential results for the niosome dispersions

It was observed in Figure C.9 that all the dispersions had zeta-potential values lower than - 45 mV, indicating stable dispersions formed, which will resist aggregation and flocculation (Silva *et al.*, 2012:860). Highly negative values are desirable as they improve permeation of the API into the skin, which may in turn lead to good topical delivery of the API during the skin diffusion studies.

Table C.2: Droplet size and Pdl results for the niosome dispersions containing the API

Niosome dispersions	Measurement			Average droplet size (d.nm)	Average Pdl
	1	2	3		
RM1	298.4	315.2	271.1	294.9 ± 22.257	0.405 ± 0.093
RM2	1124.0	1164.0	1121.0	1136.3 ± 24.007	0.616 ± 0.052
RM3	739.4	710.9	730.4	726.9 ± 14.569	0.645 ± 0.057
RM4	970.4	966.1	947.1	961.2 ± 12.399	0.712 ± 0.010
RM5	658.8	650.6	691.7	667.0 ± 21.752	0.588 ± 0.045
CD1	298.7	256.0	260.4	271.7 ± 23.486	0.351 ± 0.040
CD2	1058.0	1079.0	1058.0	1065.0 ± 12.124	0.907 ± 0.095
CD3	992.0	979.7	977.3	983.0 ± 7.886	0.746 ± 0.028
CD4	459.0	458.5	463.9	460.5 ± 2.888	0.523 ± 0.025
CD5	570.9	563.2	527.5	553.9 ± 23.156	0.622 ± 0.052
QC1	299.7	288.4	280.4	289.5 ± 9.697	0.650 ± 0.054
QC2	1340.0	1310.0	1269.0	1306.3 ± 35.642	0.998 ± 0.003
QC3	884.4	889.5	869.3	881.1 ± 10.504	0.683 ± 0.046
QC4	385.2	409.2	422.7	405.8 ± 18.993	0.383 ± 0.019
QC5	729.9	702.3	721.2	717.8 ± 14.111	0.583 ± 0.021

The sizes of the niosomes are influenced by the repulsion between the entrapped drug and the bilayers (Kumar & Rajeshwarrao, 2011:213). This can clearly be seen by the fact that the sizes of the empty vesicles containing no API are relatively smaller than the niosomes encapsulating the API. In Table C.2, it can clearly be seen that all the sizes measured for the niosomes were well within the desired ranges for topical drug delivery, and all the samples would have no problem permeating through the skin. The dispersion containing cholesterol and Span[®] 40 in the ratio of 1:1 (**RM2**, **CD2** and **QC2**) had the largest particle size, whereas the dispersion containing the excipients in the ratio of 0.5:1.0 (**RM1**, **CD1** and **QC1**) had the smallest size. None of the samples displayed a Pdl lower than 0.3, indicating the resulting niosomes do not form monodispersed systems, but rather polydispersed systems, meaning they present with variation in sizes within the dispersion. Polydispersed systems will be expected in this case because the samples were not filtered after preparation. If it had been filtered, there would be a

greater chance to obtain niosomes with a more uniform size, an aspect requiring investigation during the preparation of the final product, as it might influence the homogeneity and stability of the final formulation.

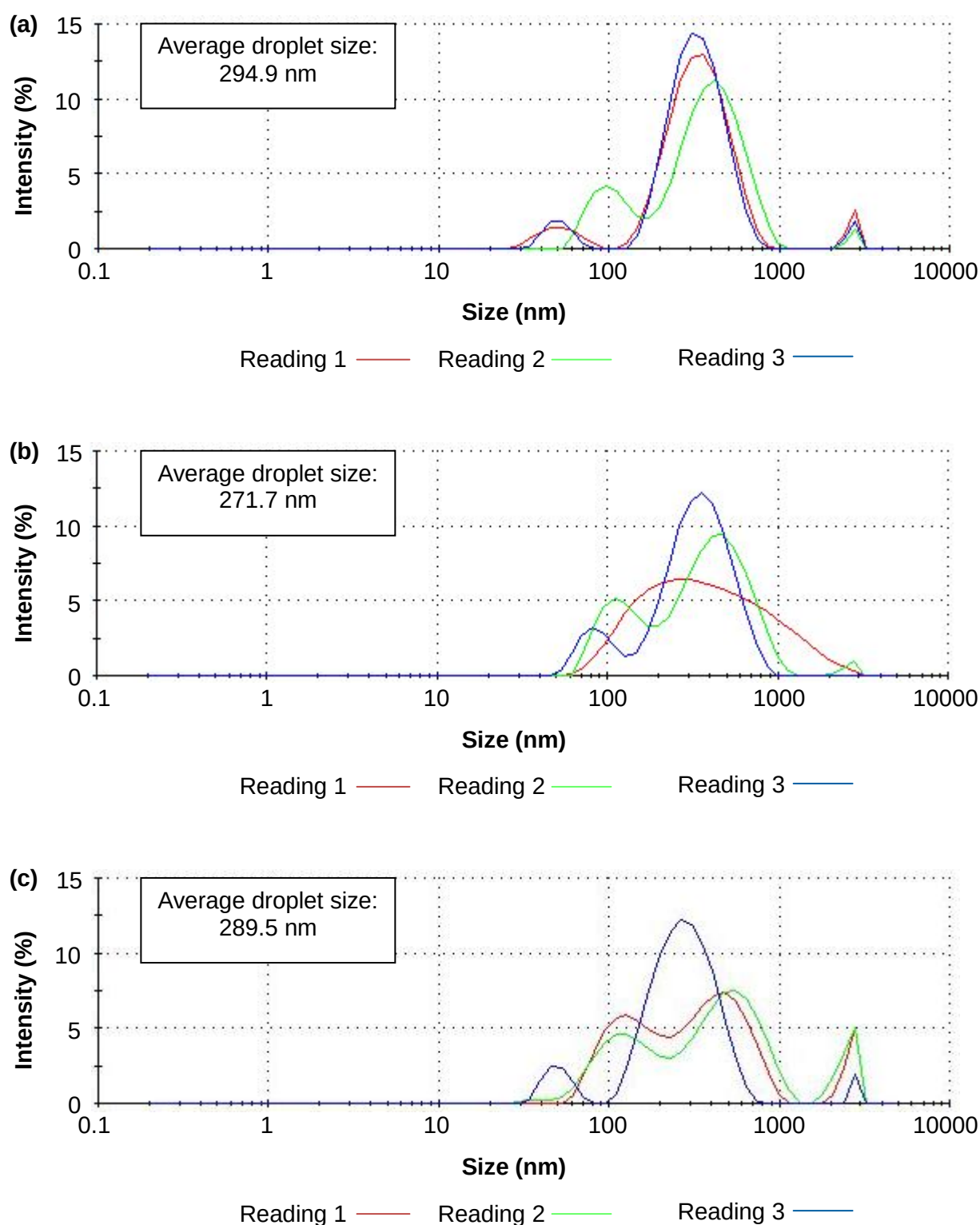


Figure C.10: Size distribution results for the niosomes of dispersion 1 containing: a) **RM**, b) **CD** and c) **QC**

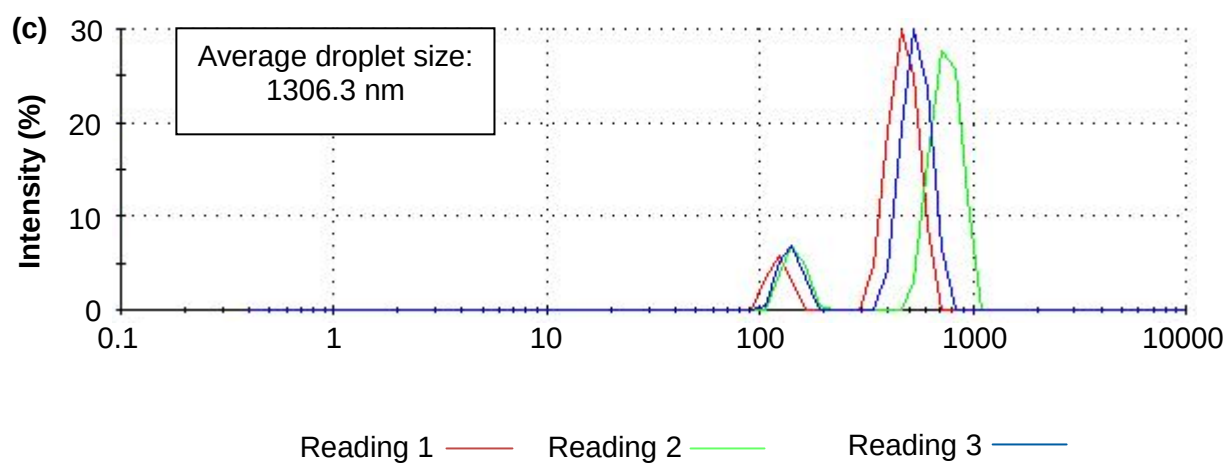
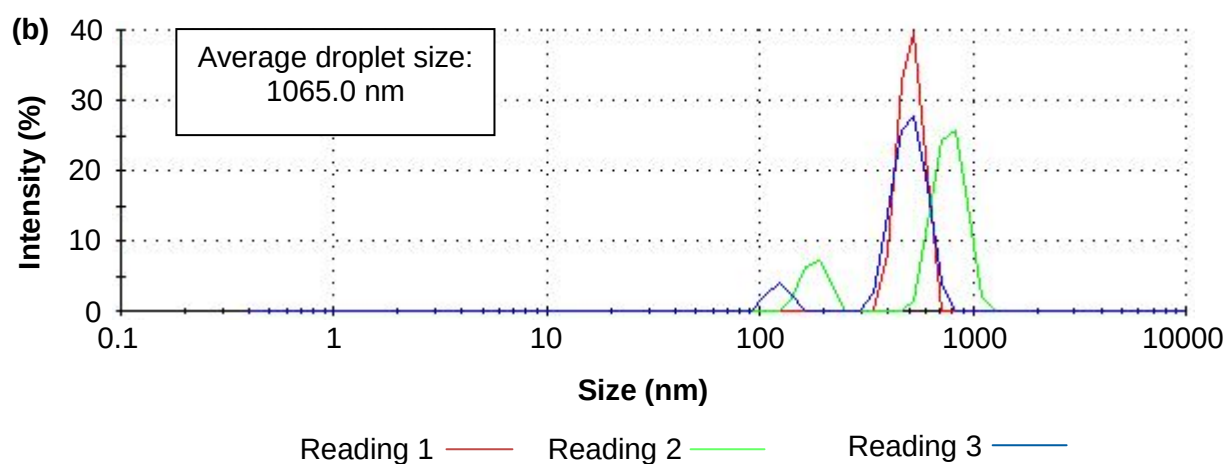
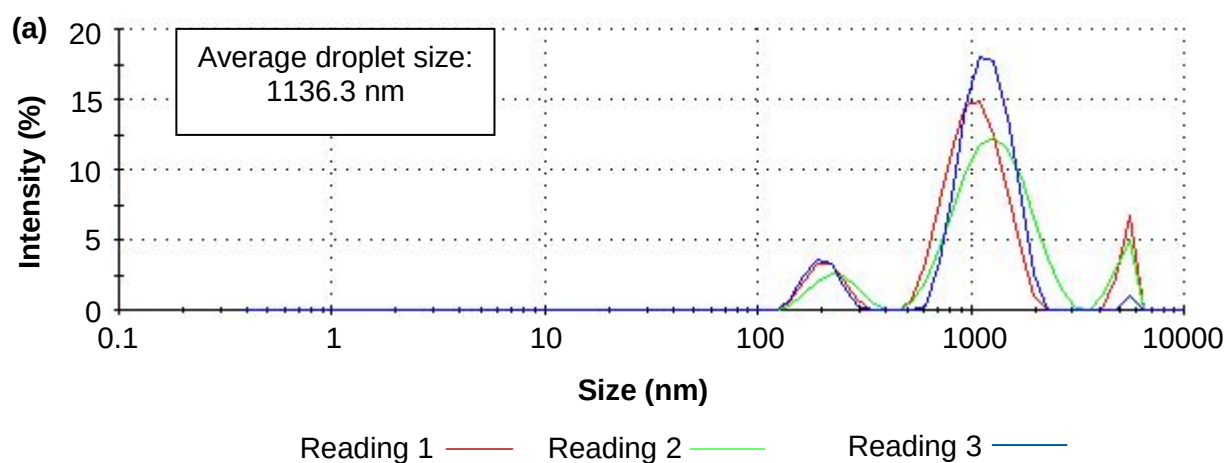


Figure C.11: Size distribution results for the niosomes of dispersion 2 containing: a) **RM**, b) **CD** and c) **QC**

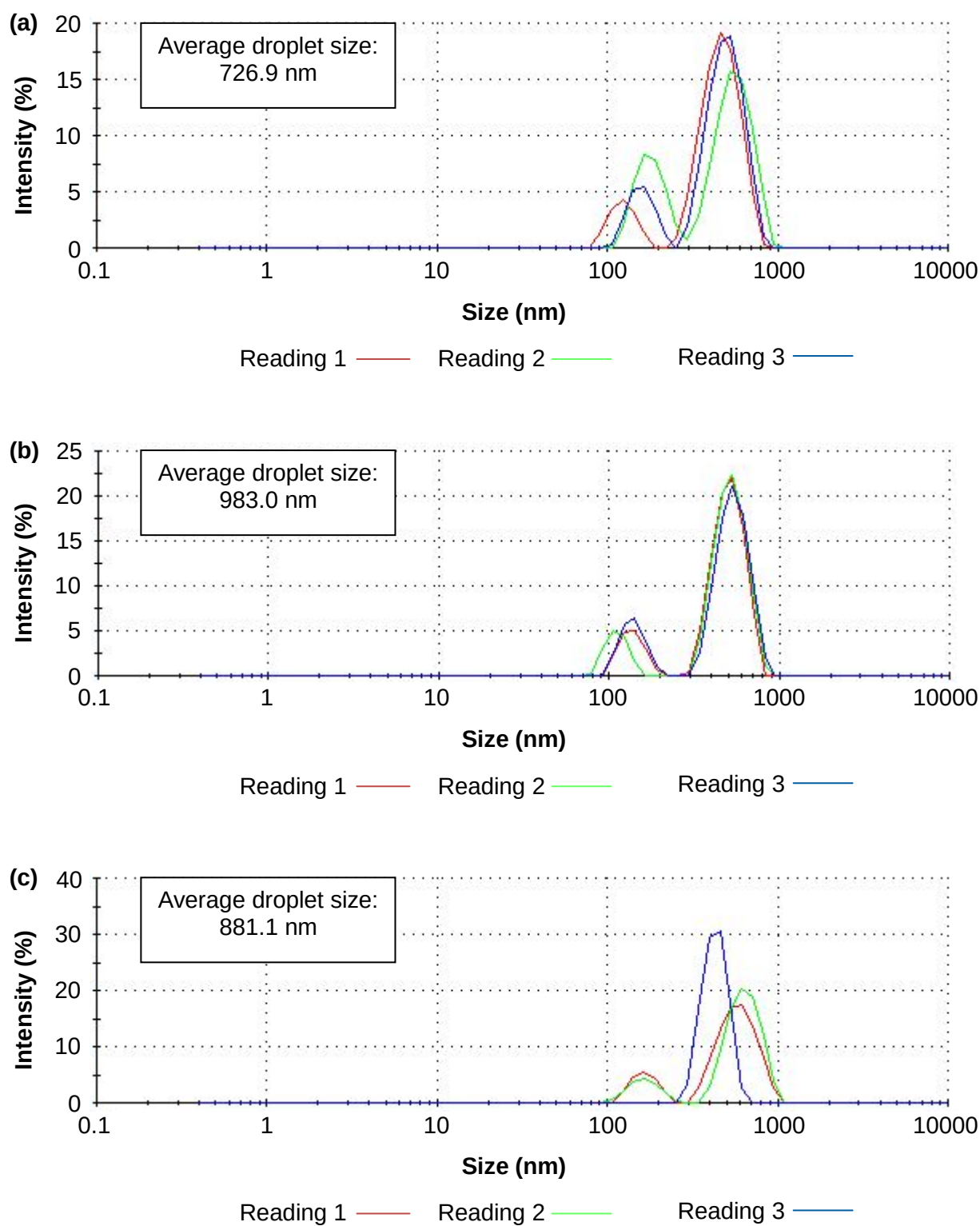


Figure C.12: Size distribution results for the niosomes of dispersion 3 containing: a) **RM**, b) **CD** and c) **QC**

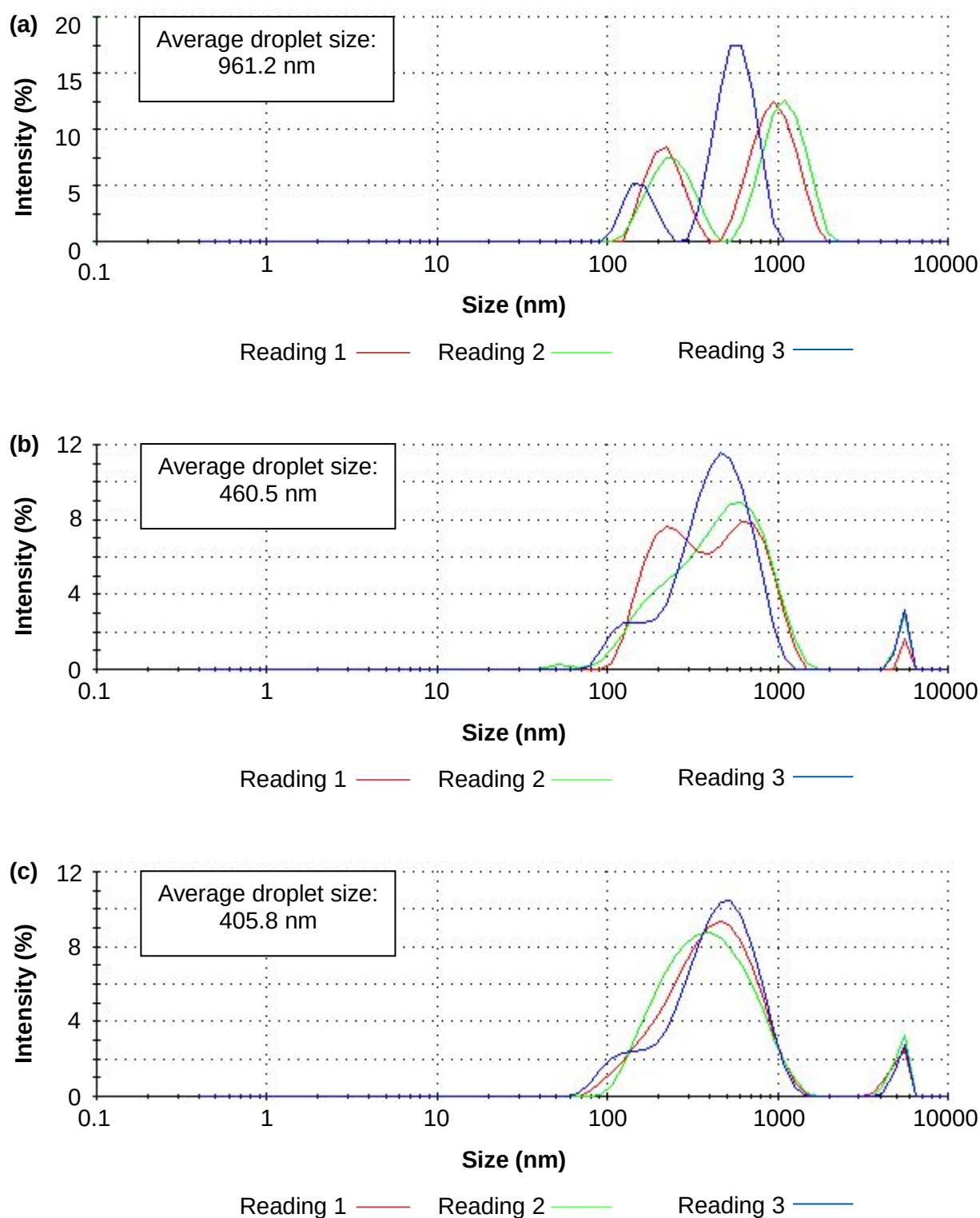


Figure C.13: Size distribution results for the niosomes of dispersion 4 containing: a) **RM**, b) **CD** and c) **QC**

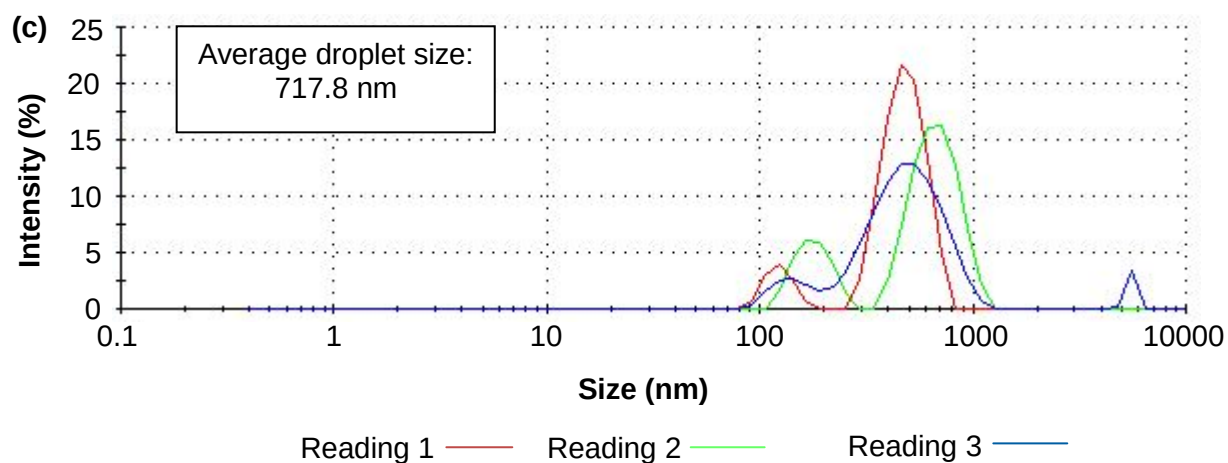
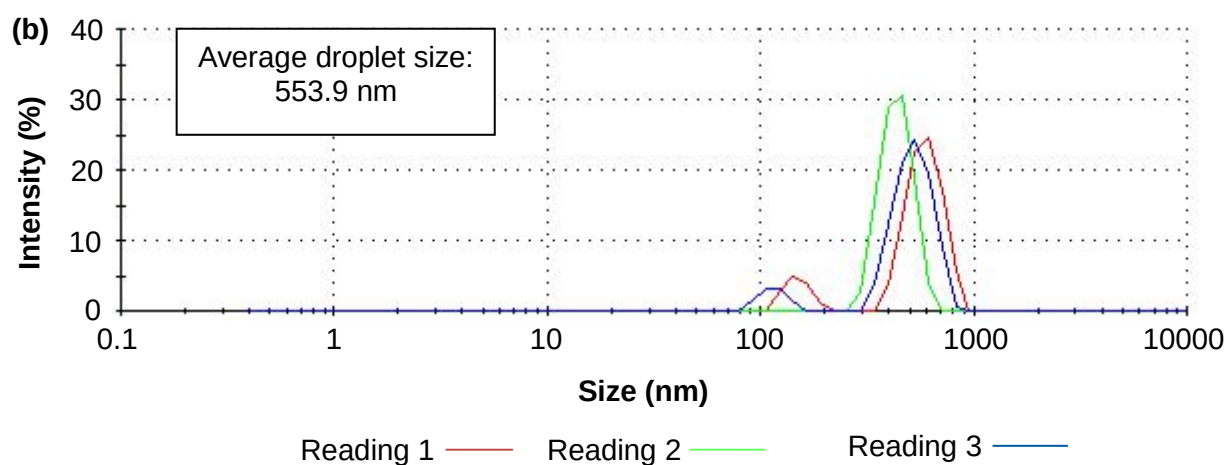
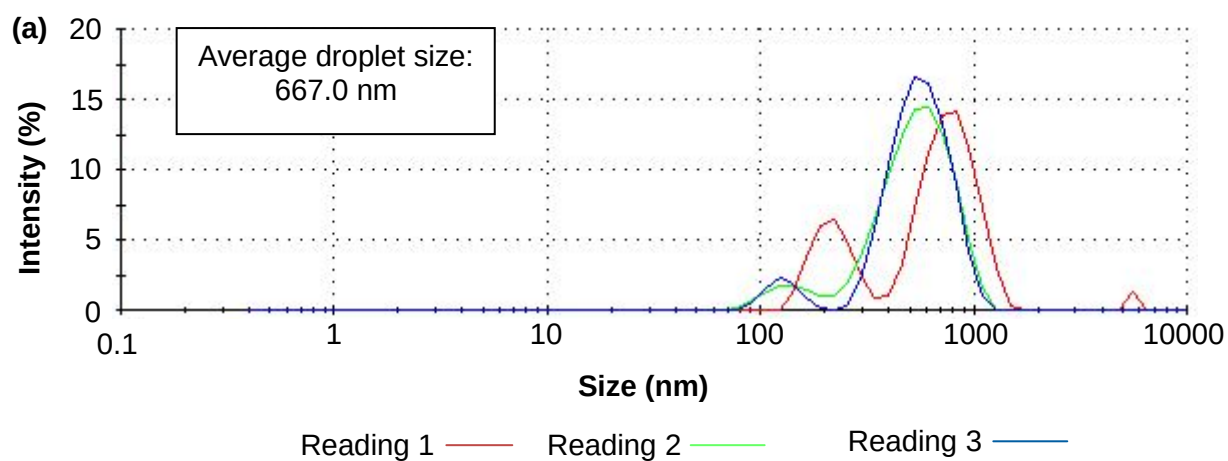


Figure C.14: Size distribution results for the niosomes of dispersion 5 containing: a) **RM**, b) **CD** and c) **QC**

C.2.5.3 pH determination

The pH of the skin varies, because the stratum corneum has a pH ranging between 4.2 and 5.6 and the viable epidermis ranges between 7.3 and 7.4 (N'Da, 2014:20787). The pH for ideal transdermal drug delivery ranges between 5.0 and 9.0 (Sharma *et al.*, 2011:75; Naik *et al.*, 2000:319). A Mettler Toledo® pH meter (Mettler Toledo, CU), equipped with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU), was used to determine the pH values of the niosomes (Figure C.15). The pH was measured by placing the probe in approximately 20 ml of each sample and waiting for the measurement to stabilise before recording the value. Each sample was measured in triplicate and the same samples were measured one week later, to ensure the pH remained stable.

Table C.3: pH measurement results for the niosomes

Niosomes	Measurement			Average pH
	1	2	3	
RM1	8.07	8.08	8.06	8.07 ± 0.010
RM2	7.93	7.92	7.94	7.93 ± 0.007
RM3	8.07	8.09	8.04	8.07 ± 0.025
RM4	7.95	7.99	7.96	7.97 ± 0.021
RM5	7.98	7.96	7.96	7.97 ± 0.012
CD1	8.23	8.18	8.18	8.20 ± 0.029
CD2	8.53	8.51	8.50	8.51 ± 0.015
CD3	8.39	8.36	8.36	8.37 ± 0.017
CD4	8.41	8.40	8.40	8.40 ± 0.006
CD5	8.22	8.17	8.17	8.19 ± 0.029
QC1	8.19	8.18	8.18	8.18 ± 0.006
QC2	8.39	8.38	8.37	8.38 ± 0.010
QC3	8.63	8.63	8.61	8.62 ± 0.012
QC4	8.20	8.18	8.16	8.18 ± 0.020
QC5	8.16	8.14	8.14	8.15 ± 0.012

It is well known that a pH of 7 is neutral and all the niosome samples measured had a slightly basic pH, ranging between 7.93 and 8.61. Hence, the pH of all the samples lay between 5.0 and 9.0 and therefore would cause no harm to the skin when applied topically. The pH values were also relatively close to the pH of the receptor phase used during the diffusion studies (pH 7.4), ensuring that no precipitation would form.



Figure C.15: A Mettler Toledo® pH meter with a Mettler Toledo® InLab® 410 electrode

C.2.5.4 Entrapment efficiency

EE% is used to determine the amount of the API, which was encapsulated into the niosomes. The EE% of niosomes are influenced by the method of preparation, the stability of the dispersion and the intrinsic properties of the vesicle, which includes nature of the membrane components, cholesterol content and the size of the vesicles (Mahale *et al.*, 2012:51).

In order to measure entrapment, the samples firstly need to be centrifuged to form a clear supernatant that can be extracted. An aliquot of each dispersion was transferred into 20 ml plastic test tubes, with each tube containing the same volume of sample. The samples were centrifuged with the assistance of Me S Lowe at the Laboratory for Applied Molecular Biology (LAMB) of the NWU, Potchefstroom Campus. An OptimaL-100 XP ultracentrifuge (Beckman Coulter, USA), with a 50.2 Ti Rotor, was used to centrifuge the samples at a rotation speed of 25 000 rpm for 30 min at a temperature of 25 ± 2 °C. This process formed two phases, the pellet at the bottom containing roxithromycin entrapped in niosomes and the clear supernatant containing the untrapped free drug (as seen in Figure C.16). The supernatant was extracted with a syringe and transferred into HPLC vials for subsequent HPLC analysis. The experiment was performed in triplicate, with freshly prepared samples each time. The following equation adapted from Xiang *et al.* (2009:186) was used to calculate the EE% of the vesicle systems.

$$EE\% = \frac{\text{Drug (total)} - \text{Drug (supernatant)}}{\text{Drug (total)}} \times 100$$

Equation C.1

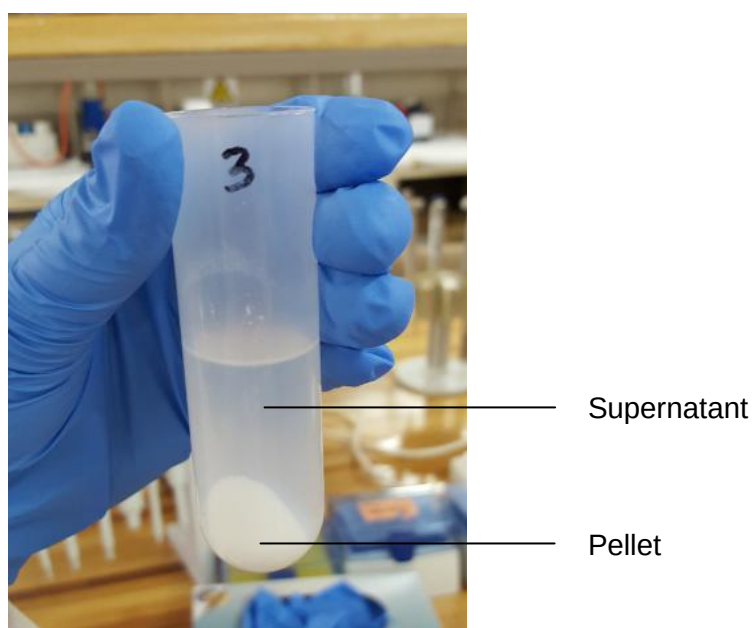


Figure C.16: A plastic test tube containing the sample after it was centrifuged

Table C.4: Entrapment efficiency results

Niosomes	Experiment			Average EE%
	1	2	3	
RM1	98.18	97.45	97.94	97.86 ± 0.372
RM2	98.25	97.81	98.16	98.07 ± 0.232
RM3	98.51	98.07	98.08	98.22 ± 0.251
RM4	98.30	98.49	97.92	98.24 ± 0.290
RM5	97.97	97.85	98.04	97.95 ± 0.096
CD1	97.73	97.67	98.97	98.12 ± 0.734
CD2	97.73	97.54	99.07	98.11 ± 0.834
CD3	97.94	97.91	99.02	98.29 ± 0.632
CD4	97.61	97.54	98.96	98.04 ± 0.800
CD5	98.11	97.84	98.90	98.28 ± 0.551
QC1	97.58	98.06	98.21	97.95 ± 0.329
QC2	97.87	98.03	98.54	98.15 ± 0.350
QC3	97.92	99.24	98.82	98.66 ± 0.674
QC4	97.15	98.09	98.47	97.90 ± 0.680
QC5	96.96	98.35	98.00	97.77 ± 0.723

The EE% was excellent, with the dispersions encapsulating more than 97% of the drug and **QC3** showing the highest encapsulation with 98.66 ± 0.674%. It is generally observed that lipophilic molecules display very high entrapment efficiencies (Bhushan *et al.*, 2014:414) and due to the lipophilic nature of the API, this could explain the high entrapment obtained. The inclusion of cholesterol increases the EE% and the phase transition temperature of the

surfactant also affects entrapment, as a result of the high transition temperature of Span 40[®], providing better entrapment (Chandu *et al.*, 2012:28-29).

C.3 Conclusion

Empty vesicles were firstly prepared to determine if the different ratios of the excipients would result in the formation of vesicles, which was characterised by means of light microscopy and TEM, after which the sizes were also determined. All the niosome dispersions formed vesicles therefore none of the ratios could be eliminated. The vesicles were prepared once again, but this time the API was entrapped inside the vesicles. Characterisation tests were performed on the loaded niosomes with more tests conducted to evaluate all the properties of the vesicles including determination of the size, zeta-potential, pH and EE%.

All the niosome dispersions showed good or fairly good results with some doing slightly better than did others. The difference was not great enough to rule out any dispersion and therefore, the fifteen niosomes were further studied by means of membrane release and skin diffusion studies. The results of these studies are recorded in Appendix D, after which a choice was made to see which niosome formed the optimal and final vesicle dispersion.

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Appendix D:

Diffusion studies of roxithromycin encapsulated niosomes for topical delivery

D.1 Introduction

The primary purpose of topical delivery is for the drug to have a local effect on the skin, compared to transdermal delivery, where the drug is intended for systemic effects, with the skin only being the point of entry (Goyal *et al.*, 2016:77). In this study, the topical delivery of roxithromycin was the aim, as it treats acne at the site of administration, in and on the affected area of the skin. The need for topical treatment of diseases located in the deep layers of the skin, like acne, is increased as conventional dosage forms have poor retention in the skin and therefore, are powerless to effectively treat these conditions (More *et al.*, 2016:197).

The stratum corneum is a highly organised structure and acts as the rate-limiting barrier against the penetration of drugs through the skin (Foldvari, 2000:418). As a result, the API and vesicle systems need to have certain characteristics to permeate through the skin. Roxithromycin is slightly soluble in water (BP, 2016) and was therefore encapsulated into niosome vesicle systems, to help improve the solubility problems and also enhance topical delivery. The two amorphous forms were also investigated to see if they would provide better diffusion results, as they are more soluble than the crystalline form (Biradar *et al.*, 2006:22).

Niosomes are the best vesicle system for topical delivery, according to Kumar and Rasjeshwarrao (2001:214), because they enhance skin penetration and act as a drug reservoir over a prolonged period of time. By localising the drug action, it reduces systemic toxic effects and enhances the effectiveness and the bioavailability of the drug (Karim *et al.*, 2010:375). Niosomes are said to, 1) increase the time of the drug in the stratum corneum and epidermis and 2) reduce the absorption of the drug into the systemic circulation (Uchechi *et al.*, 2014:212), which is exactly characteristic of topical delivery.

Diffusion studies consisted of membrane release and skin diffusion studies. Membrane release studies were performed prior to skin diffusion studies to determine if the encapsulated roxithromycin was being released from the niosome dispersions. Skin diffusion studies followed by tape stripping were performed to determine whether any transdermal and/or topical delivery into the skin had been achieved for all the dispersions, respectively.

The solubility and log D values for crystalline roxithromycin and the two amorphous forms were previously determined by Csongradi (2015:177-178), therefore it was not necessary to determine it again.

D.2 Methods

D.2.1 HPLC analysis of the roxithromycin concentration

The concentration of the different roxithromycin solid-state forms during membrane release and skin diffusion studies was determined by means of HPLC analysis. An HPLC method was adapted and validated with the assistance of Prof JL du Preez at the ATL of the NWU, Potchefstroom Campus (See Sections A.2. and A.3), in a controlled environment at a temperature of $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$. The receptor phase, collected at predetermined times, was analysed during the membrane release studies. For the skin diffusion studies, the receptor phase as well as the samples collected from the tape strips and cut skin pieces were analysed.

HPLC analysis was performed using a Venusil XBP C₁₈ (2) reverse phase 150 x 4.6 mm column with a 5 μm particle size (Agela Technologies, Newark, DE). The mobile phase consisted of 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ buffer solution, with the pH adjusted to 7.0 with diluted ammonia (7% w/v) solution. The buffer was mixed with methanol in the ratio 15:85. The mobile phase was filtered through a 0.22 μm nylon membrane filter (Membrane Solutions[®], Kent, USA) before use. The flow rate was 1 ml/min with a default injection volume of 50 μl , the run time was set at 10.0 min, the detection wavelength was 205 nm and the retention time for roxithromycin was ± 5.0 min.

A fresh standard solution was prepared before every diffusion study by weighing 10 mg of API and dissolving it in a 100 ml volumetric flask with methanol. A dilution was further made for the skin diffusion studies by transferring 10 ml of the solution into a 100 ml volumetric flask and making it up to volume with methanol. The standard solution was injected in duplicate in different injection volumes (2.0, 10.0, 20.0, 30.0, 40.0 and 50.0 μl) to create a standard curve for analysis.

D.2.2 Preparation of the receptor phase

The receptor phase consisted of phosphate buffer solution (PBS, pH 7.4), which was prepared by weighing 6.5 g of potassium phosphate (KH_2PO_4) and dissolving it in 250 ml of Milli-Q[®] water, then weighing 1.5 g of NaOH pellets and dissolving them in 400 ml of Milli-Q[®] water. The NaOH was added to the KH_2PO_4 and stirred with a magnetic stirrer. The mixture was filled to 1000 ml with Milli-Q[®] water, after which the pH was measured using a Mettler Toledo[®] pH meter (Mettler Toledo, CU) equipped with a Mettler Toledo[®] InLab[®] 410 electrode (Mettler Toledo, CU) and adjusted to 7.4 with phosphoric acid (H_3PO_4). To remain fresh, the receptor phase was kept in a refrigerator ($2 - 8\text{ }^{\circ}\text{C}$) between experiments.

D.2.3 Preparation of the donor phase

The donor phase consisted of the niosome vesicle systems encapsulating the different solid-state forms of roxithromycin. During the membrane release and the skin diffusion studies, loaded niosome dispersions, as well as empty niosomes (placebo), are needed and were prepared as seen in Sections C.2.4 and C.2.1, respectively. Before each experiment, the niosome samples were freshly prepared, covered with Parafilm® and left at room temperature. Approximately 1 h before the diffusion study would start, the niosome dispersions were placed in a water bath set at 32 °C.

D.2.4 Membrane release studies

Membrane release studies were performed to determine whether the three forms of roxithromycin were released from the vesicle system in which it was encapsulated. Each of the three forms consisted of 5 formulations and therefore a total of 15 membrane release studies were performed. Every study comprised of 12 Franz cells, 10 of which contained the formulation with API and 2 containing the placebo (the same formulation but with no API). The placebos were used as the control group during each experiment. The method for the membrane release study was as follows:

- The receptor phase, PBS at pH 7.4, was placed in a water bath, 1 h before the study would start at 37 °C, to represent the temperature of the blood in the human body (Williams, 2013:685).
- The donor phase was placed in a separate water bath, 1 h before the study, at 32 °C to represent the temperature of the surface of the skin (Williams, 2013:685) (Figure D.1.a).
- The even side of the donor and receptor compartments of the 12 Franz cells were thinly greased using Dow Corning® (Sigma-Aldrich, Germany) high vacuum grease.
- A small magnetic stirring rod was placed inside the receptor compartment and then a hydrophilic polyvinylidene fluoride (PVDF) (Pall® Life Sciences, Michigan, USA) membrane filter, with a pore size of 0.45 µm and diameter of 25 mm, was placed onto the receptor compartment with the rough side facing upwards.
- The donor compartment was securely placed on top of the receptor compartment and sealed on the sides with vacuum grease (Figure D.1.c).
- The Franz cell was clamped together with a horseshoe clamp to hold the two compartments tightly together to prevent any leakage of the cells (Figure D.1.d).
- While holding the Franz cells downwards, the receptor compartment was filled with 2 ml of PBS (pH 7.4) using a syringe and needle with a small tube attached to it, ensuring no

air bubbles formed. A permanent marker was used to make a line at the 2 ml mark, to ensure it was filled to the same point every time.

- 1 ml of the niosomes containing the API was placed into the donor compartment of the 10 Franz cells and 1 ml of the placebo was placed into the remaining 2 Franz cells. Each donor compartment was covered with a piece of Parafilm® and a cap to prevent evaporation or loss of the donor phase.
- The 12 Franz cells positioned on a tray were placed into the 37 °C water bath (Grant Instruments, UK) onto a Variomag® (Variomag, USA) magnetic stirrer plate, with the receptor compartments fully submerged in the water. A thermometer was placed into the bath to ensure the temperature was constantly maintained and the lid of the water bath was closed (Figure D.1.e).
- The receptor phase of each Franz cell was extracted and refilled with new PBS (pH 7.4, 37 °C), with extraction times at 1, 2, 3, 4, 5 and 6 h, respectively (Figure D.1.f). Before the extraction took place, the level of the PBS for every cell was noted, to determine whether it stayed the same, decreased or increased.
- Each Franz cell had its own syringe with a needle and tube to extract the PBS. The extracted receptor phase was placed into marked HPLC vials respectively (Figure D.1.b).
- The samples were analysed through HPLC analysis, as mentioned in Section D.2.1.



Figure D.1: Diffusion study work area: (a) water bath set at 32 °C, (b) syringes with needles and tubes attached used for extraction of the receptor phase, Dow corning® vacuum grease, PVDF membranes and marked HPLC vials, (c) assembled and greased vertical Franz cells, (d) Franz cells clamped with horse shoe clamps, (e) Franz cells placed inside the water bath, as well as the PBS (pH 7.4) and a thermometer and (f) water bath set at 37 °C showing the time it was placed inside and the 6 extraction times

D.2.5 Skin diffusion studies

D.2.5.1 Skin ethics and collection

The skin used during the skin diffusion studies was obtained from female Caucasian patients after undergoing cosmetic surgery on the abdomen. An informed consent form was completed by each patient giving us permission to use their skin for research purposes, with patient information remaining confidential. Ethical approval for the use of biological material (i.e. human skin) during experiments was obtained from the Research Ethics Committee of the North-West University, with NWU-00114-11-A5 as the reference number. After the surgery was performed, the skin was collected in plastic bags, placed in an icebox containing icepacks and transported to the university where the full-thickness skin was frozen at -20 °C in the Transdermal laboratory (TDL).

D.2.5.2 Skin preparation

The preparation of the skin was performed in the TDL. A day before the preparation would commence, the skin was taken out of the freezer and left in containers to defrost. The skin was dermatomed using a Zimmer™ electric dermatome Model 8821 (Zimmer, Ohio, USA) (Figures D.2.a & D.2.b), after it was visually examined for any stretch marks, lesions or hair, as this could affect diffusion in that specific area. The dermatome was used to cut the skin into pieces with a thickness of 400 µm and the skin was placed on Whatman® filter paper, with the stratum corneum facing upwards. The skin was stretched out tightly, avoiding any fold as seen in Figure D.2.c. The filter paper with the skin sample was wrapped in aluminium foil and stored in a clear plastic bag in a freezer at -20 °C until used. The bag was clearly marked with the preparation date and the name of the person who prepared the skin (Figure D.2.d). The unused skin or that had stretch marks or lesions were placed into black bags and put into the freezer until the waste removal company approved by the NWU came to collect the waste. All the gloves and paper towels that came into contact with the skin were discarded into the bio-hazardous bins, which are reserved for biological materials that need incinerating. Due to the limited amount of skin that can be used from one donor, more than one donor was used to complete all fifteen skin diffusion studies, but each sample with the 12 Franz cells consisted of the same donor.



Figure D.2: Skin preparation: (a) An electric Zimmer™ dermatome, (b) dermatome power station, (c) 400 μ m dermatomed skin and (d) clear plastic bag with information about the skin preparation on it

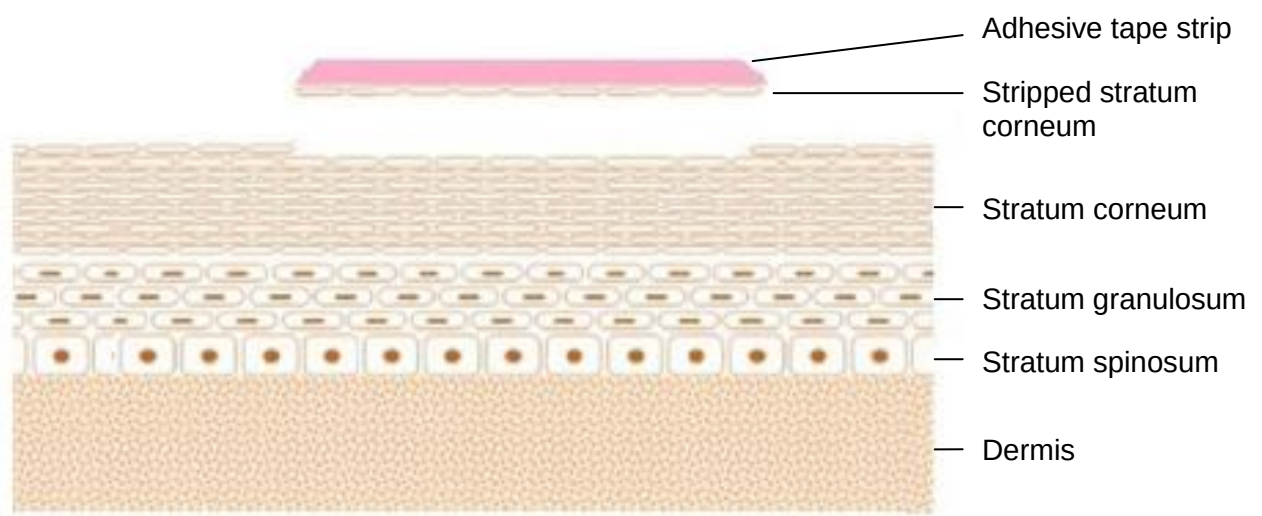


Figure D.3: Tape stripping of the stratum corneum (Adapted from Nair *et al.*, 2013:426).

D.2.5.3 *In vitro* skin diffusion studies

The same method discussed during the membrane release studies (Section D.2.4) was used in the skin diffusion studies, with a few exceptions. The prepared skin samples were placed on the receptor compartment of the Franz cell with the stratum corneum facing upwards rather than the membranes. The receptor phase (PBS, pH 7.4) was extracted only once at 12 h, and then the skin samples were further used for tape stripping.

D.2.5.4 Tape stripping

The tape stripping method was used to determine the amount of roxithromycin that diffused through the stratum corneum after 12 h (Pellett *et al.*, 1997:91). After completion of the diffusion studies, the skin sample was carefully removed from the Franz cell and pinned with needles to a solid surface on a piece of Parafilm® (Figure D.5.a). The skin was then gently dabbed with clean paper towel to remove any excess formulation (Figure D.5.b). The diffusion area of the skin was visible through the indent made by the Franz cell. The SCE, the top layer of the skin, was removed using 3M Scotch® Magic™ tape (Figure D.4.e). The tape was cut into pieces the size of the diffusion area on the skin and sixteen pieces were needed for each skin sample (a total of 192 strips per skin study). The tape was placed on the skin and removed consecutively until the skin had a shiny appearance (Figure D.5.c). The first piece of tape was discarded, as it could possibly still contain excess donor phase. Each set of fifteen strips containing parts of the SCE was placed in a polytop with 5 ml of absolute ethanol (99.9% analytical grade), making sure it covered all the strips. After the tape stripping, the remaining skin (ED) was cut into smaller pieces (Figure D.5.d) and placed in a polytop with 5 ml of absolute ethanol. These steps were done for all 12 Franz cells and resulted in 24 polytops (Figure D.5.e), all of which were stored in the refrigerator at 2 – 8 °C for a period of 8 h. Thereafter the samples were filtered with a 0.45 µm PVDF syringe filter (Figure D.4.d) with a glass fibre pre-filter (Agela Technologies) into vials (Figure D.5.f) and analysed using an HPLC.



Figure D.4: Skin diffusion components: (a) cut up skin pieces on the receptor compartment of the Franz cell with the skin facing up and the filter paper facing towards the receptor phase, (b) scissor and tweezers, (c) HPLC vials for the receptor phase, SCE and ED extraction fluid, (d) Clarinert™ syringe filter and (e) 3M Scotch® Magic™ tape

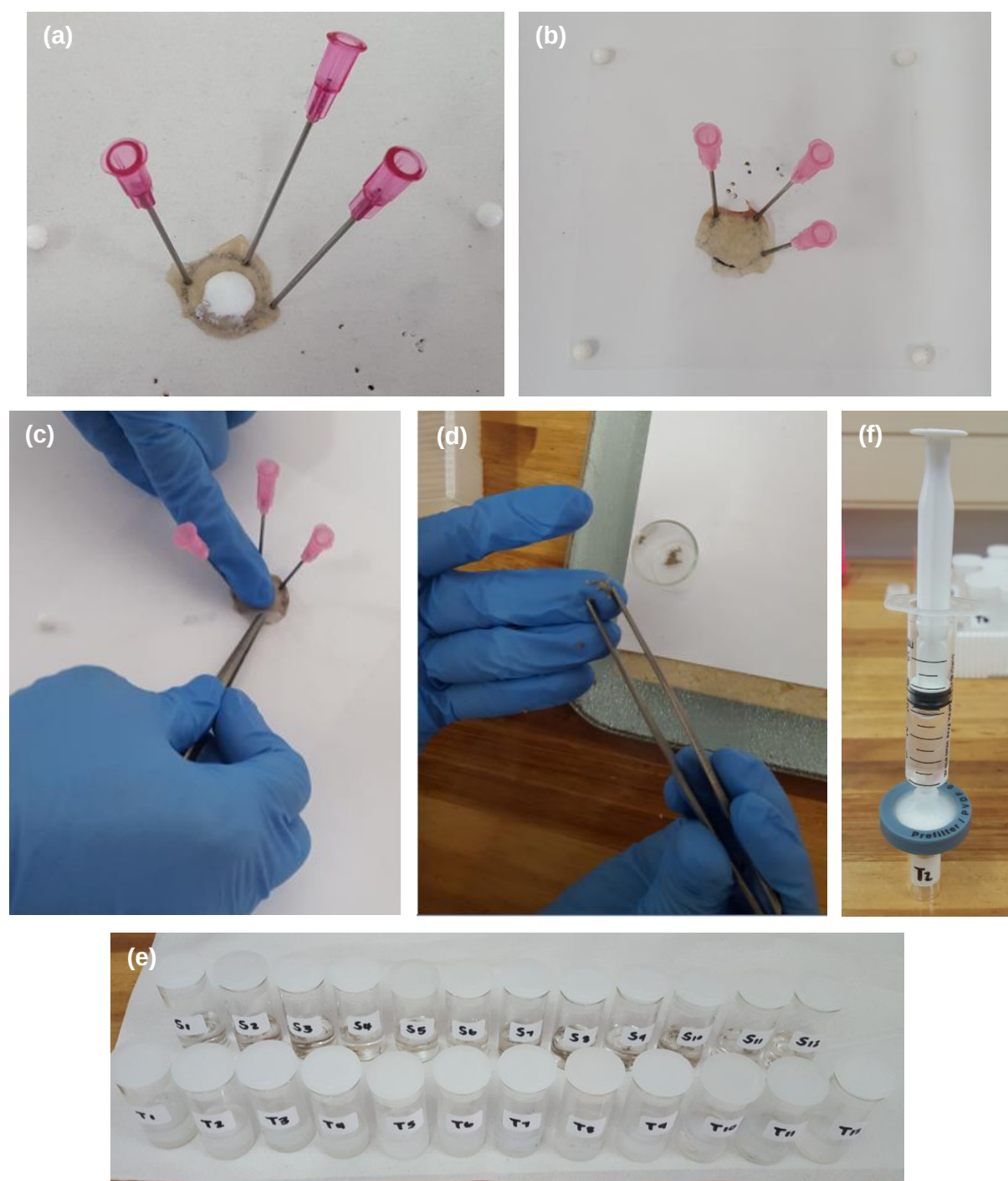


Figure D.5: Skin diffusion study: (a) skin sample with the dispersion still on it, (b) skin sample after all the excess dispersion was removed, (c) tape stripping, (d) cut up skin pieces, (e) polytops containing tape strips (T1 – T12) and the skin pieces (S1 – S12) and (f) sample being filtered into an HPLC vial

D.3 Results and discussion

D.3.1 Aqueous solubility

The experimental aqueous solubility of roxithromycin monohydrate was previously determined in a study by Aucamp *et al.* (2013:26) and was reported to be 0.0335 mg/ml in water at 25 °C. The solubility of roxithromycin in PBS (pH 7.4) at 32 °C was determined in an earlier study by Csongradi (2015:177-178), since the receptor phase consisted of PBS (pH 7.4). The values were reported as 1.98 mg/ml for the monohydrate, 1.92 mg/ml for chloroform desolvate and 1.88 mg/ml for the quench cooled form. The values of Csongradi (2015:177-178) differed from those reported by Aucamp *et al.* (2013:26) because a higher temperature and a different medium were used. For successful topical delivery, the ideal solubility should be greater than 1 mg/ml (Naik *et al.*, 2000:319) thus indicating that roxithromycin is a favourable candidate.

D.3.2 *n*-Octanol-water partition coefficient

The log P is a good indication of the separation of the drug between the lipophilic stratum corneum and the core hydrophilic regions of the epidermis. The log D value is the log P value at a specific pH, and is a good indication as to whether a drug would be able to be delivered transdermally or not (N'Da, 2014:20786). In Csongradi's study (2015:178), the log D values for the three solid-state forms of roxithromycin was said to be 1.52. The conclusion that was made from this value is that roxithromycin was found in the *n*-octanol phase and proves it is lipophilic. The log P reported for roxithromycin was predicted as 2.9, but the experimental log P was reported as 1.7 (Drugbank, 2013). The value obtained was close to the value that was reported. If the log P values are between 1 and 4, dermal absorption is favourable, as values ranging between 2 and 3 more optimal (Akhlaiq *et al.*, 2014:178).

D.3.3 Membrane release study

The aim of the membrane release study was to determine whether the API was released from the niosome vesicle systems. The amount of Franz cells reported can vary for the experiments, due to some cells showing signs of leakage, therefore these were removed from the dataset to prevent inaccurate results. The flux is an indication of the concentration of the API which diffused through the membrane per hour during the 6 h release studies. The results are summarised in Table D.1 and displays the average flux values ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) and average percentage (%) of roxithromycin released through the PVDF membranes after a period of 6 h.

Table D.1: Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) and the average percentage of roxithromycin released (%) through the membranes for each dispersion after 6 h (n represents the amount of Franz cells)

Dispersion	n	Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)	Average percentage released (%)
RM1	10	56.63 ± 8.651	0.892 ± 0.153
RM2	9	55.24 ± 9.742	0.902 ± 0.154
RM3	9	55.84 ± 9.153	0.822 ± 0.162
RM4	10	52.25 ± 4.283	0.782 ± 0.079
RM5	10	46.36 ± 5.794	0.732 ± 0.073
CD1	10	47.13 ± 10.408	0.693 ± 0.129
CD2	10	45.21 ± 11.295	0.711 ± 0.203
CD3	10	22.52 ± 4.187	0.318 ± 0.050
CD4	10	35.79 ± 7.802	0.507 ± 0.109
CD5	10	48.28 ± 6.472	0.738 ± 0.103
QC1	10	73.69 ± 17.002	1.025 ± 0.196
QC2	10	132.43 ± 14.053	1.794 ± 0.196
QC3	10	299.81 ± 72.626	4.098 ± 0.919
QC4	10	65.71 ± 9.906	0.898 ± 0.139
QC5	10	34.44 ± 4.820	0.409 ± 0.066

The results showed that all fifteen of the dispersions released the API through the membranes, with some more than others. The dispersion which displayed the highest flux value was **QC3**, which was ± 2.3 times higher than **QC2**, the second highest flux of all the dispersions. The dispersions can thus be ranked from highest to lowest as follows: **QC3, QC2, QC1, QC4 RM1, RM3, RM2, RM4, CD5, CD1, RM5, CD2, CD4, QC5** and **CD3**. From the results obtained from the membrane release studies, it can be seen the dispersions containing the amorphous **QC** form of the API displayed the best flux values and highest percentage released when compared to the **RM** and the **CD** over the period of 6 h.

The dispersion containing cholesterol and Span[®] 40 in the ratio of 2:1, respectively, showed the best results (dispersion 3). This was predicted in Appendix B. This dispersion had the highest entrapment efficiency ($98.66 \pm 0.674\%$), as seen in Section C.2.5.4 and could therefore be the reason it had the highest release of roxithromycin, since it contained the highest amount of drug encapsulated into the niosome vesicle system.

Figures D.6 to D.35 depicts the results from the membrane release studies as obtained for all fifteen the dispersions. Every dispersion has two figures, the first shows the average cumulative amount of roxithromycin per area ($\mu\text{g}/\text{cm}^2$) released from the niosome vesicle

system over a period of 6 h. The second figure displays the cumulative amount per area ($\mu\text{g}/\text{cm}^2$) released for each individual Franz cell.

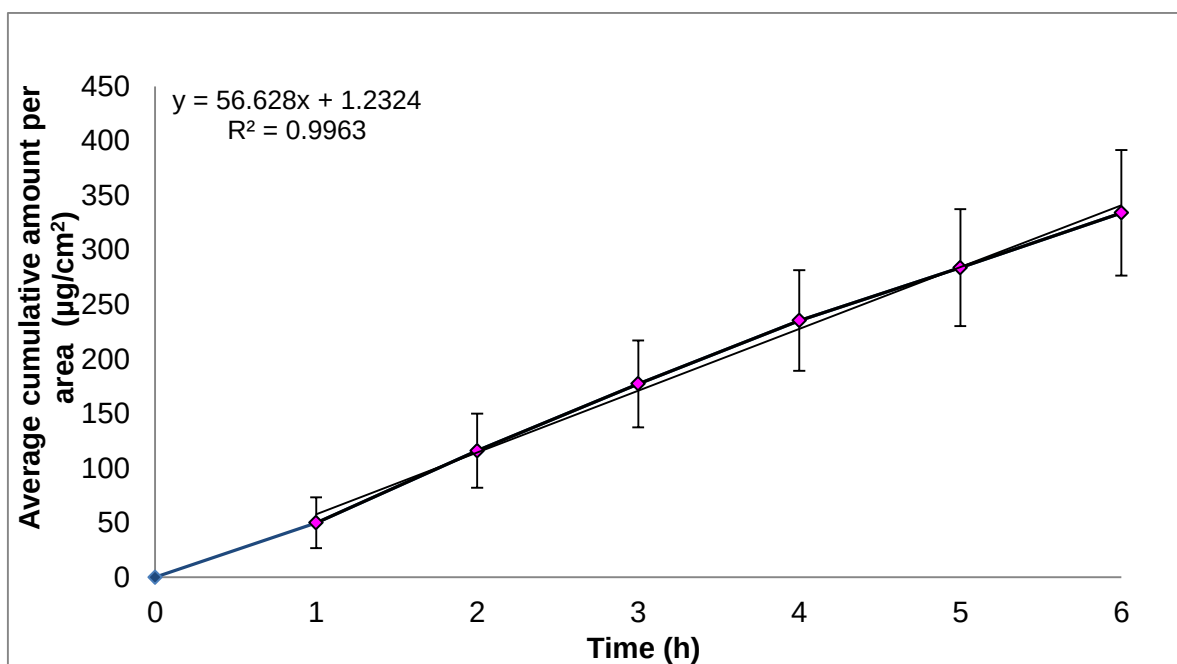


Figure D.6: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM1** that permeated through the membrane into the receptor phase over a period of 6 h (n = 10)

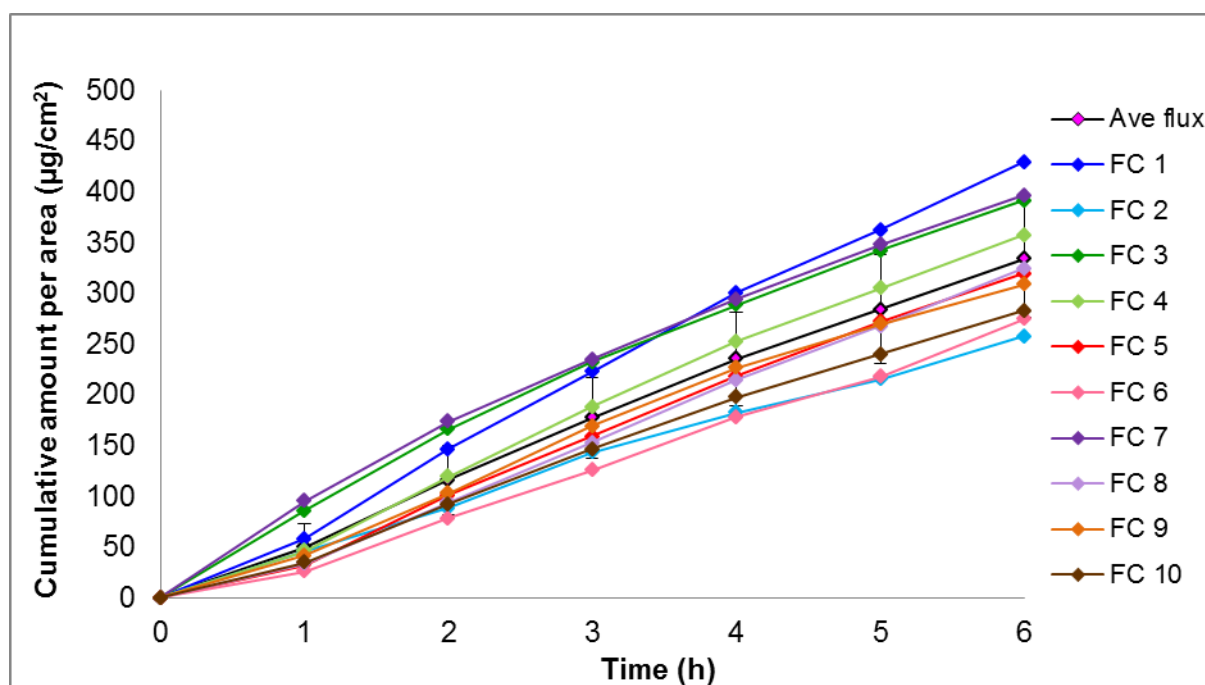


Figure D.7: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM1** that permeated through the membrane over a period of 6 h for each individual Franz cell (n = 10)

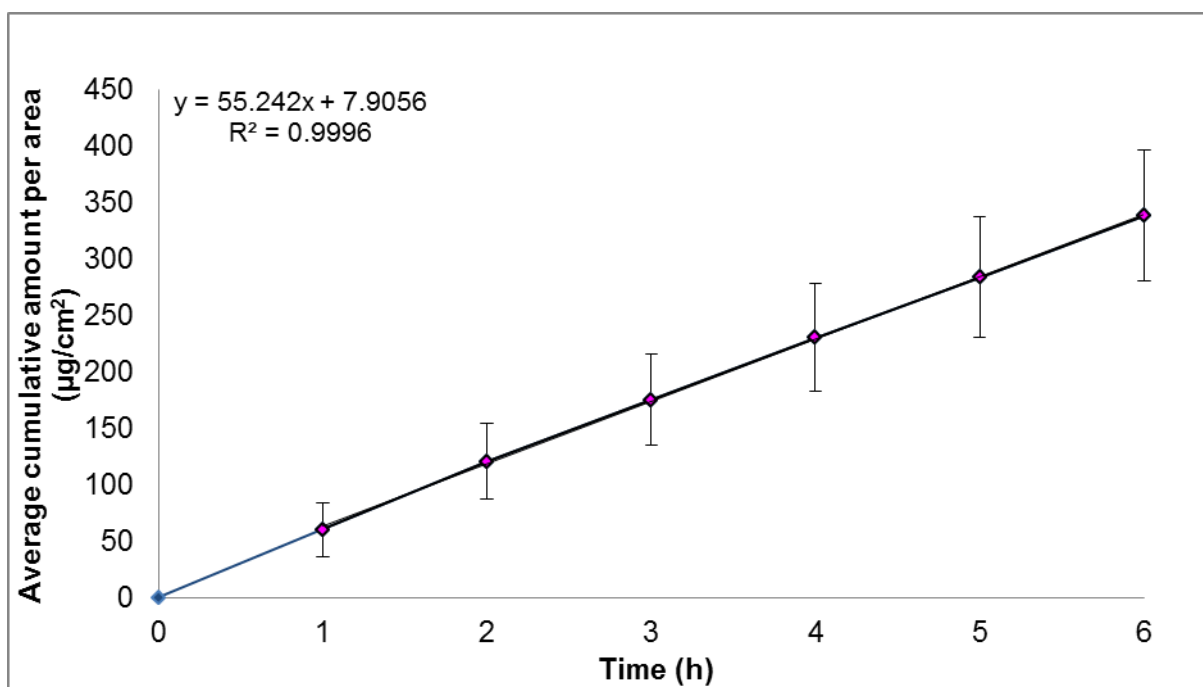


Figure D.8: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM2** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 9$)

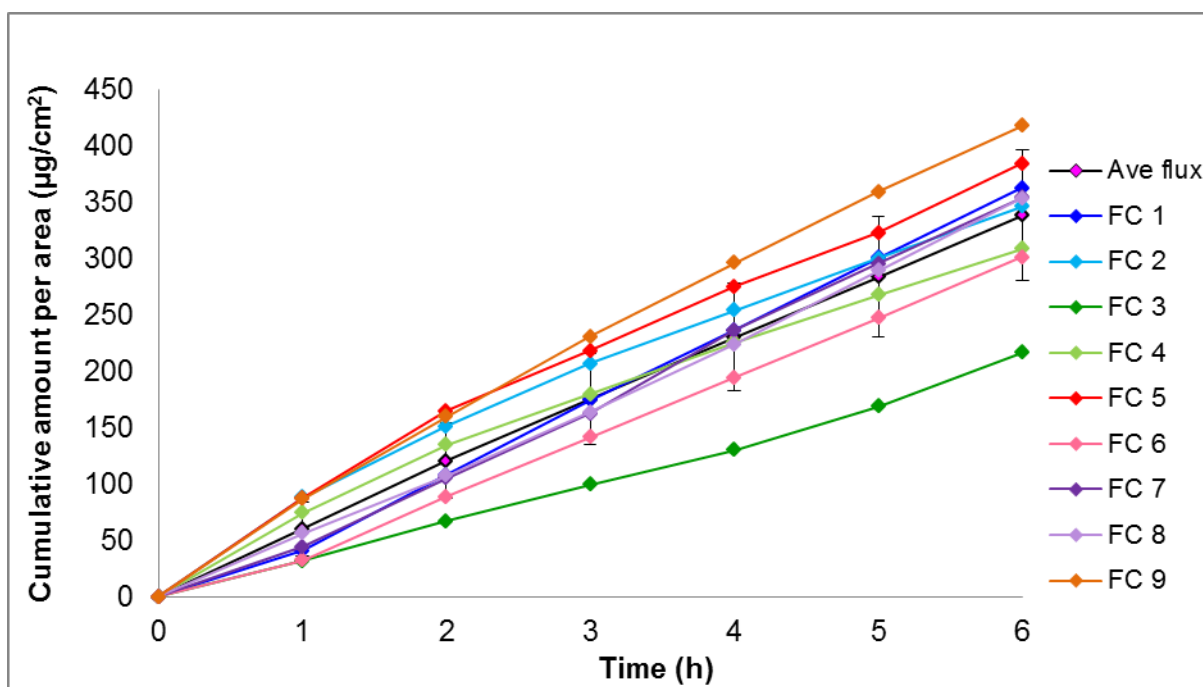


Figure D.9: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM2** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 9$)

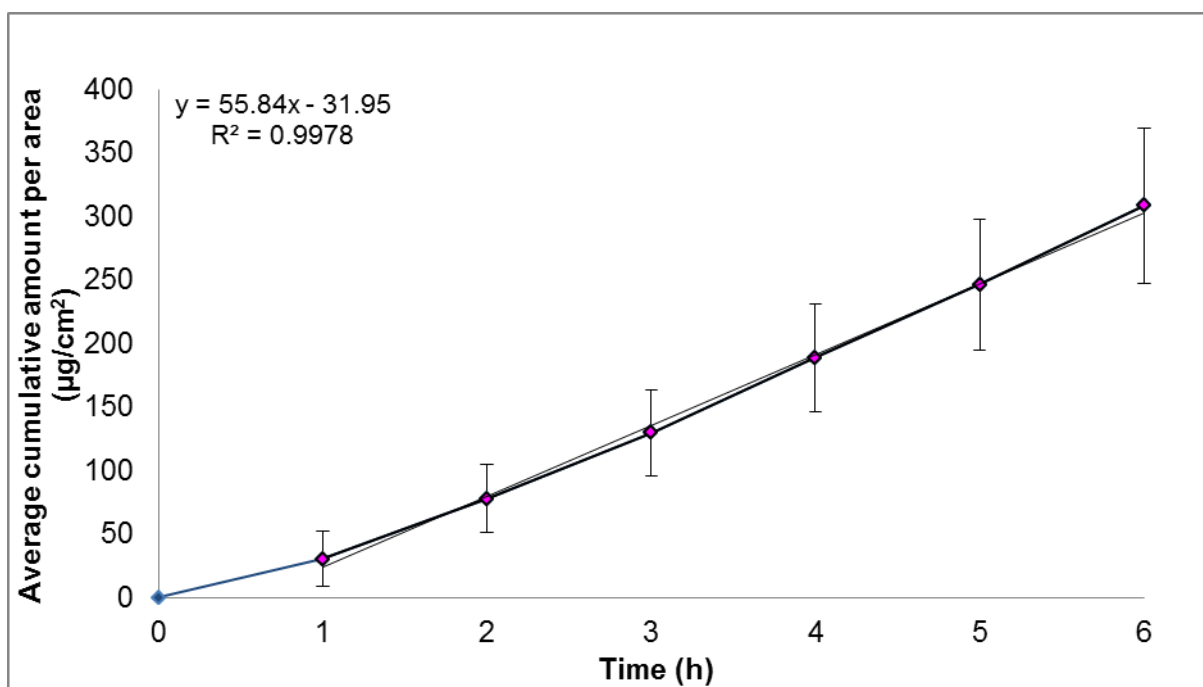


Figure D.10: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM3** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 9$)

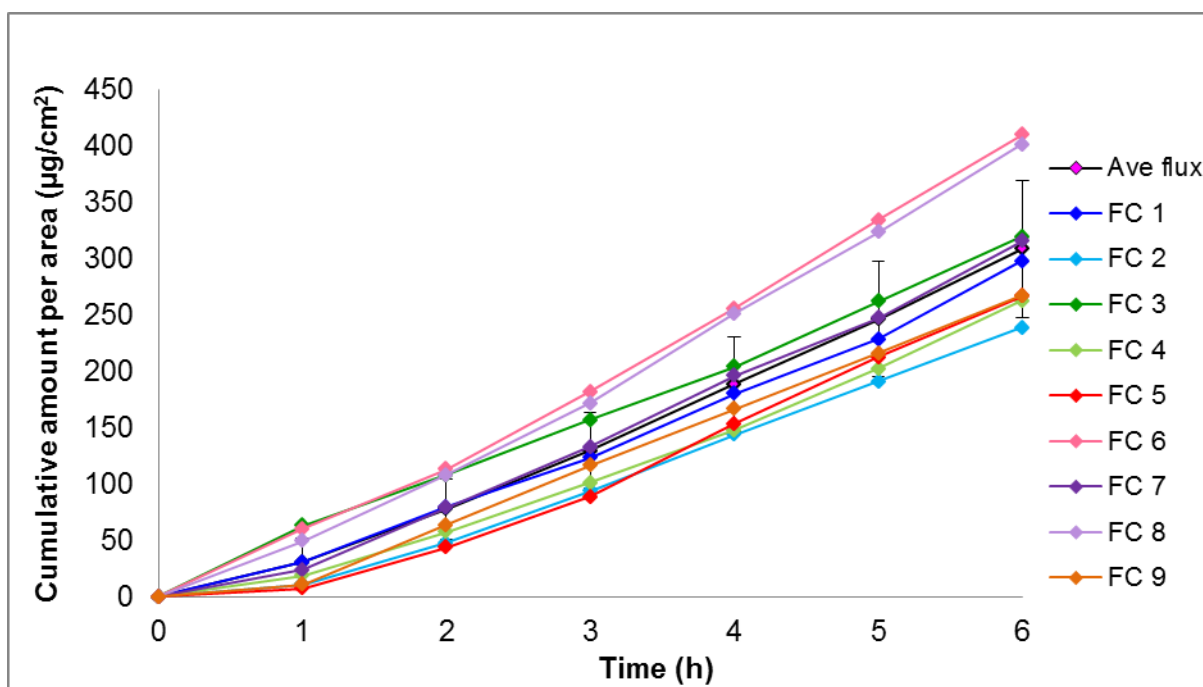


Figure D.11: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM3** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 9$)

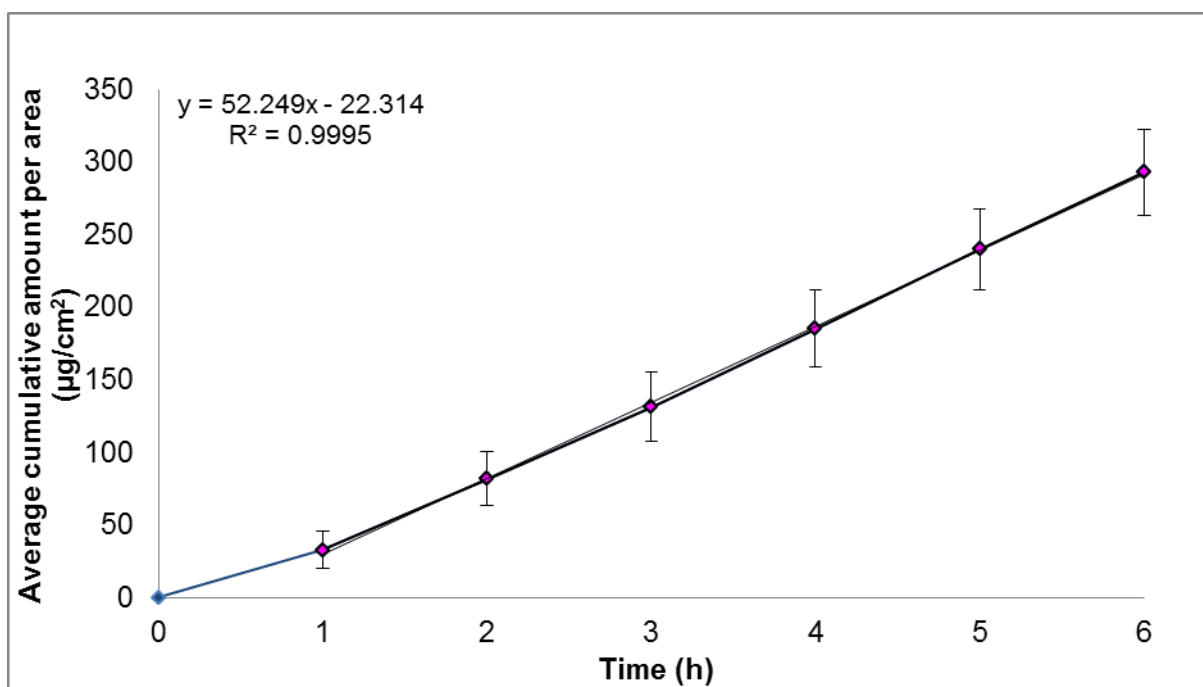


Figure D.12: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM4** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

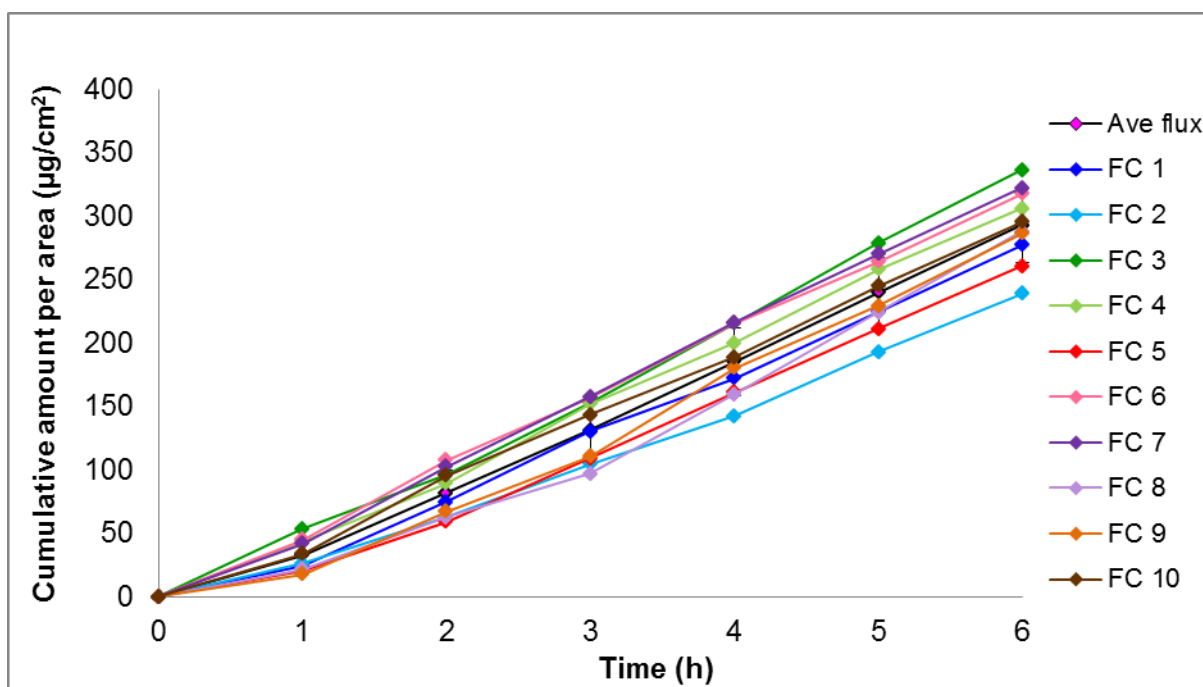


Figure D.13: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM4** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

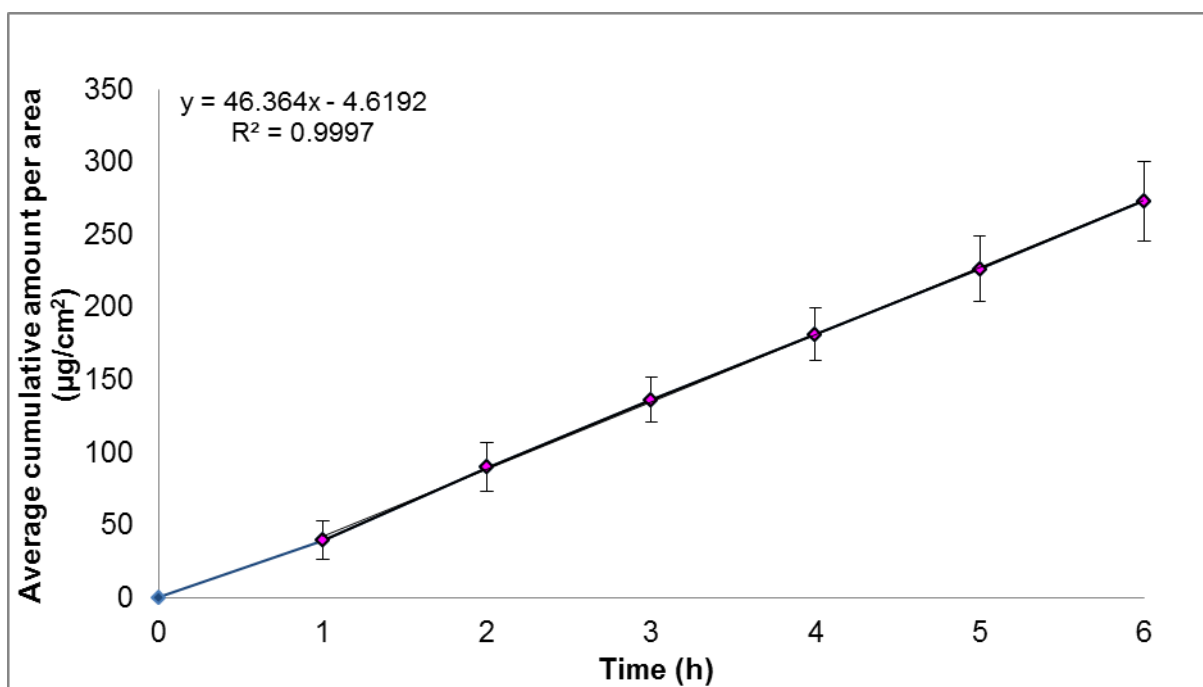


Figure D.14: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM5** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

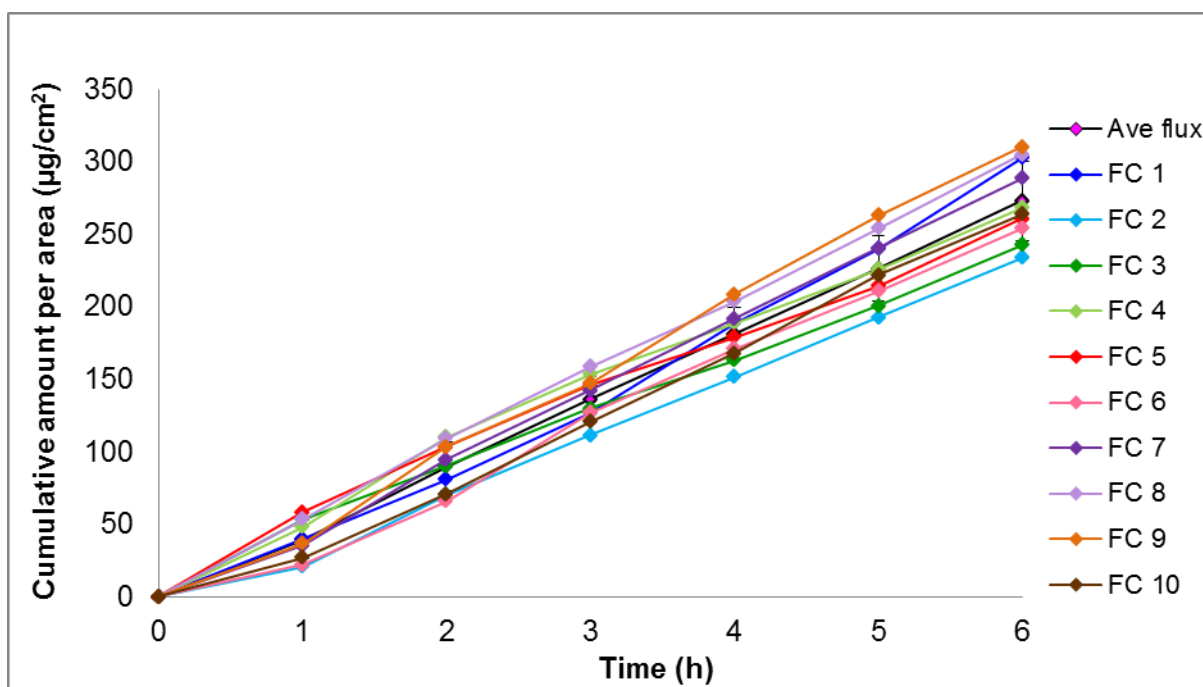


Figure D.15: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **RM5** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

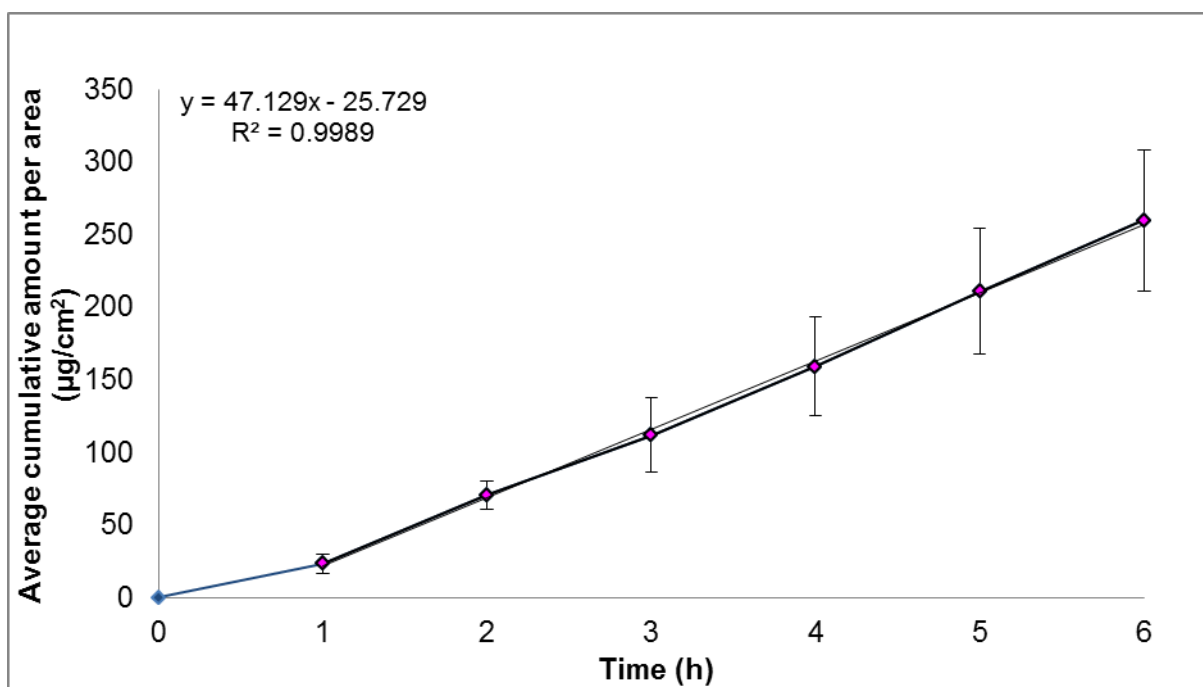


Figure D.16: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD1** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

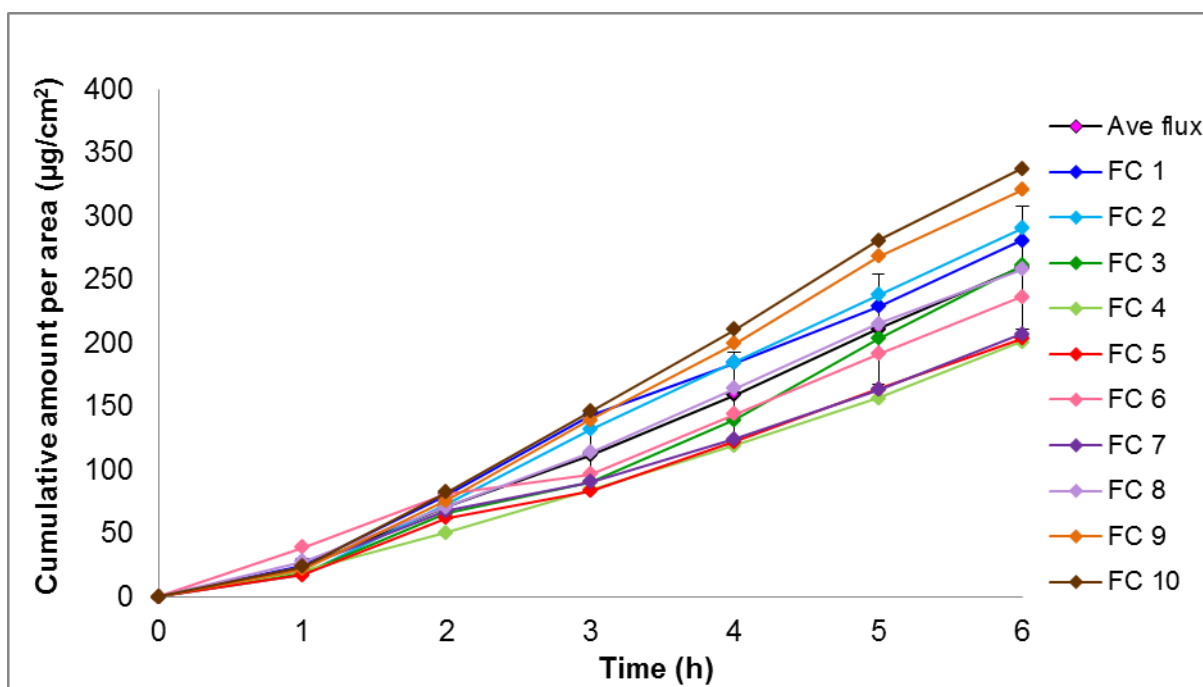


Figure D.17: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD1** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

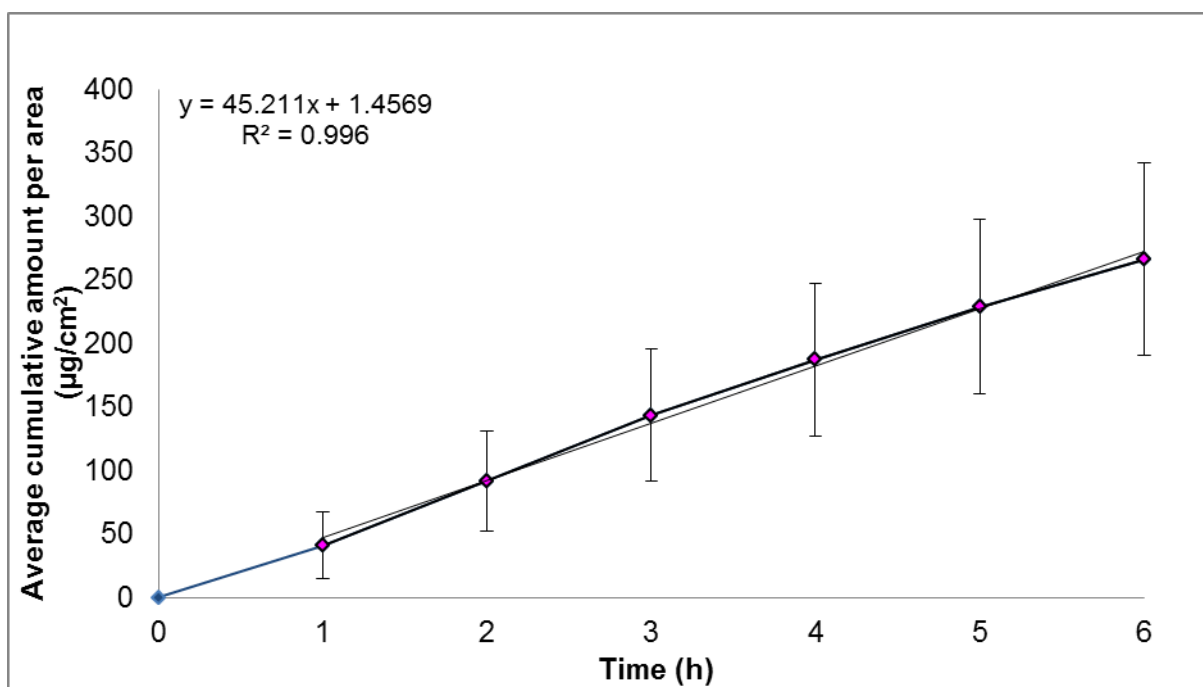


Figure D.18: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of CD2 that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

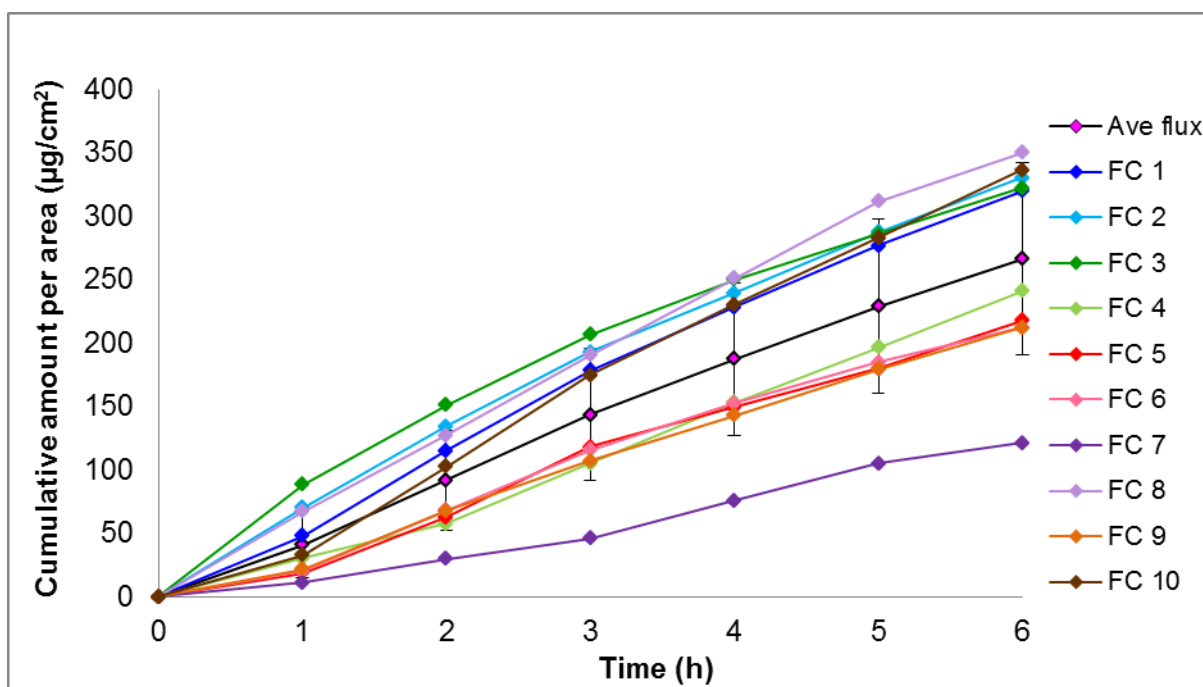


Figure D.19: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of CD2 that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

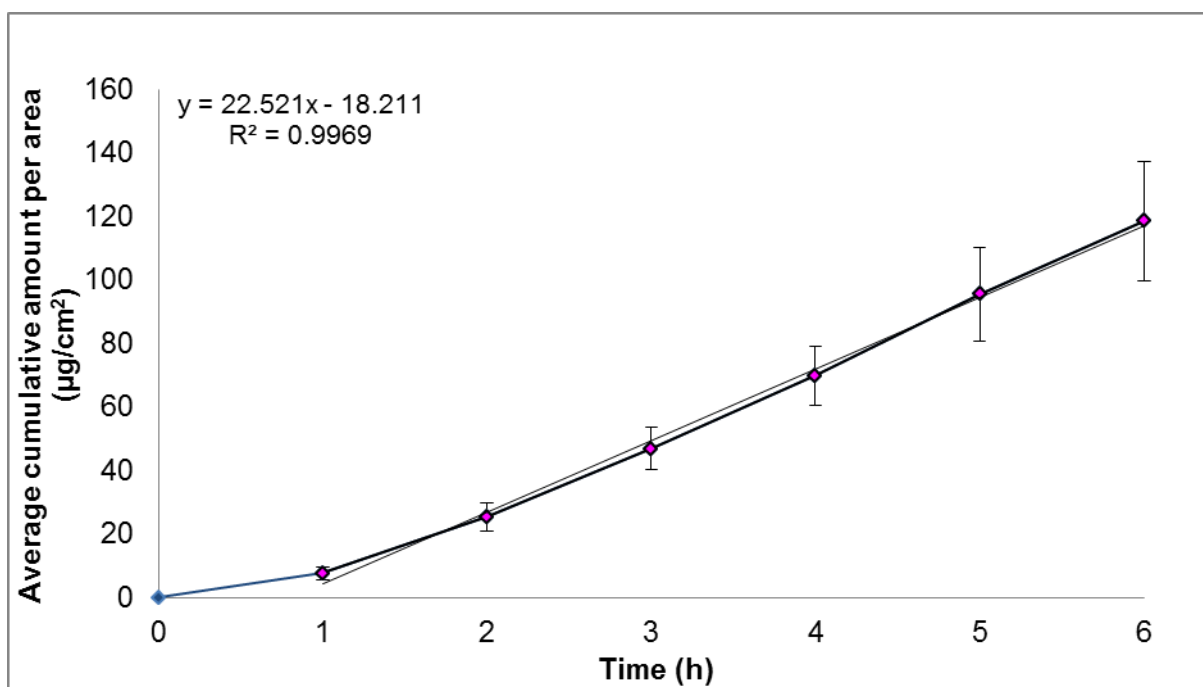


Figure D.20: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD3** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

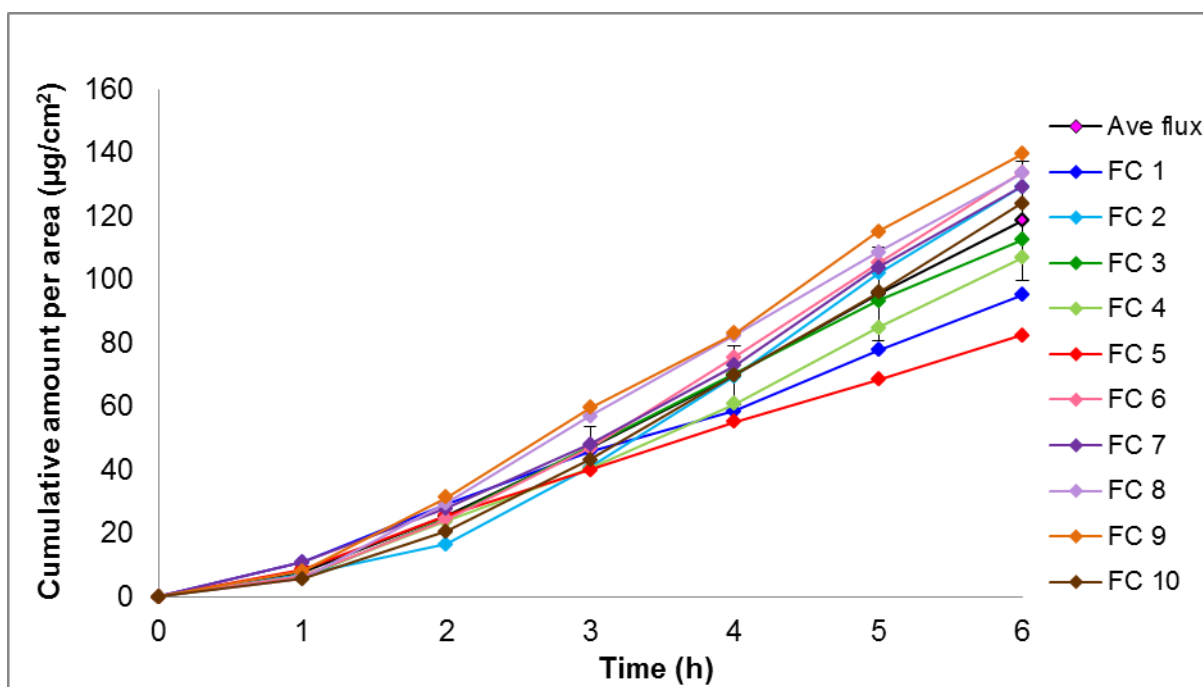


Figure D.21: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD3** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

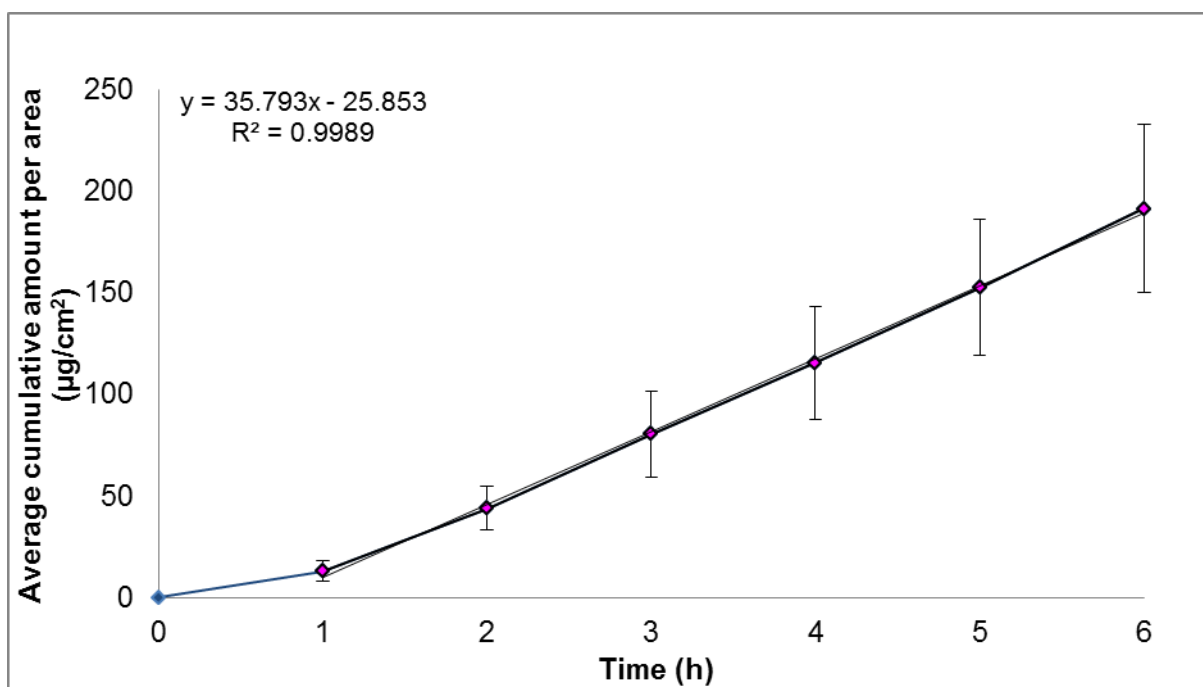


Figure D.22: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD4** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

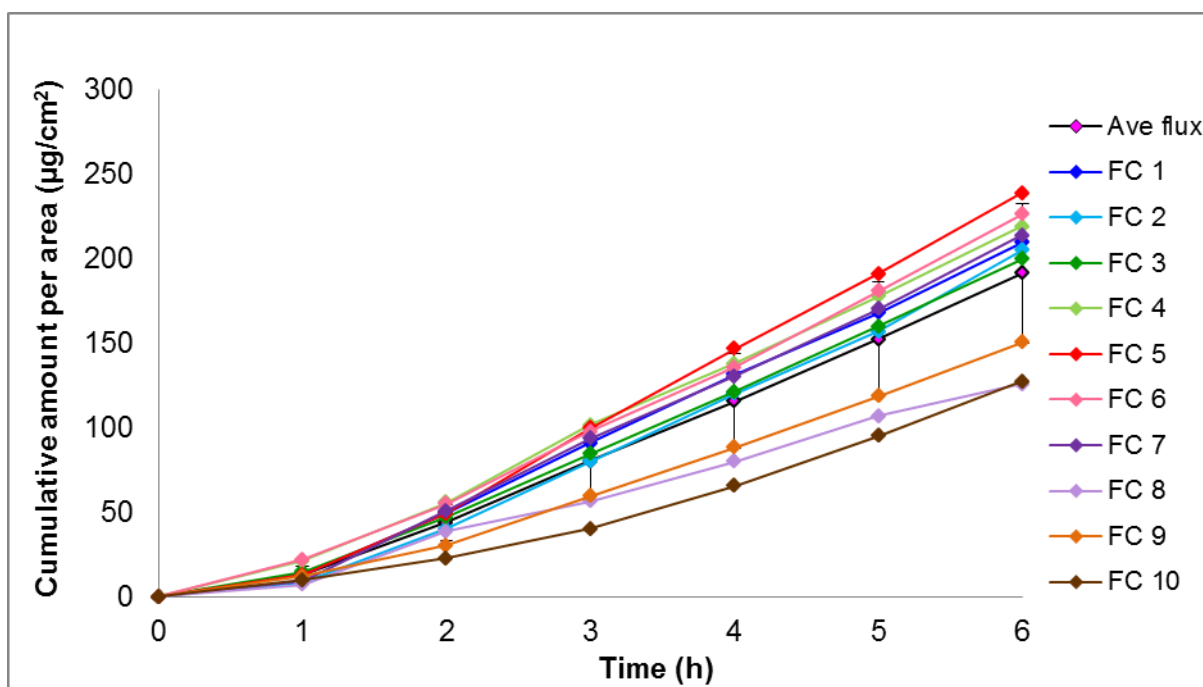


Figure D.23: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD4** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

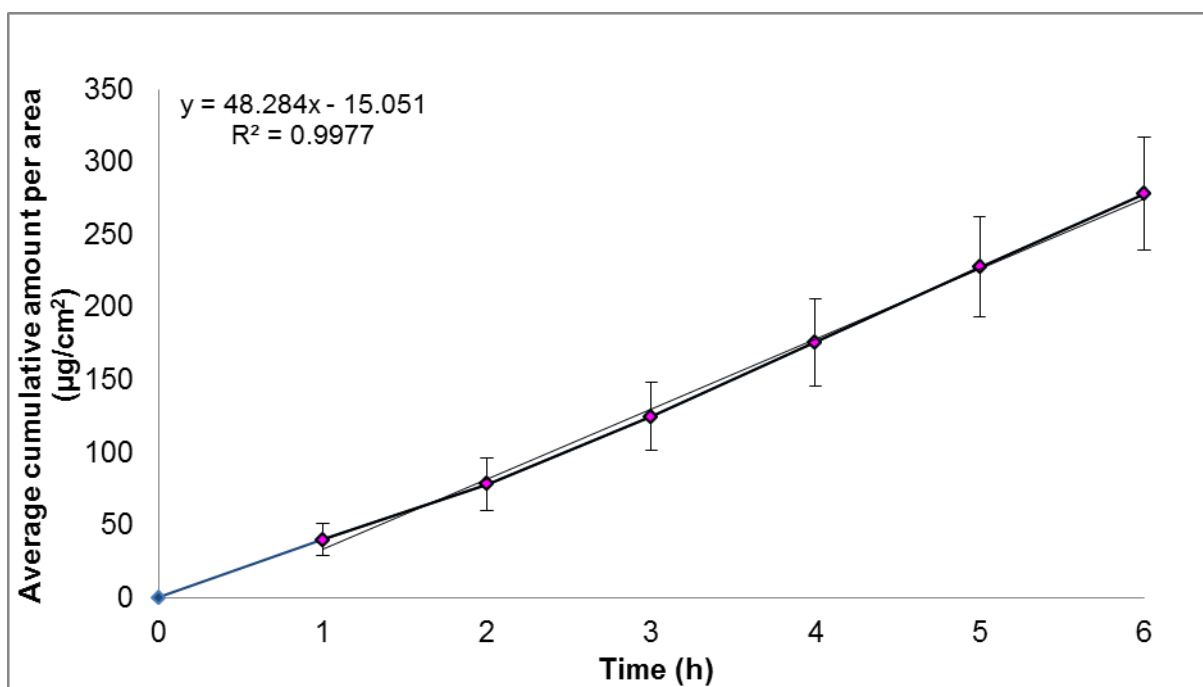


Figure D.24: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD5** that permeated through the membrane into the receptor phase over a period of 6 h (n = 10)

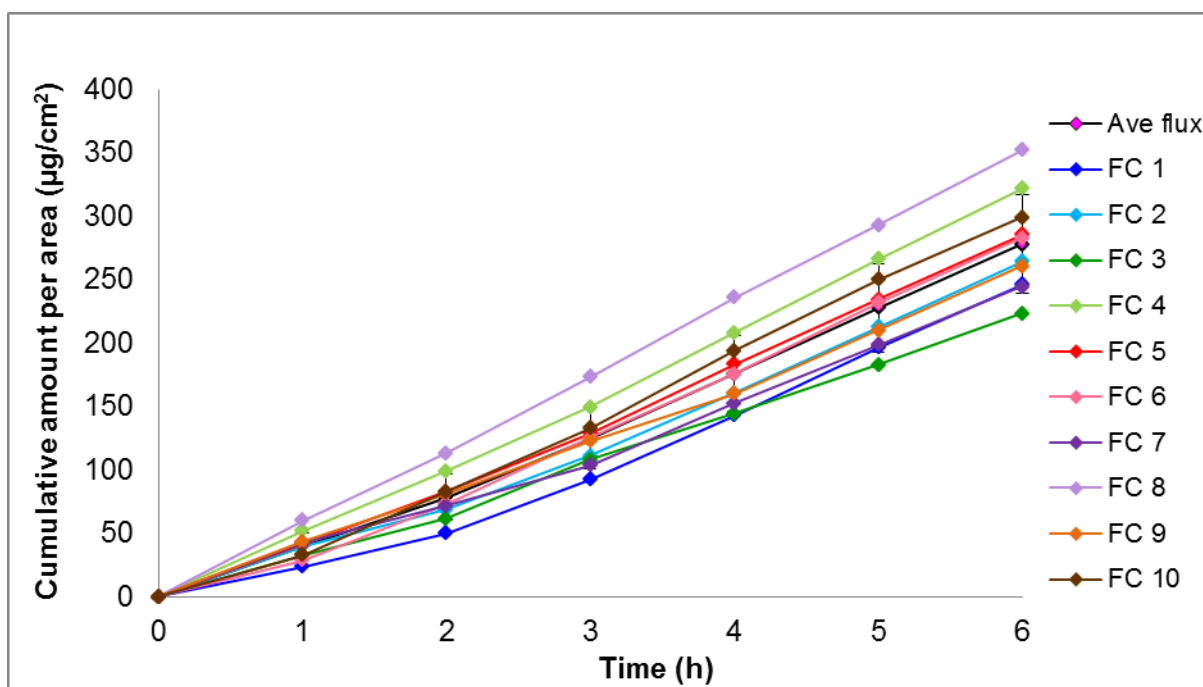


Figure D.25: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **CD5** that permeated through the membrane over a period of 6 h for each individual Franz cell (n = 10)

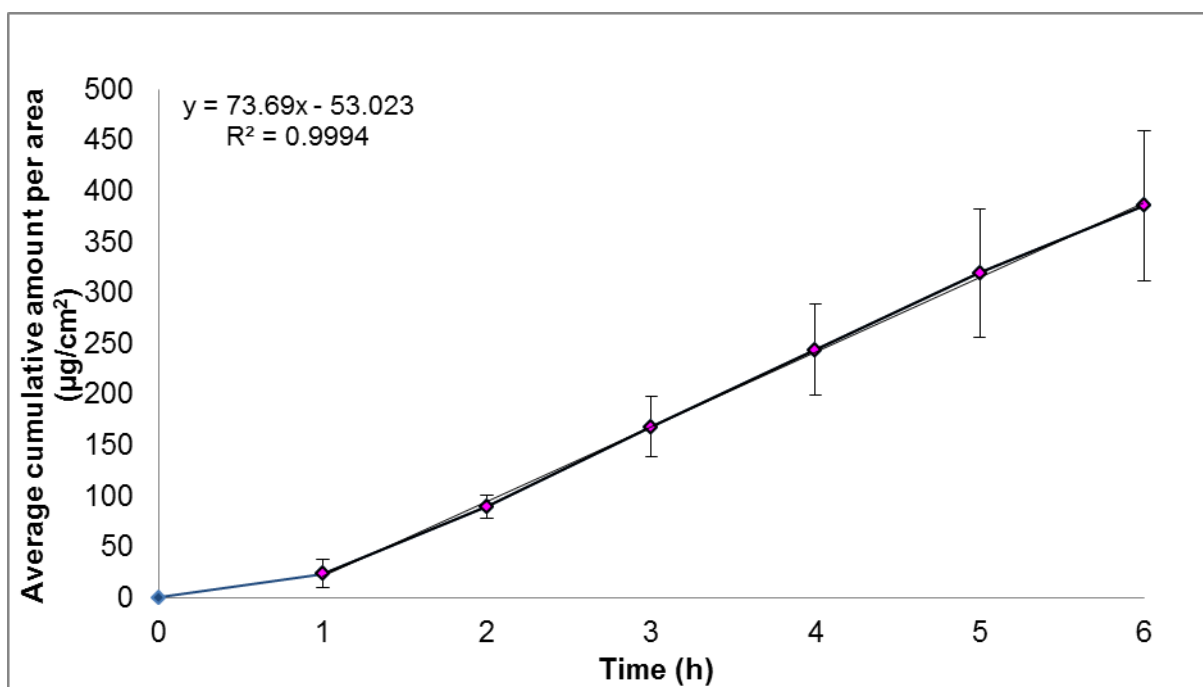


Figure D.26: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC1** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

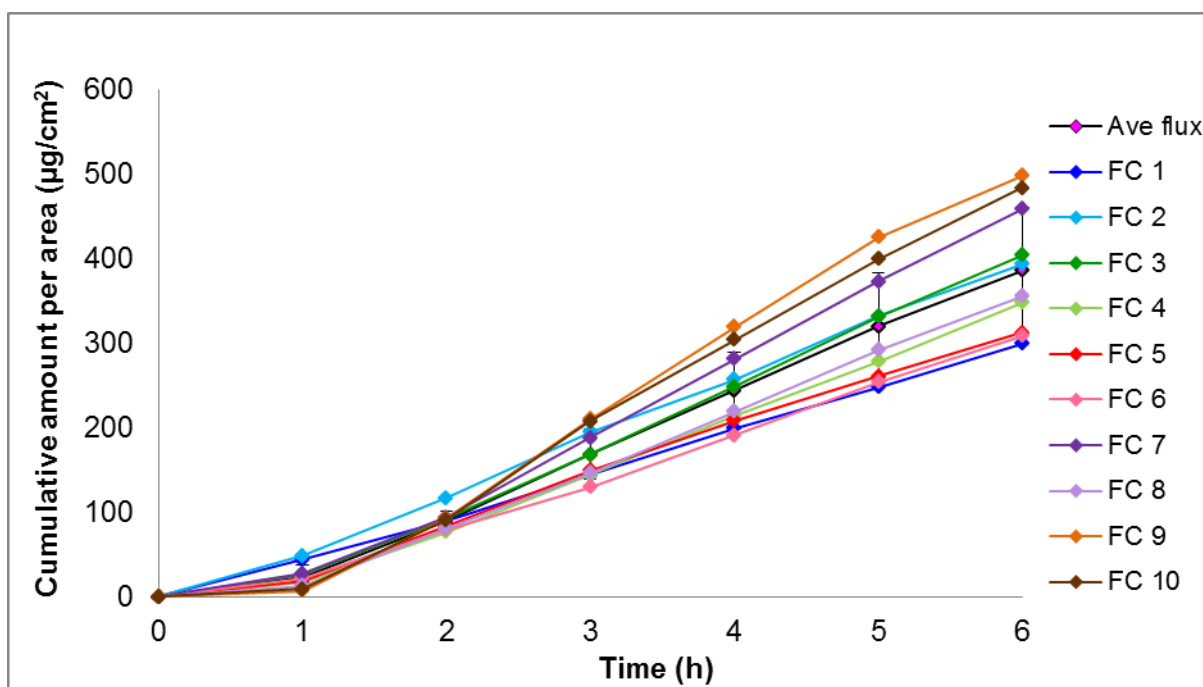


Figure D.27: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC1** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

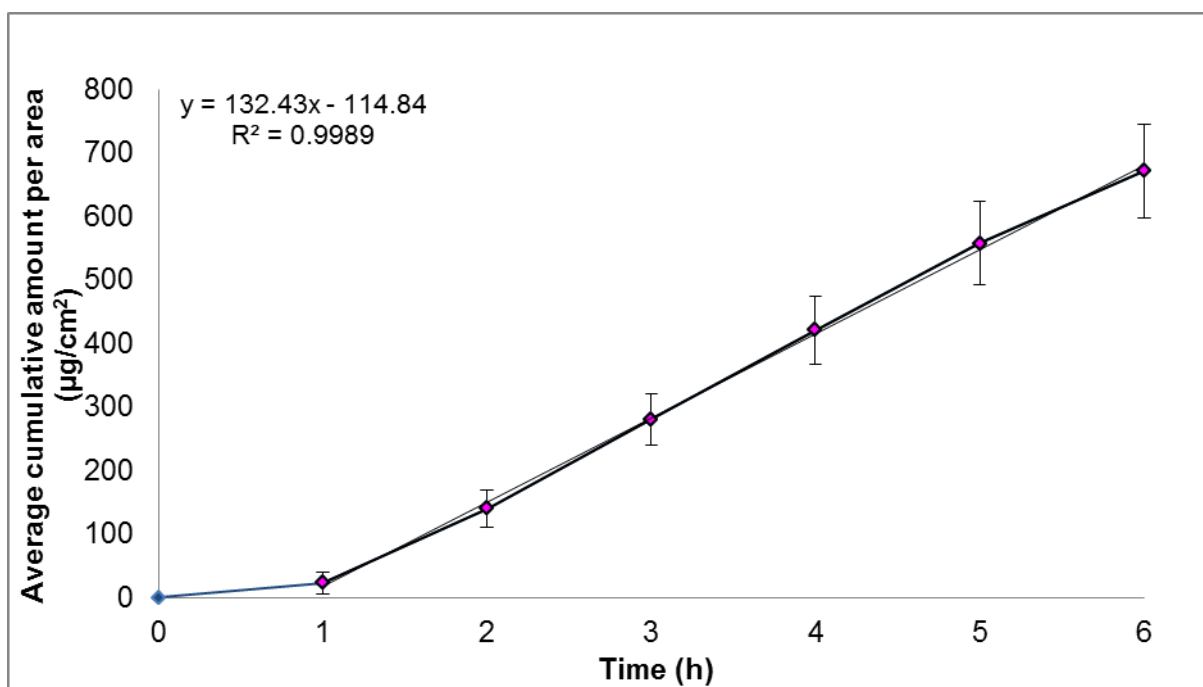


Figure D.28: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC2** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

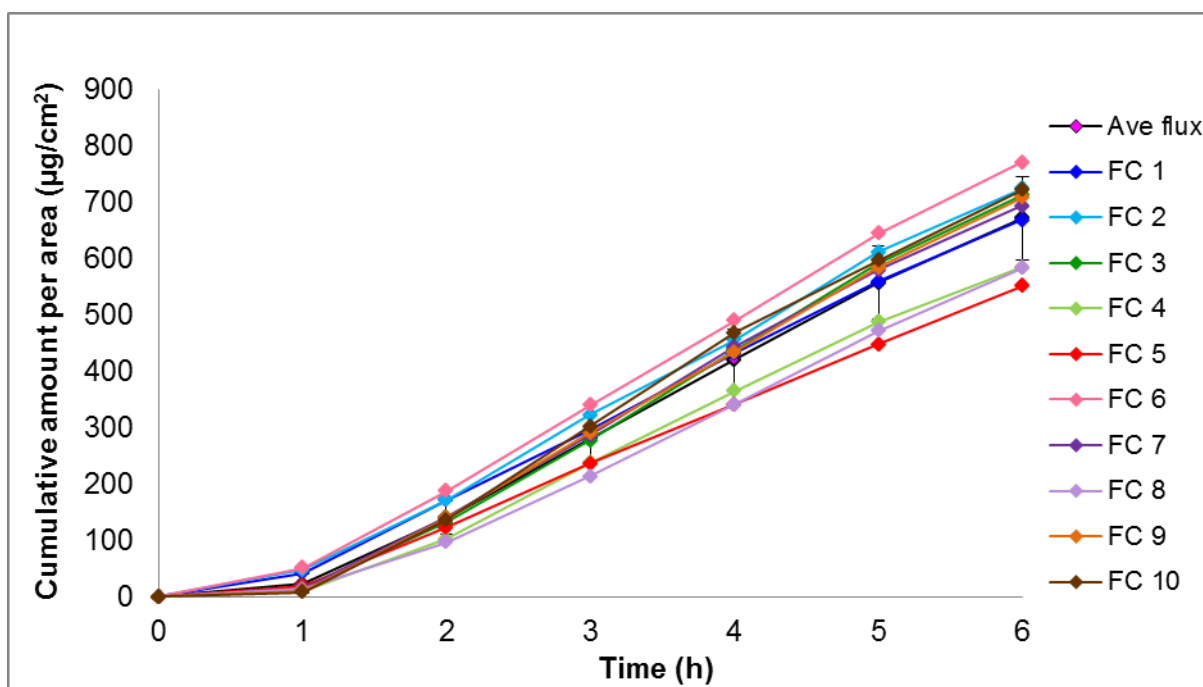


Figure D.29: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC2** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

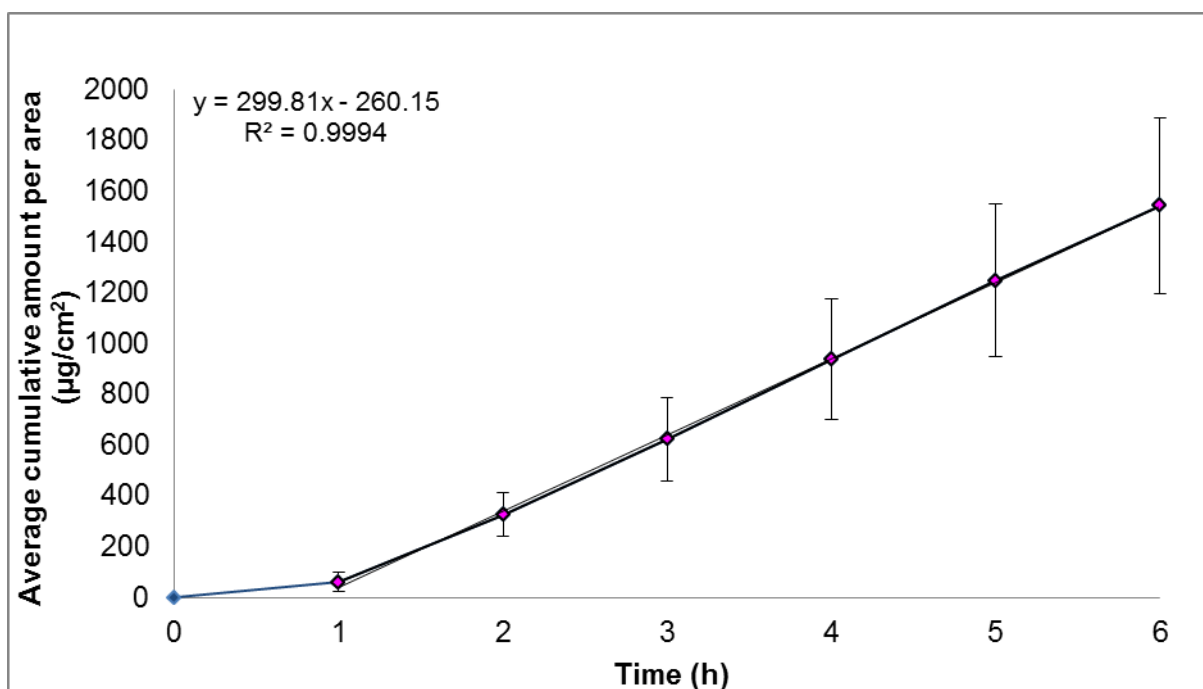


Figure D.30: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC3** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

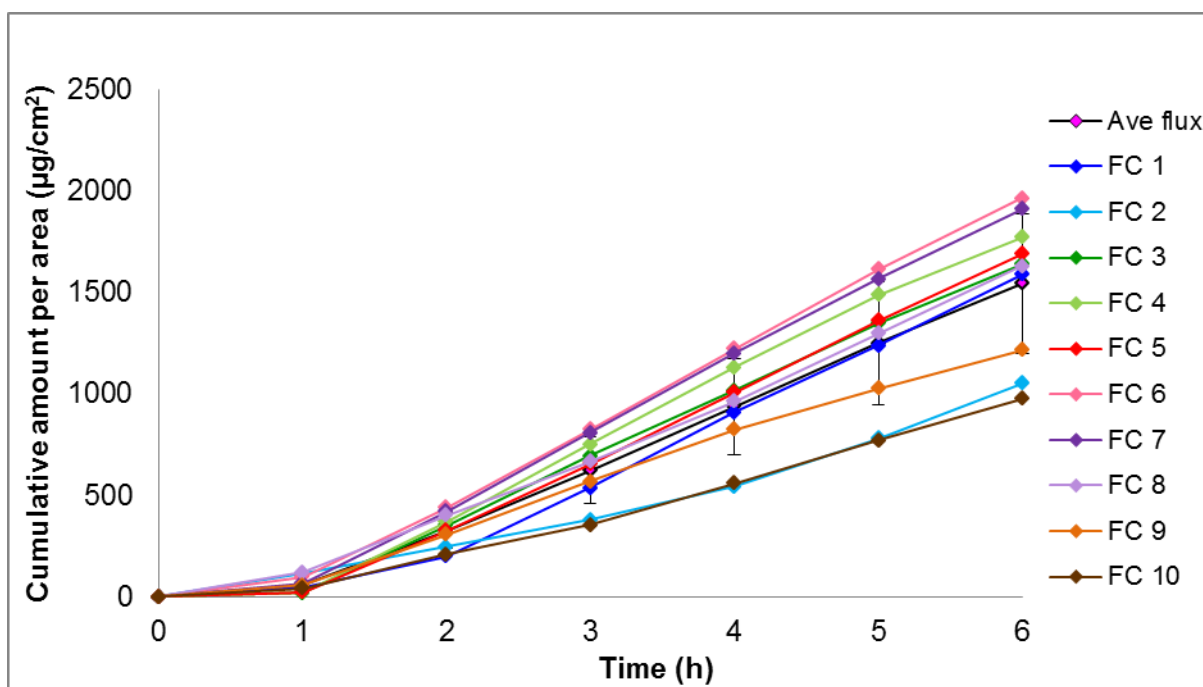


Figure D.31: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC3** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

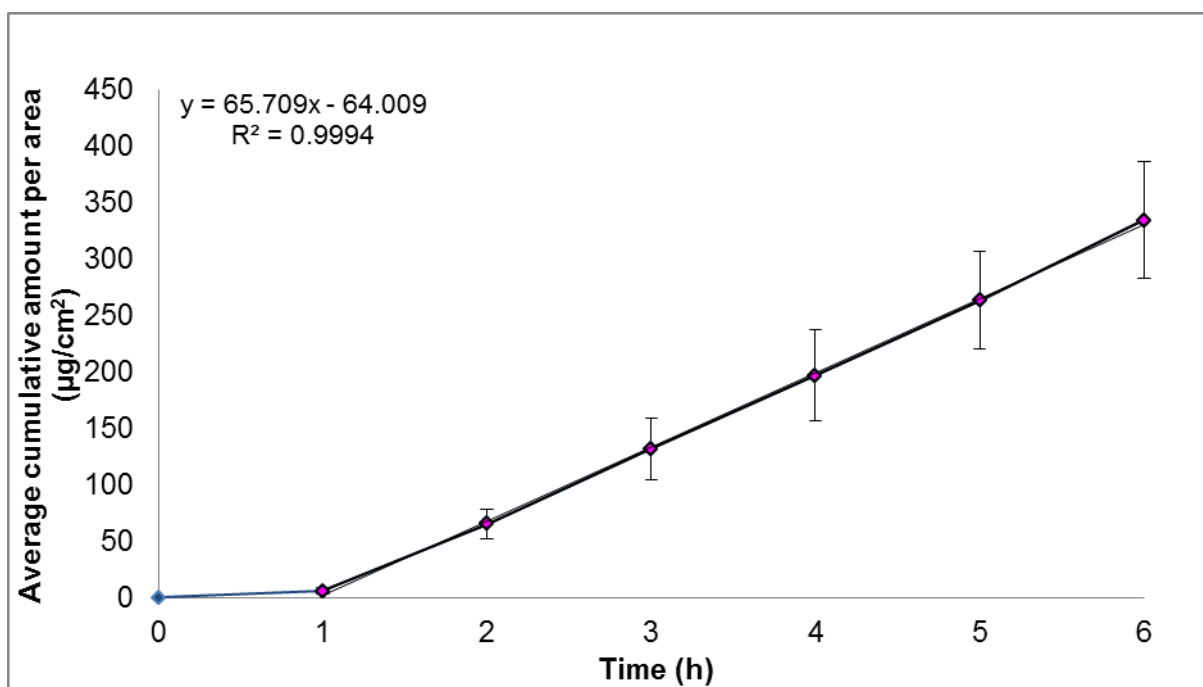


Figure D.32: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC4** that permeated through the membrane into the receptor phase over a period of 6 h (n = 10)

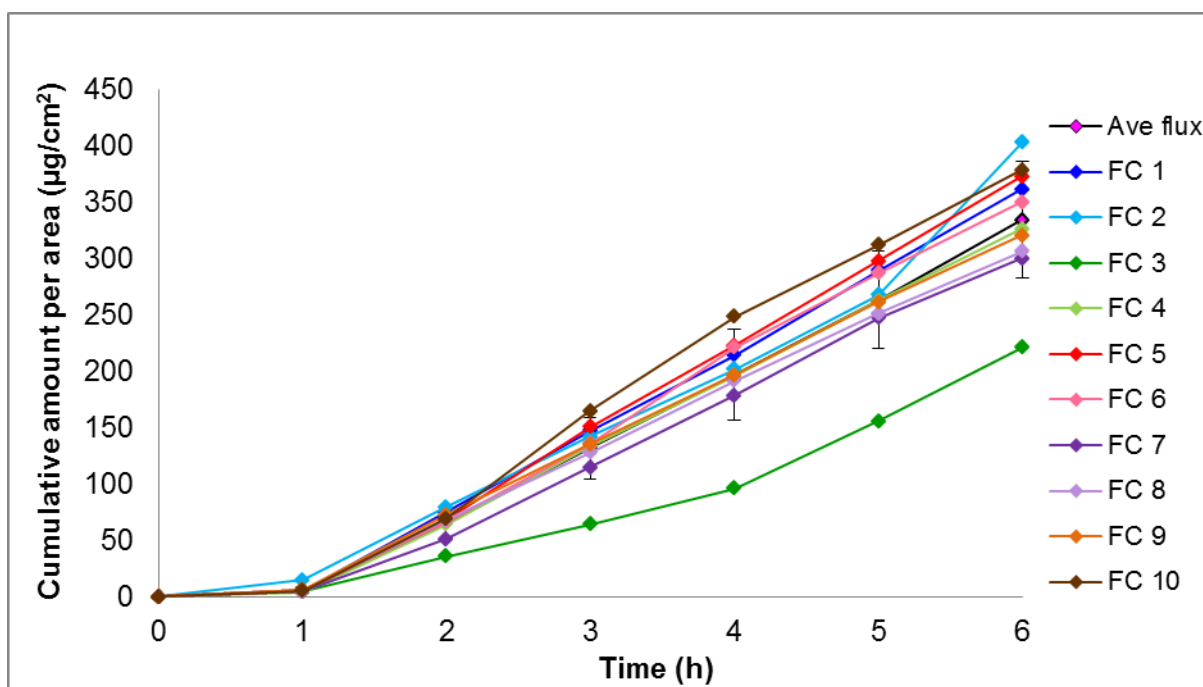


Figure D.33: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC4** that permeated through the membrane over a period of 6 h for each individual Franz cell (n = 10)

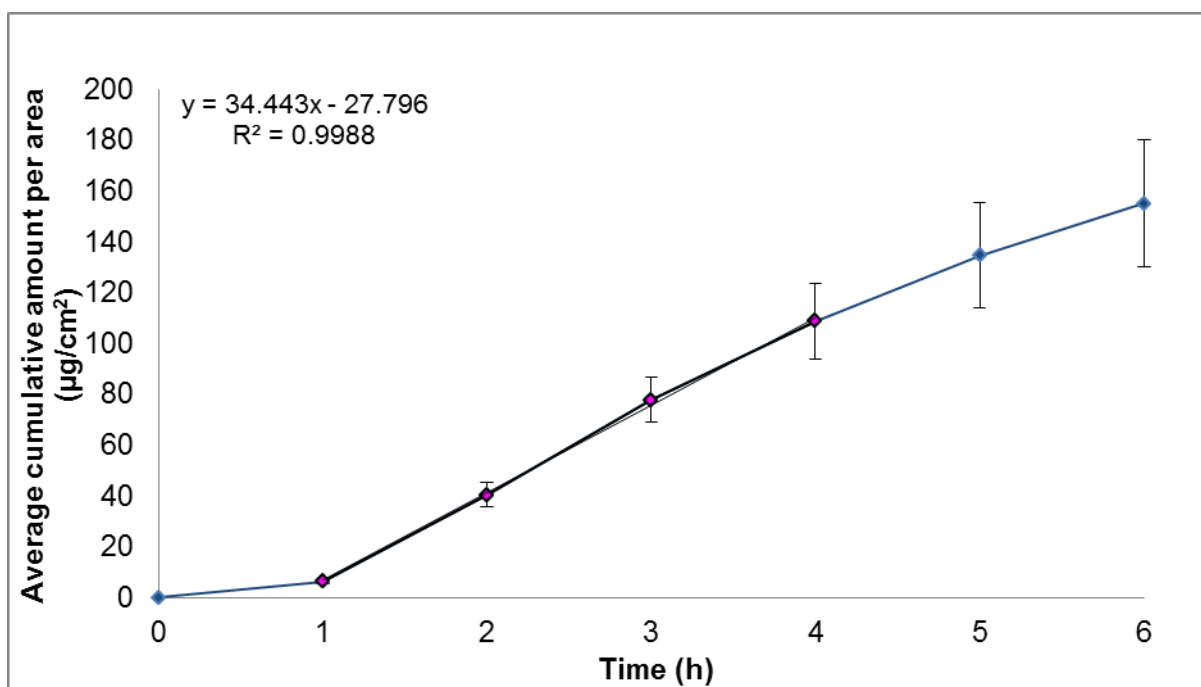


Figure D.34: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC5** that permeated through the membrane into the receptor phase over a period of 6 h ($n = 10$)

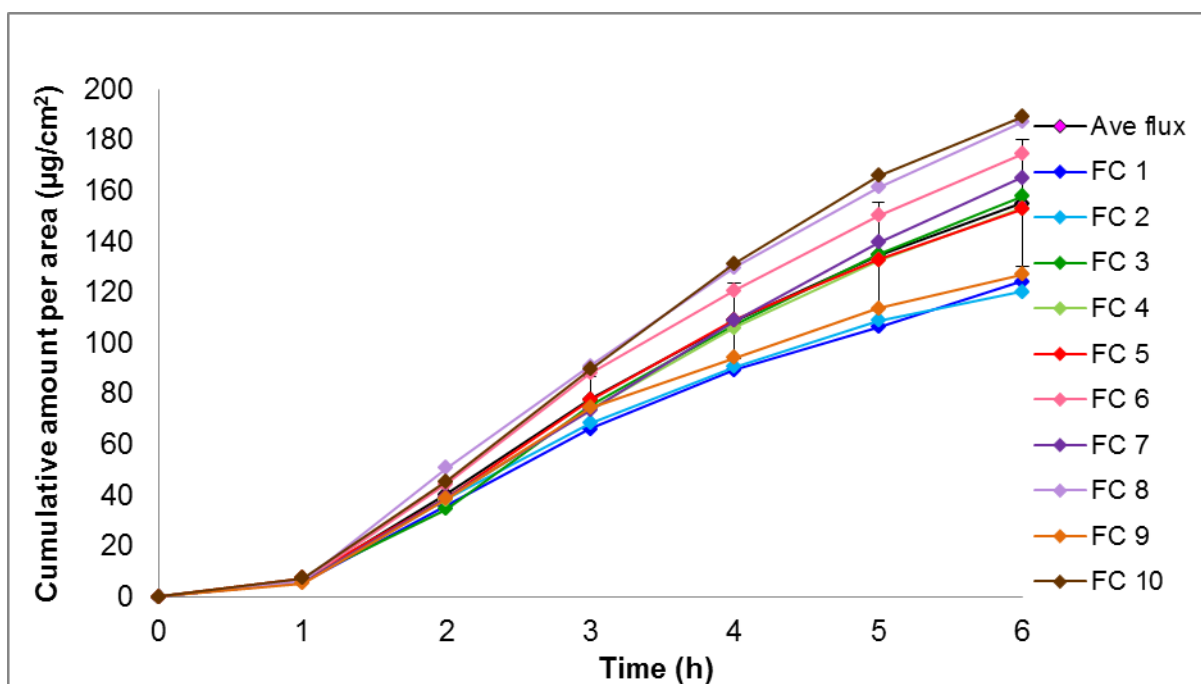


Figure D.35: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of roxithromycin released from the niosome vesicle of **QC5** that permeated through the membrane over a period of 6 h for each individual Franz cell ($n = 10$)

It is however important to remember that the dispersion displaying the best release through the membranes during the membrane release studies, are not necessarily an indication of the

dispersion that will perform best during the skin diffusion studies. The membrane release studies were only performed to determine whether API releases from the niosome vesicle systems and was confirmed. Therefore if no transdermal or topical delivery took place during the skin diffusion studies; failure of API release from the vesicles could be eliminated as a possible cause.

D.3.4 *In vitro* skin diffusion studies

After the membrane release studies were concluded, skin diffusion studies and tape stripping were conducted to determine whether the API was delivered transdermally and/or topically. The data obtained during the experiments indicated whether roxithromycin was found in the receptor phase, which represents transdermal delivery, or if it was retained within the SCE or ED, thus topical delivery. Due to the limited amount of usable skin from one donor, two skin donors had to be used to complete all fifteen of the skin studies. The studies of **RM1 – RM4**, **CD1 – CD4** and **QC1 – QC3** were completed using donor 1, while the studies of **RM5**, **CD5** and **QC4 – QC5** were done using donor 2. Once again all the experiments were started with 10 Franz cells containing the dispersions with the APIs, but where Franz cells showed signs of leakage, these were removed from the dataset to prevent the use of inaccurate data.

D.3.4.1 Transdermal diffusion

The receptor phase for each of the 12 Franz cells were extracted after 12 h and analysed through HPLC analysis. The average percentage diffused (%) and the average amount diffused per area ($\mu\text{g}/\text{cm}^2$) after 12 h were obtained and given in Table D.2.

The samples are ranked from highest to lowest average percentage diffused as follows: **QC2**, **RM2**, **RM1**, **CD2**, **RM3**, **RM4**, **CD1**, **RM5**, **QC3**, **QC1**, **CD3**, **CD4**, **CD5**, **QC4** and **QC5**. Although, for an experiment to be reliable and reproducible at least six Franz cells are required per diffusion study. Many of the dispersions had values below the LOD and LLOQ. The LOD and LLOQ were established and validated during the method validation in Section A.4.2 and were 0.851 and 1.703 $\mu\text{g}/\text{ml}$, respectively. If values lower than the LOD and LLOQ are detected, which could be due to noise and impurities, they are unreliable and although they can be quantified and reported, they will not be used (ICH, 2005:5).

From the set of fifteen dispersions, only 4 were acceptable, according to the aforementioned statements and are highlighted in Table D.2, which included **RM1** (n = 7 of 8), **RM2** (n = 9 of 10), **CD2** (n = 7 of 10) and **QC2** (n = 10 of 10). For the transdermal results, the dispersion containing **RM** had the highest values compared to the other solid-state forms. Out of the four dispersions that reached the systemic circulation, three contained cholesterol and

Span® 40 in the ratio of 1:1. It could be deduced that this dispersions was lipophilic enough to diffuse through the stratum corneum, yet had adequate hydrophilic properties to permeate into the more aqueous epidermis in order to reach the underlying systemic circulation (Perrie *et al.*, 2012:393).

Table D.2: Concentration of roxithromycin found in the receptor phase

Dispersion	n	n ≥ LLOQ	Average percentage diffused (%)	Average amount diffused per area (µg/cm ²)
RM1	8	7	0.0134 ± 0.0030	2.687 ± 1.1211
RM2	10	9	0.0147 ± 0.0061	2.959 ± 2.2976
RM3	10	5	0.0113 ± 0.0108	2.265 ± 4.0422
RM4	9	3	0.0087 ± 0.0098	1.748 ± 3.6632
RM5	10	0	0.0065 ± 0.0004	1.301 ± 0.1627
CD1	10	5	0.0074 ± 0.0062	1.484 ± 2.3261
CD2	10	7	0.0127 ± 0.0095	2.544 ± 3.5569
CD3	9	1	0.0024 ± 0.0039	0.483 ± 1.4411
CD4	9	1	0.0020 ± 0.0033	0.402 ± 1.2425
CD5	9	0	0.0009 ± 0.0006	0.174 ± 0.2360
QC1	9	1	0.0049 ± 0.0053	0.971 ± 1.9600
QC2	10	10	0.0246 ± 0.0173	4.945 ± 6.4713
QC3	10	3	0.0049 ± 0.0052	0.994 ± 1.9342
QC4	10	0	0.0003 ± 0.0006	0.054 ± 0.2138
QC5	10	0	0.0001 ± 0.0005	0.029 ± 0.1734

The small amount of roxithromycin found transdermally could be attributed to the topical targeting of the vesicle system or to the lipophilic nature of the API. Niosomes are said to reduce the absorption of drugs into the systemic circulation (More *et al.*, 2016:201) and therefore could be a possible reason why only four out of the fifteen dispersions were delivered transdermally. The APIs are more lipophilic in nature and poorly aqueous soluble, which could lead to the retention of the drug in the more lipophilic areas of the SCE, than the mostly hydrophilic systemic circulation (Perrie *et al.*, 2012:392).

The values obtained for the dispersions of **RM5**, **CD5**, **QC4** and **QC5** were low compared to the other values and can possibly be attributed to the fact that a different skin donor was used during the skin diffusion studies of these experiments. The skin, being biological material is very variable and unpredictable and any physiological condition can change the absorption of a drug through the skin, as it could affect the properties of the skin (Jhawar *et al.*, 2013:49).

It should however be remembered that the aim of this study was the topical delivery of the APIs, since the target site is the dermis and therefore the desired outcome is not to obtain transdermal penetration (diffusion into the receptor phase).

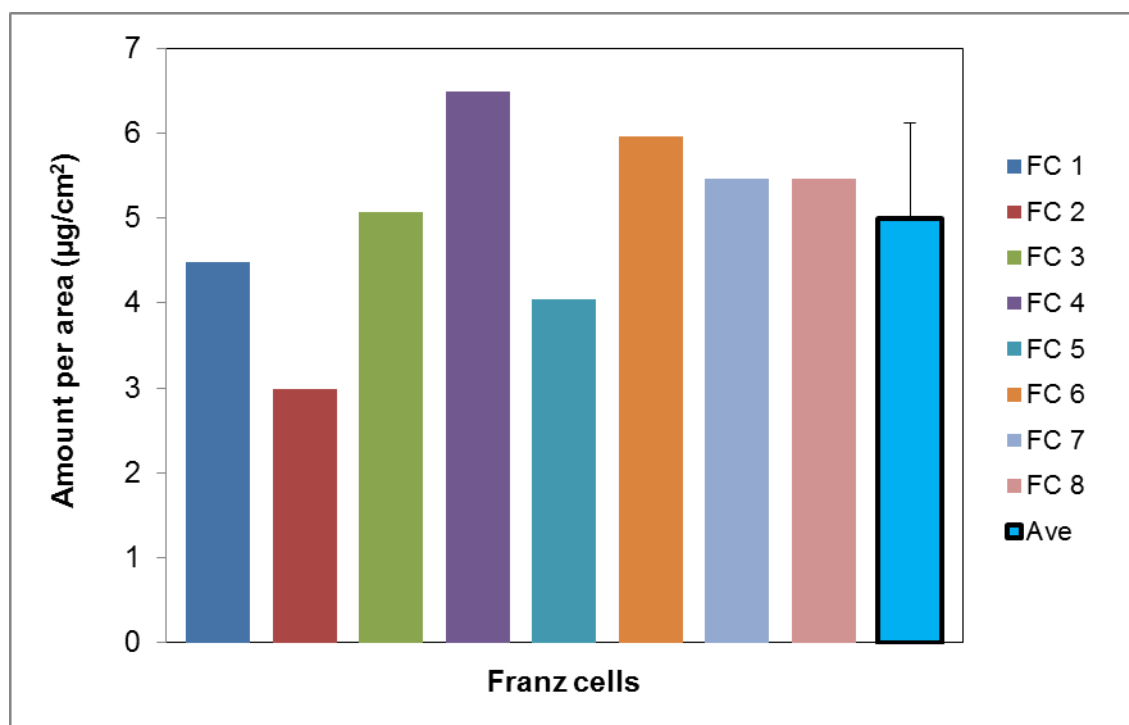


Figure D.36: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **RM1**

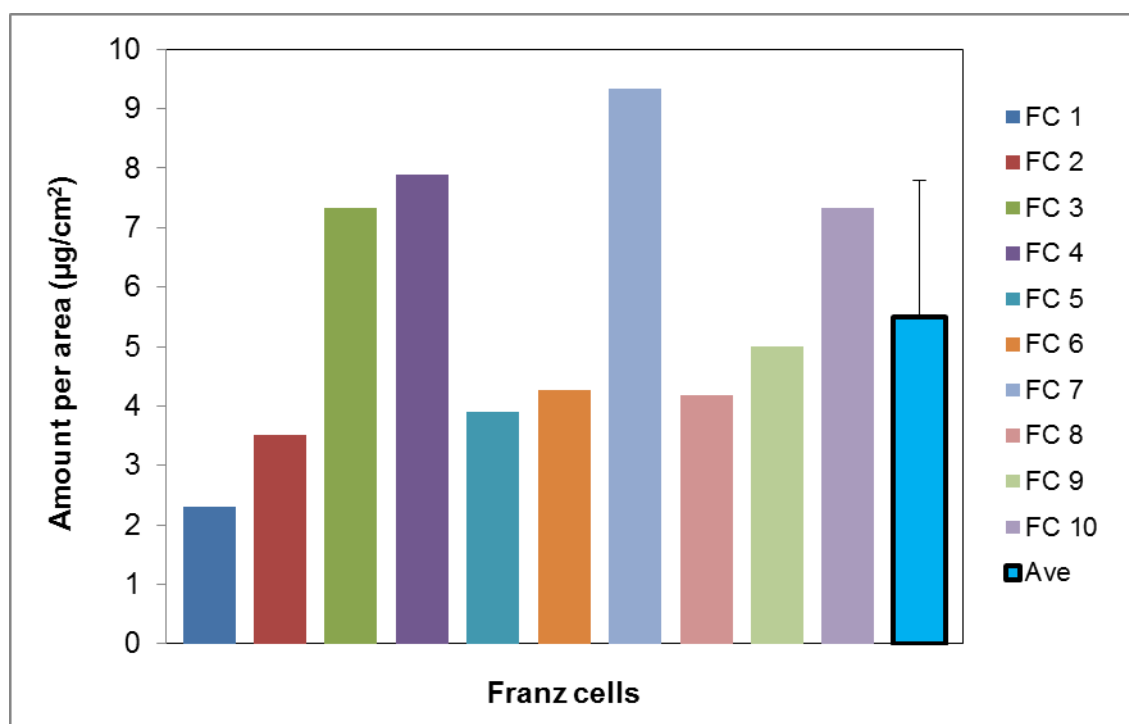


Figure D.37: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **RM2**

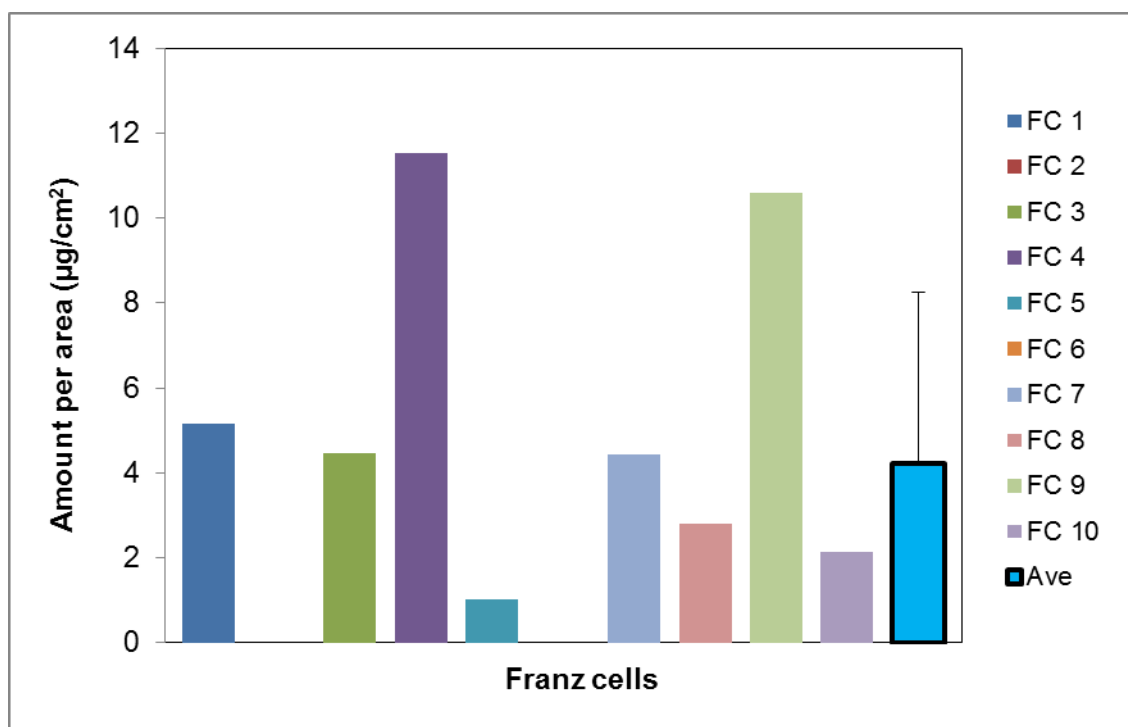


Figure D.38: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **RM3**

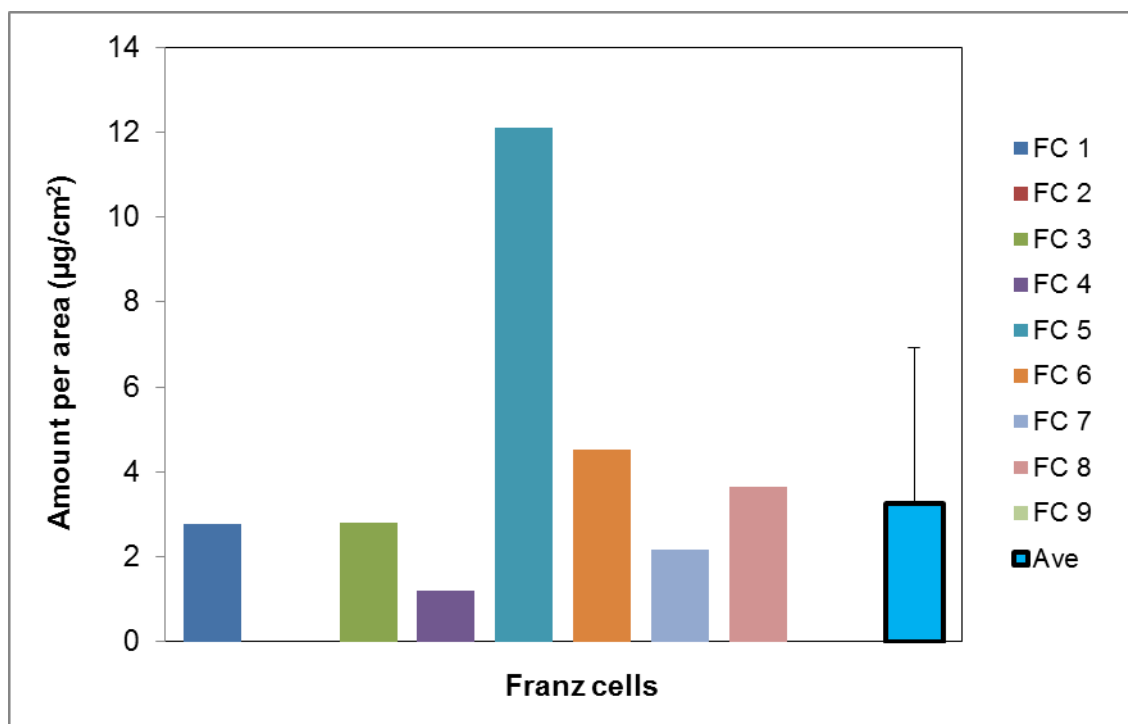


Figure D.39: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **RM4**

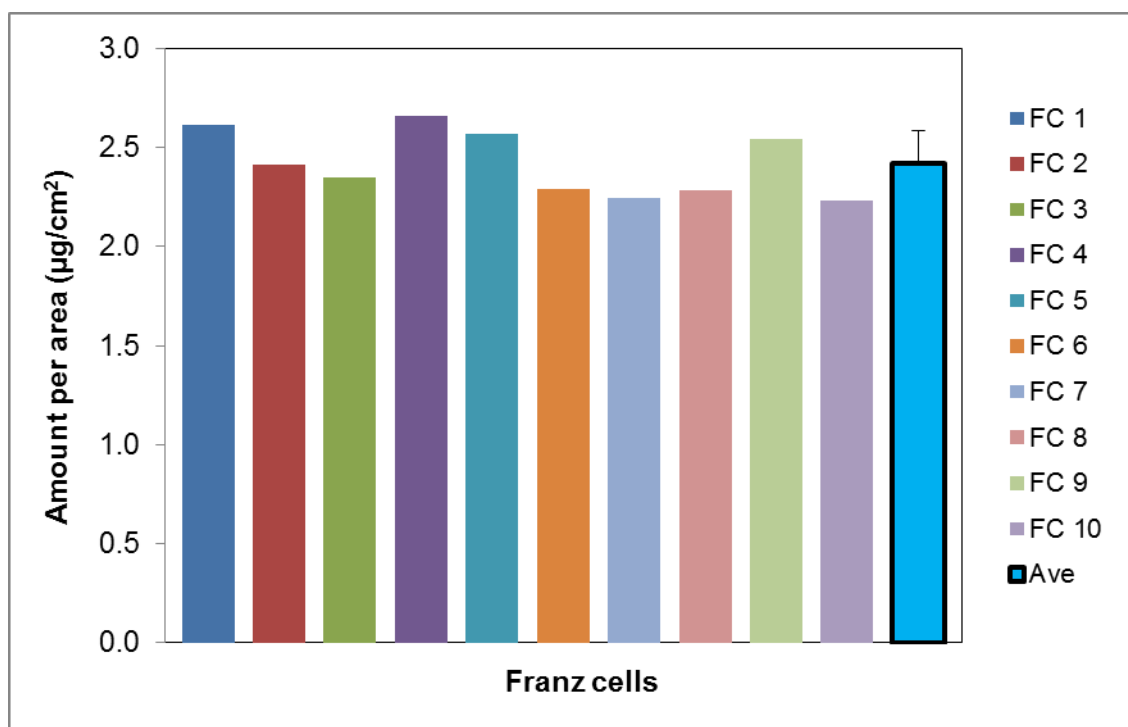


Figure D.40: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **RM5**

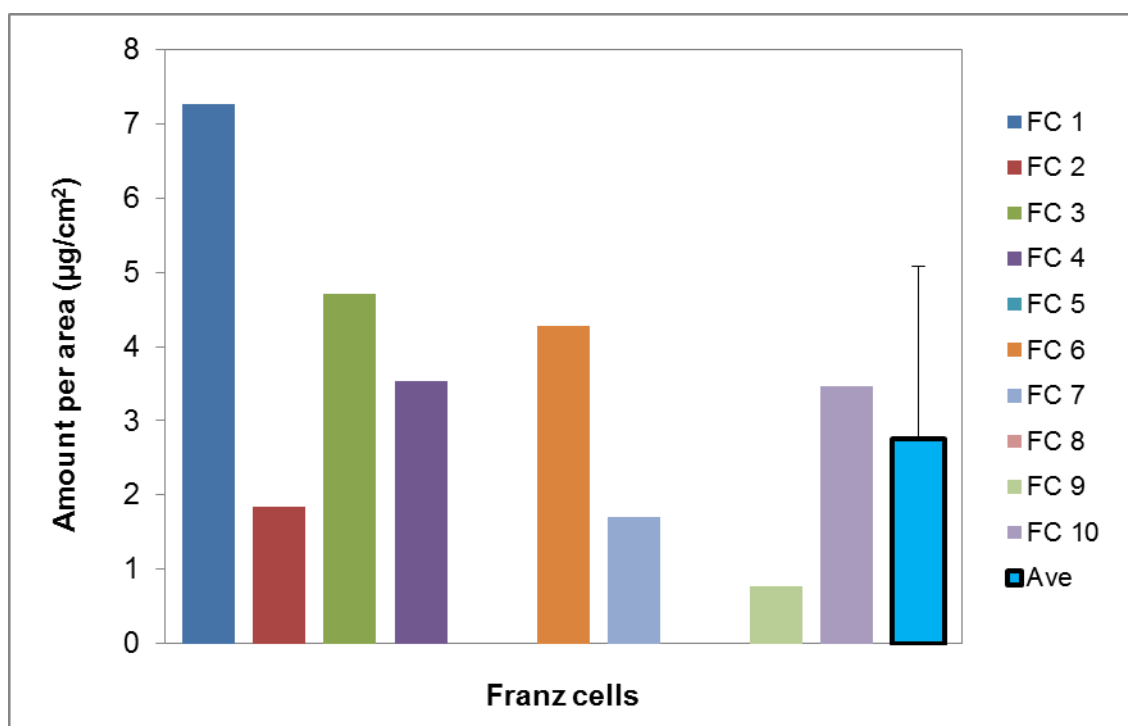


Figure D.41: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **CD1**

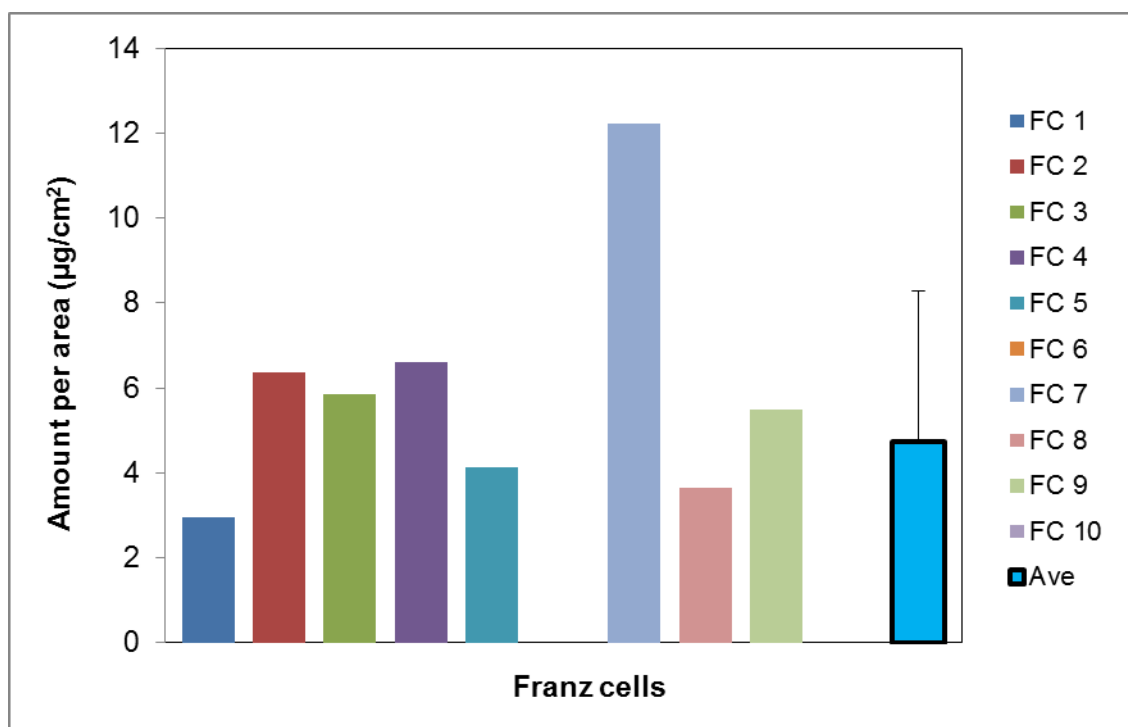


Figure D.42: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **CD2**

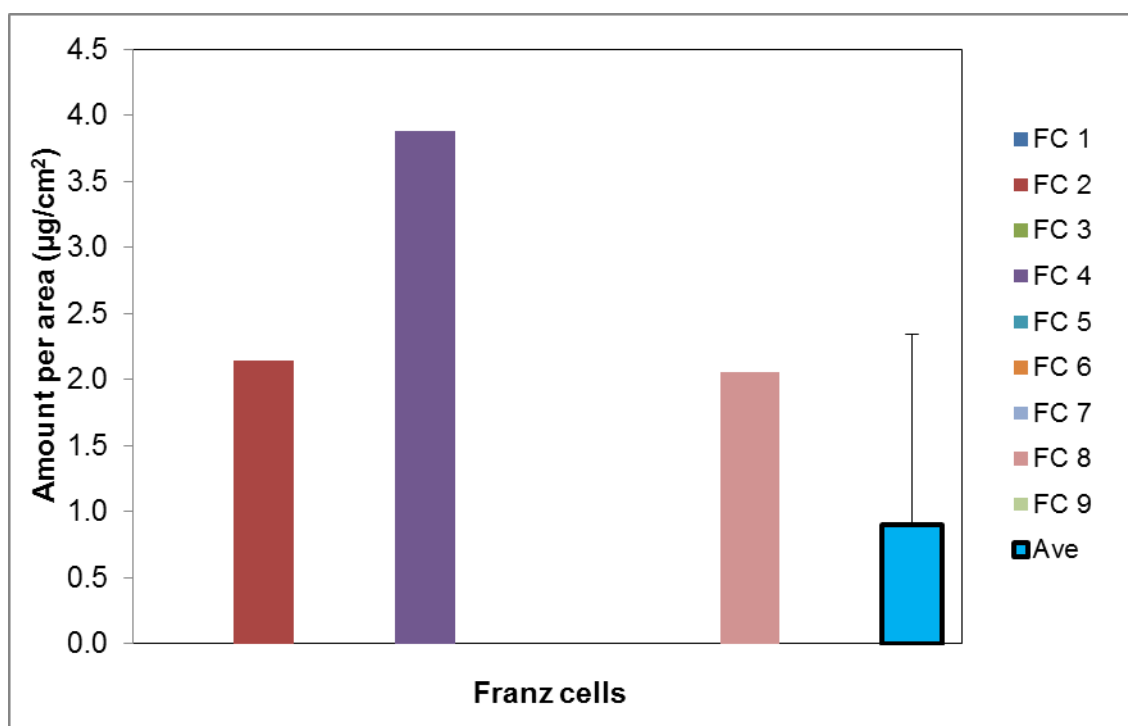


Figure D.43: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **CD3**

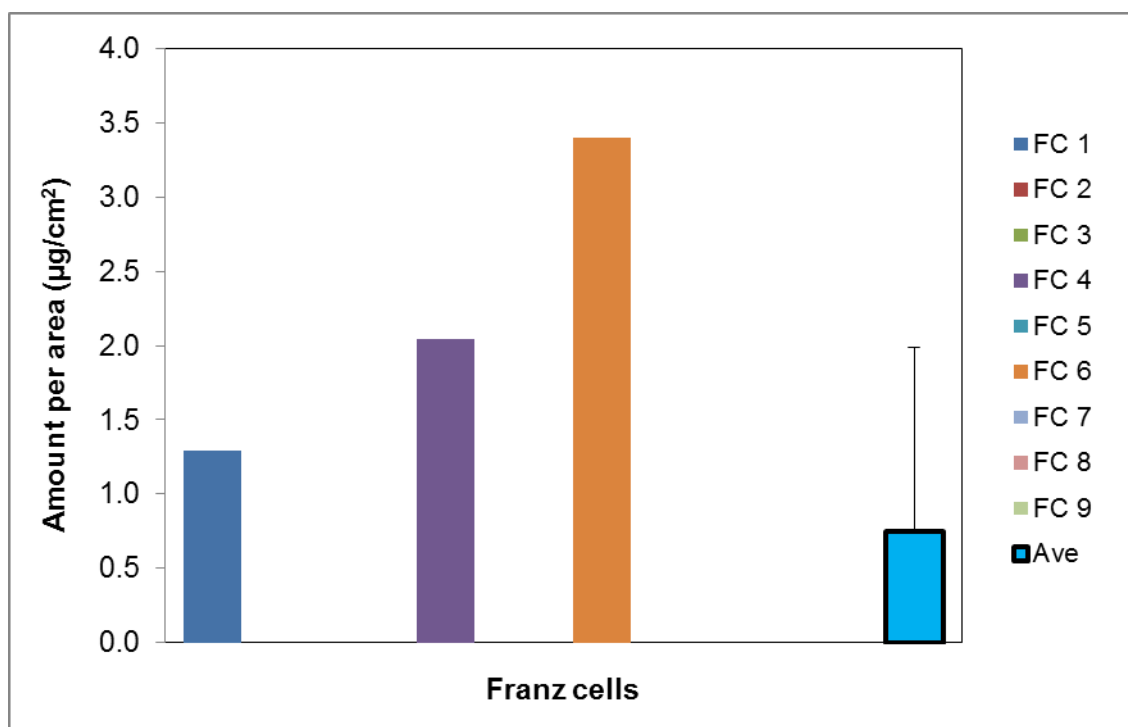


Figure D.44: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **CD4**

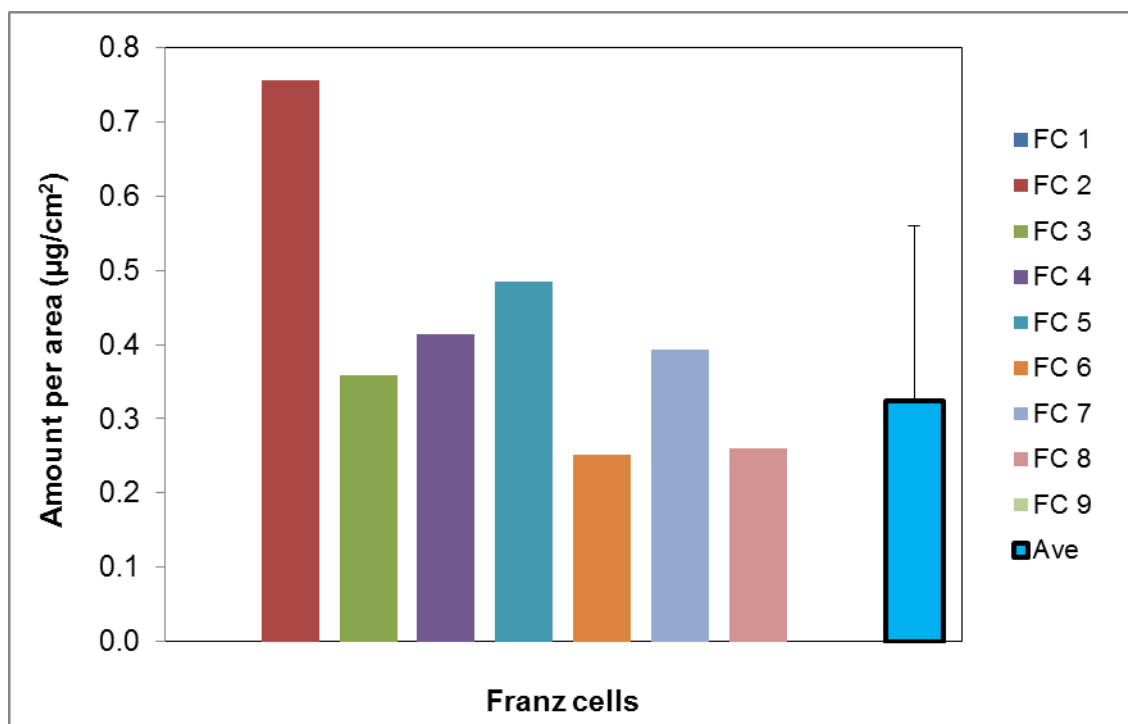


Figure D.45: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **CD5**

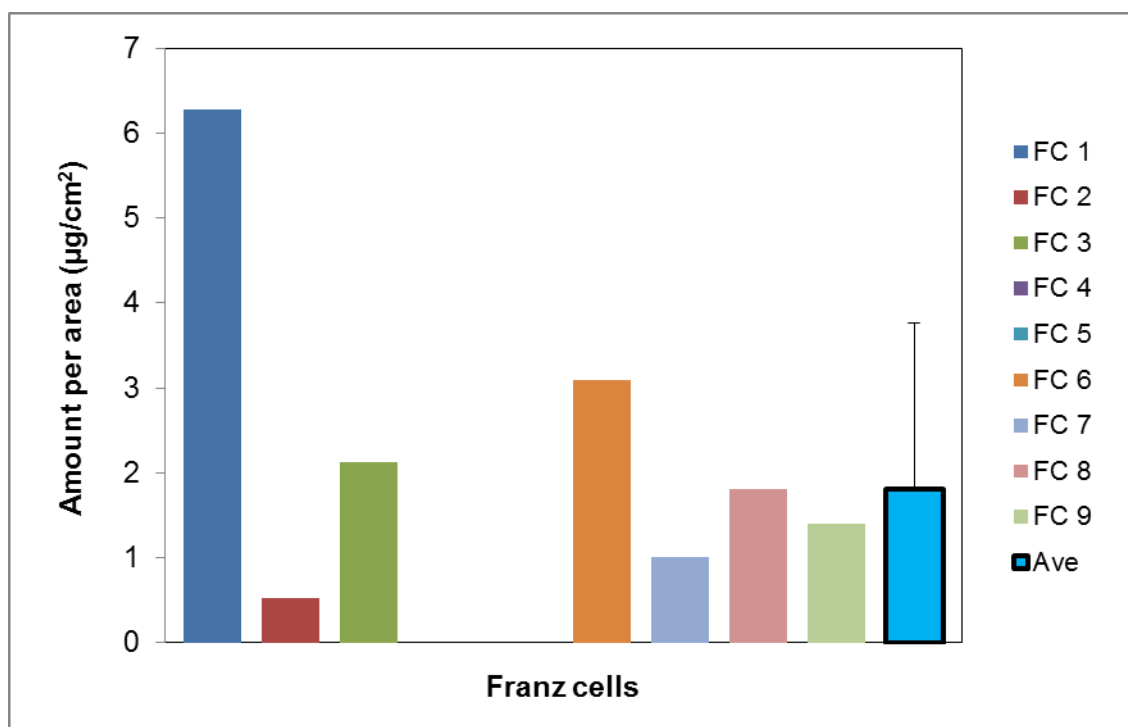


Figure D.46: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **QC1**

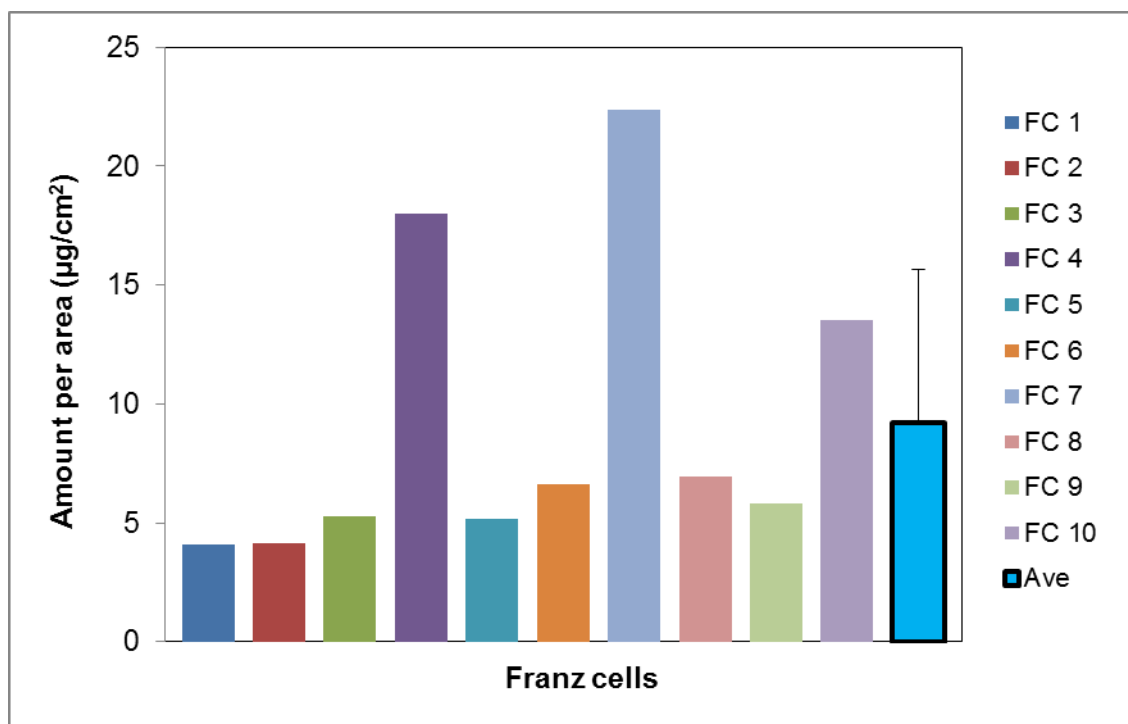


Figure D.47: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **QC2**

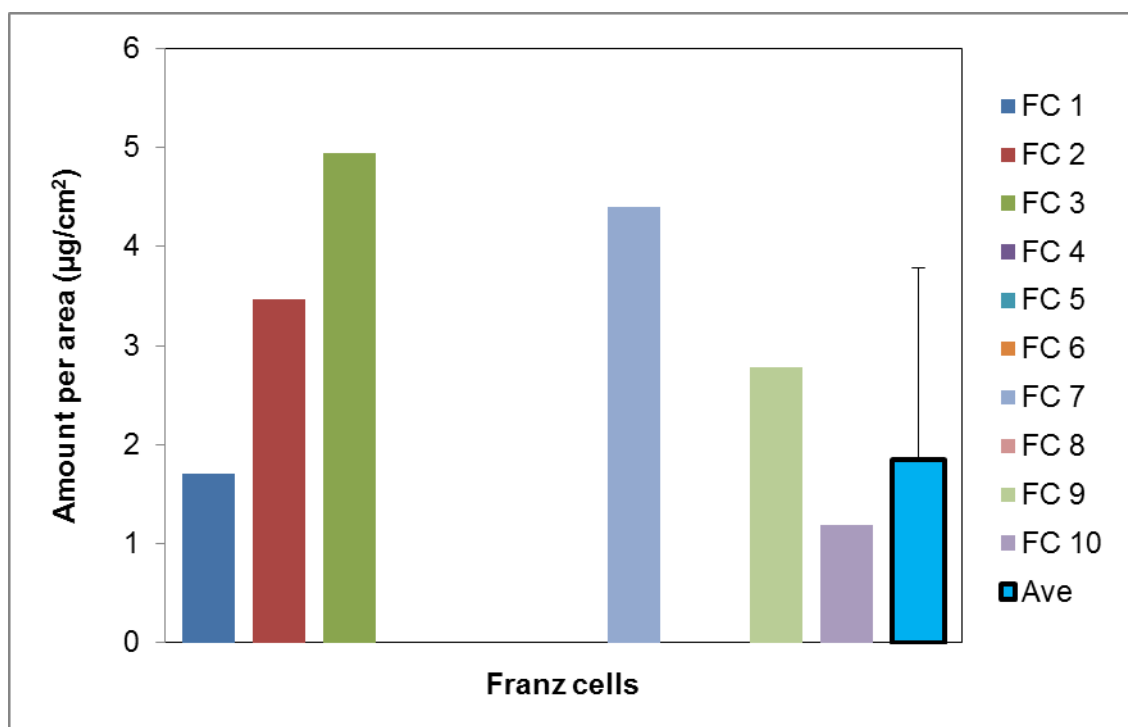


Figure D.48: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **QC3**

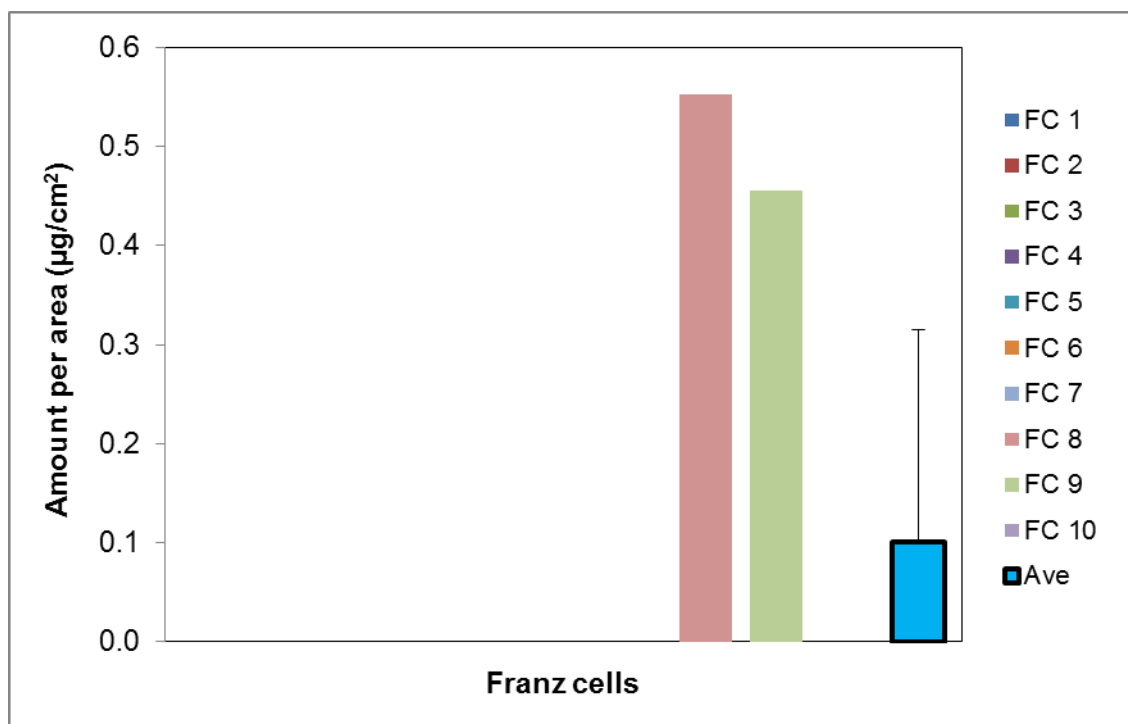


Figure D.49: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **QC4**

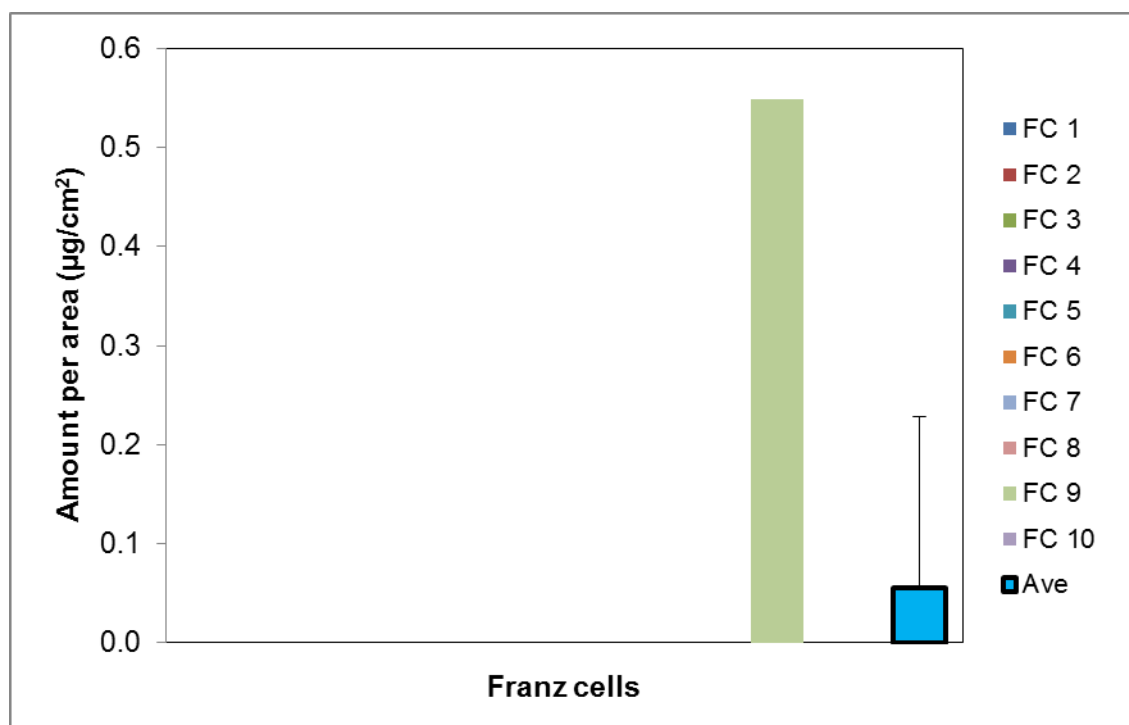


Figure D.50: Roxithromycin concentration in the receptor phase of the Franz cells during the diffusion study performed on niosome vesicle of **QC5**

D.3.4.2 Tape stripping

Tape stripping was performed to determine if the API encapsulated into the niosomes diffused into the skin. The target site for this study was therefore the skin and if the API resided in the skin layers, the aim would be reached. The results obtained through tape stripping are represented in Table D.3 and illustrate the average concentration of roxithromycin (µg/ml) present in the SCE and ED. It also displays $n \geq \text{LLOQ}$, which shows the amount of Franz cells that had values above the LOD and LLOQ values. Figures D.51 to D.62 depict the results from the SCE as obtained during the skin diffusion studies.

Table D.3: The average concentration of roxithromycin present in the SCE and the ED obtained through tape stripping after the 12 h skin diffusion studies

Dispersion	n	n $\geq$ LLOQ (for SCE)	n $\geq$ LLOQ (for ED)	Average concentration in SCE ($\mu\text{g/ml}$)	Average concentration in ED ($\mu\text{g/ml}$)
RM1	8	8	7	2.762 ± 0.618	2.419 ± 0.698
RM2	10	0	4	0.300 ± 0.415	1.652 ± 0.450
RM3	10	1	9	0.896 ± 0.699	2.587 ± 1.013
RM4	9	0	9	0.000 ± 0.000	3.044 ± 1.488
RM5	10	9	3	3.375 ± 1.615	1.697 ± 0.254
CD1	10	7	2	2.469 ± 1.325	1.415 ± 0.504
CD2	10	1	8	0.957 ± 0.593	2.531 ± 1.174
CD3	9	0	9	0.000 ± 0.000	2.585 ± 0.736
CD4	9	0	0	0.000 ± 0.000	0.757 ± 0.384
CD5	9	6	1	2.364 ± 1.599	1.043 ± 0.506
QC1	9	9	5	5.442 ± 3.125	2.189 ± 0.940
QC2	10	9	10	2.793 ± 0.905	6.277 ± 2.367
QC3	10	0	4	0.349 ± 0.596	1.770 ± 1.211
QC4	10	0	0	0.059 ± 0.076	0.396 ± 0.182
QC5	10	0	0	0.245 ± 0.235	0.588 ± 0.328

D.3.4.2.1 Stratum corneum-epidermis

In the SCE, the results obtained for the concentration of roxithromycin are ranked as follows: **QC1 > RM5 > QC2 > RM1 > CD1 > CD5 > CD2 > RM3 > QC3 > RM2 > QC5 > QC4 > RM4 = CD3 = CD4**. From the concentration of roxithromycin found in the SCE, only 6 dispersions displayed values above the LOD and LLOQ, as highlighted in Table D.3. The 6 dispersions ranked from highest to lowest include: **QC1, RM5, QC2, RM1, CD1** and lastly, **CD5**. It was noticed dispersion 1 was represented three times, while dispersions 5 and 2 appeared twice and once, respectively. Dispersions 1 (0.5:1.0) and 5 (2.0:2.5) contained more Span[®] 40 than cholesterol. It is evident the dispersions containing a higher amount of Span[®] 40 were able to reach and reside in the SCE. A possible explanation is the fact that Span[®] 40, with an HLB value of 6.7, is more oil soluble, therefore residing in the lipophilic regions of the SCE (CRODA, 2015:45).

The effect of surfactants in biological systems are very complex and for this reason the permeation of the dispersions containing different amounts of Span[®] 40 (surfactant) may vary considerably (Som *et al.*, 2012:3).

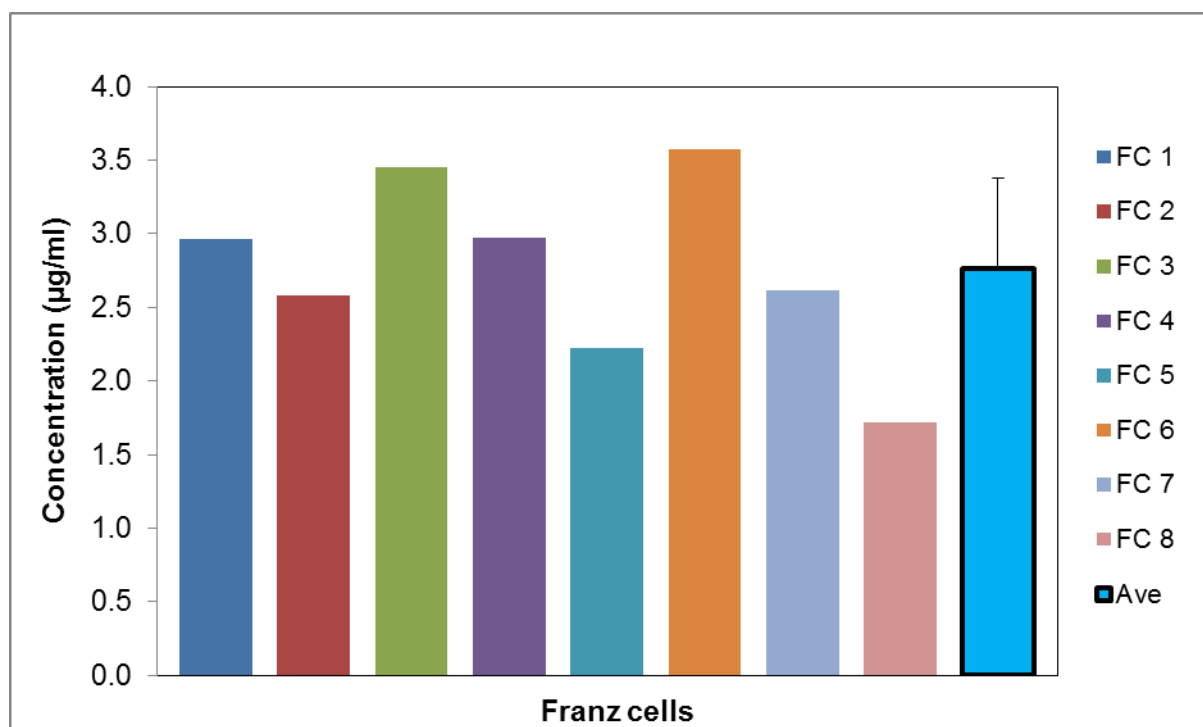


Figure D.51: Roxithromycin concentration (µg/ml) from niosome vesicle **RM1** in the SCE after tape stripping

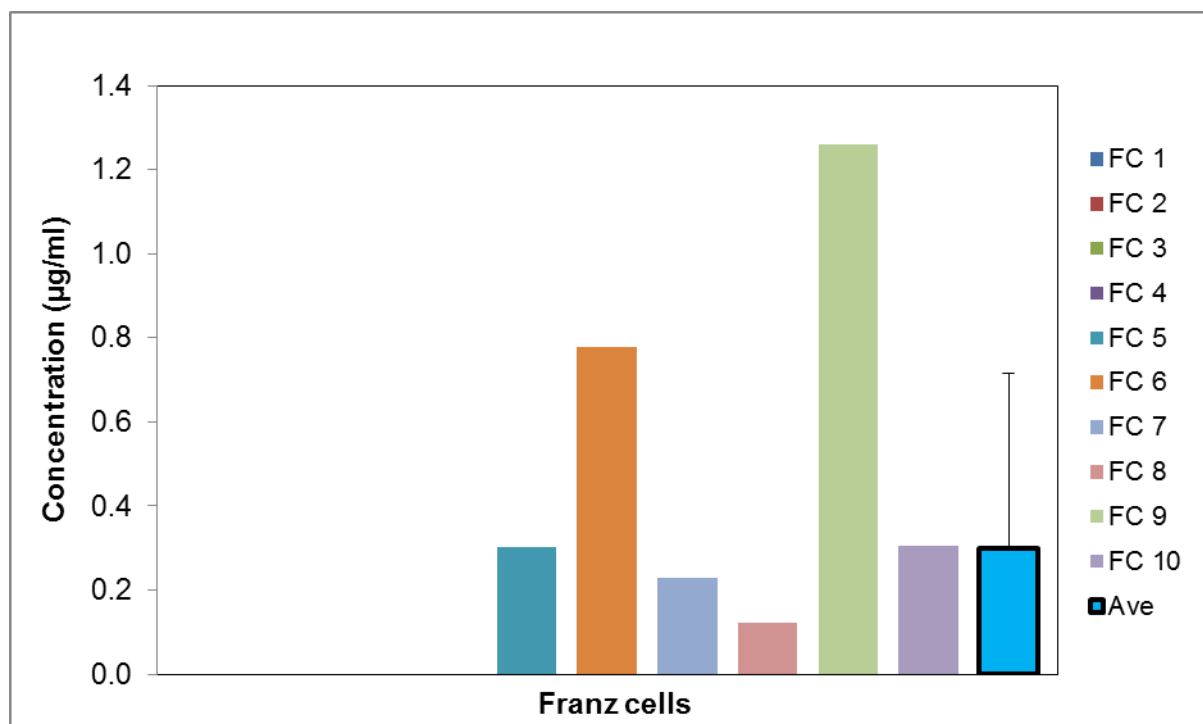


Figure D.52: Roxithromycin concentration (µg/ml) from niosome vesicle **RM2** in the SCE after tape stripping

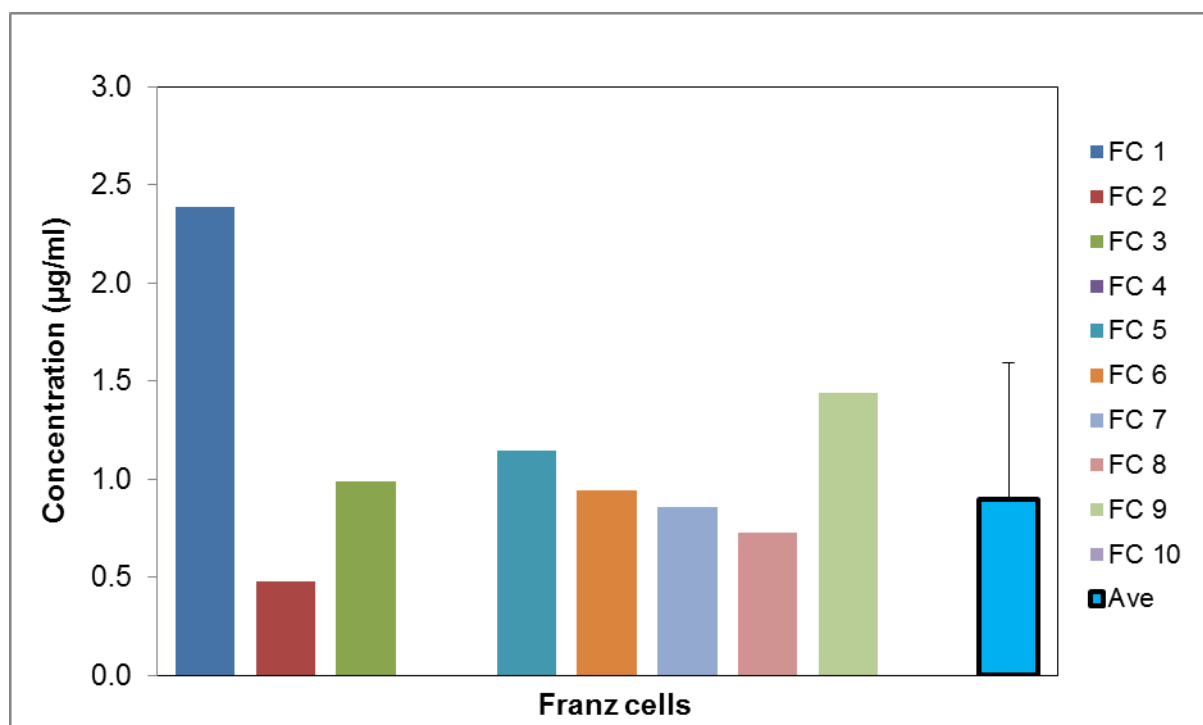


Figure D.53: Roxithromycin concentration (µg/ml) from niosome vesicle **RM3** in the SCE after tape stripping

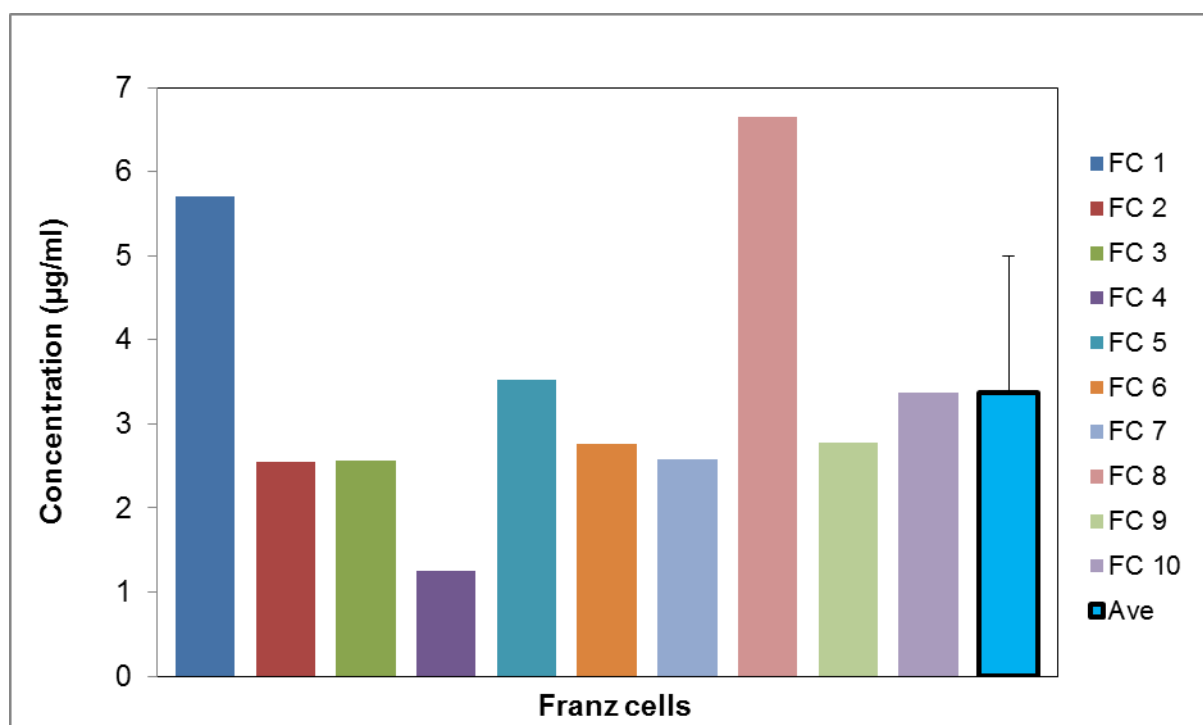


Figure D.54: Roxithromycin concentration (µg/ml) from niosome vesicle **RM5** in the SCE after tape stripping

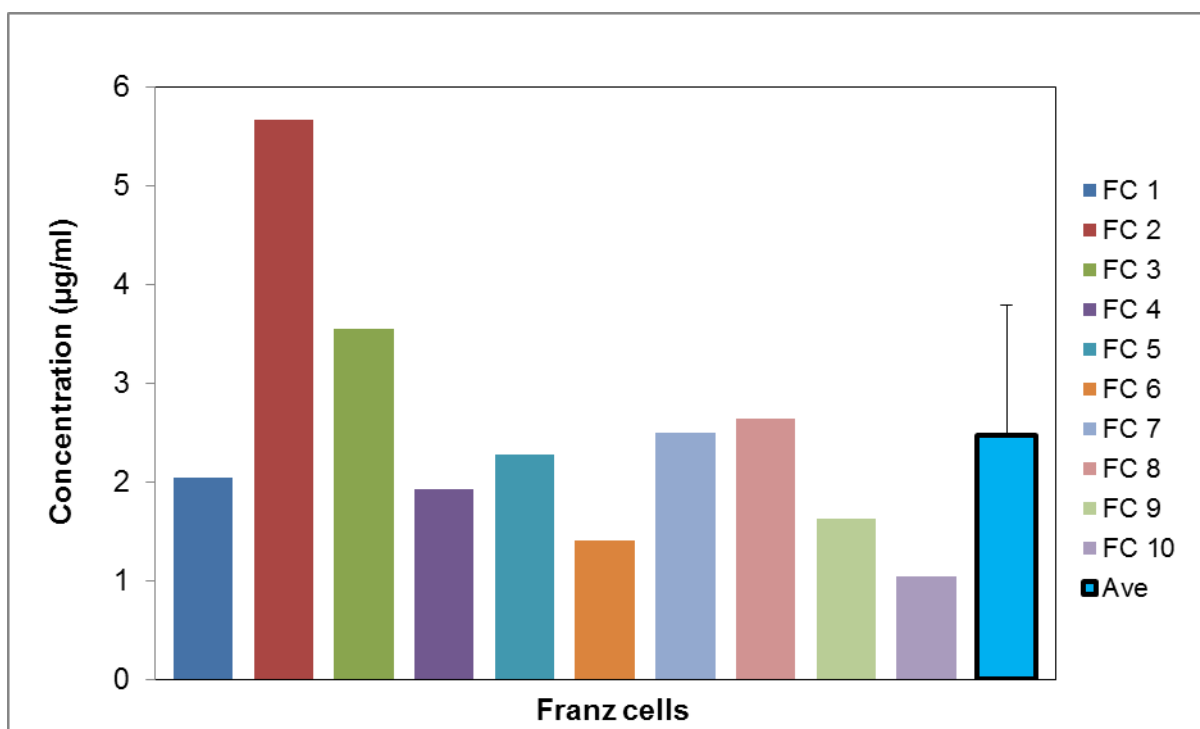


Figure D.55: Roxithromycin concentration (µg/ml) from niosome vesicle **CD1** in the SCE after tape stripping

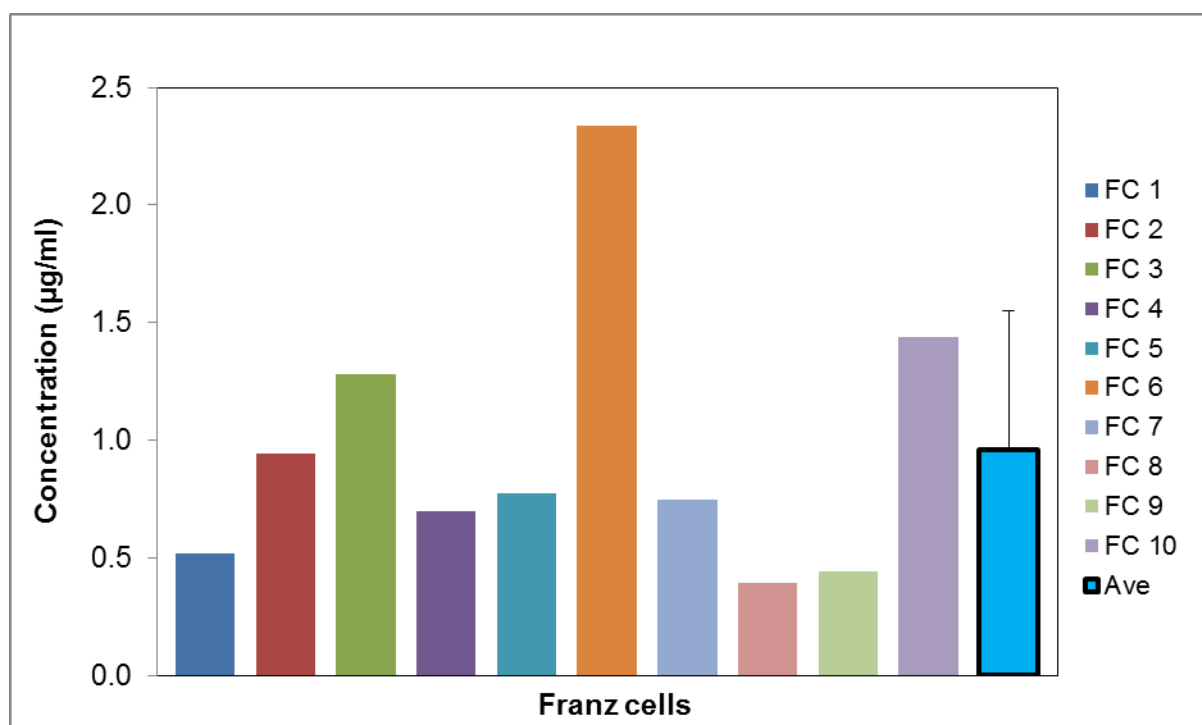


Figure D.56: Roxithromycin concentration (µg/ml) from niosome vesicle **CD2** in the SCE after tape stripping

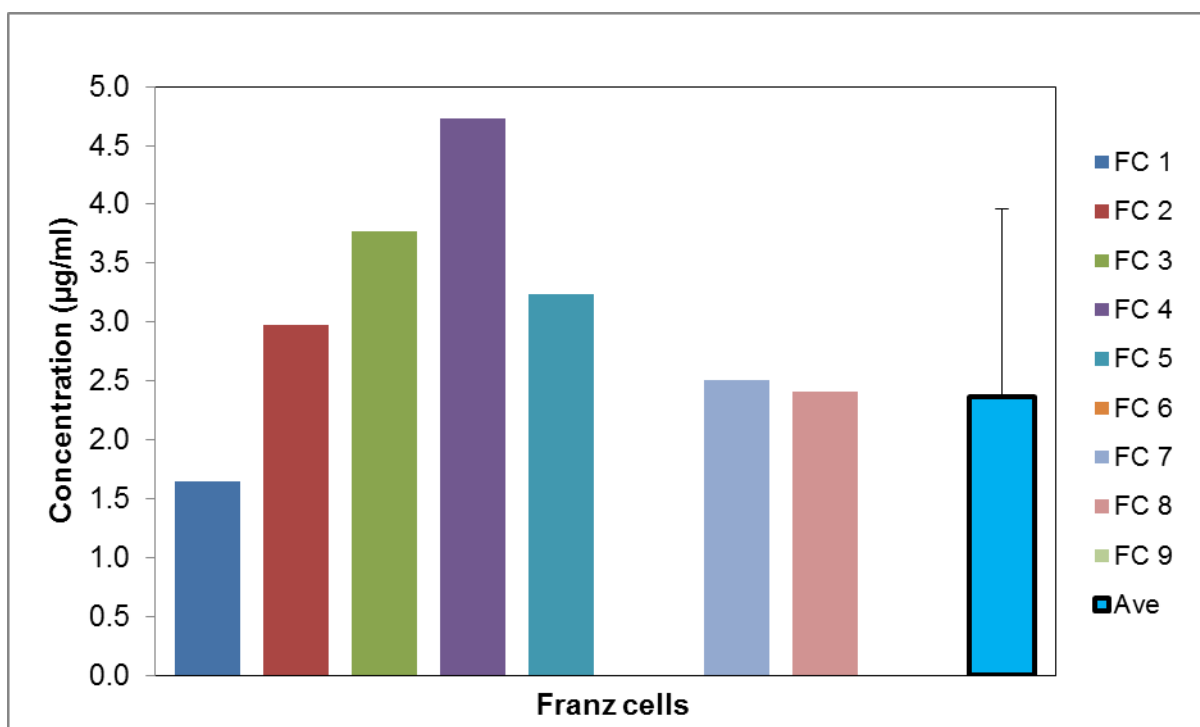


Figure D.57: Roxithromycin concentration (µg/ml) from niosome vesicle **CD5** in the SCE after tape stripping

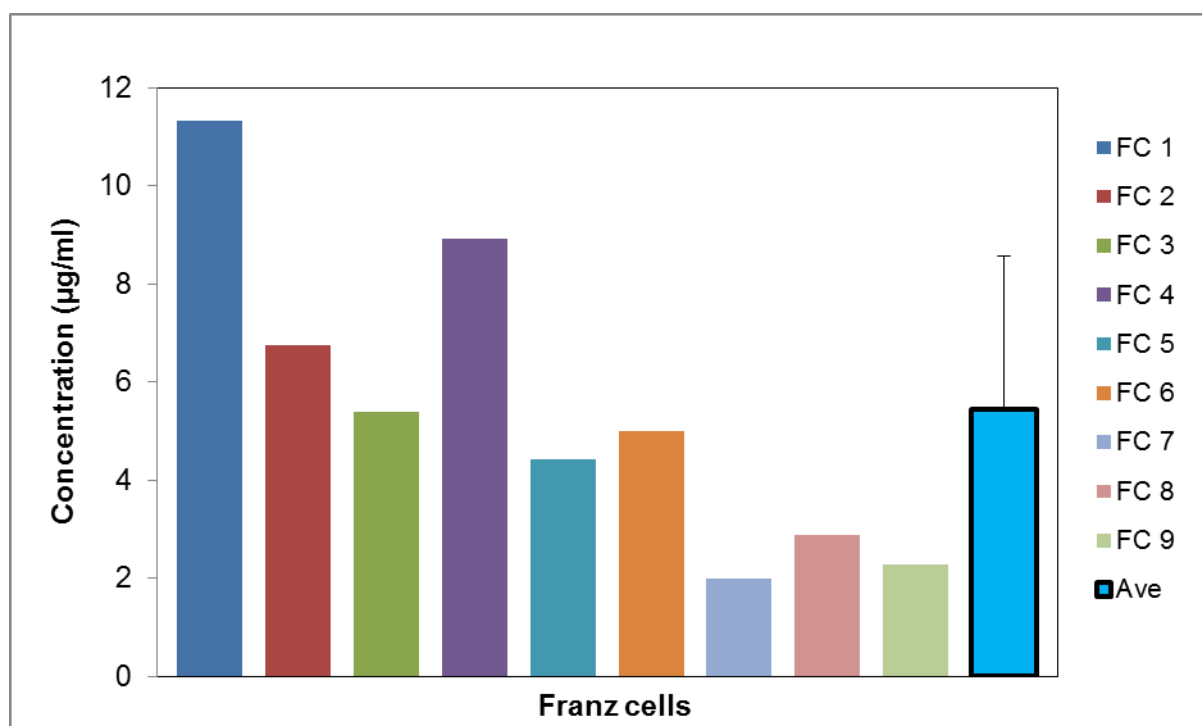


Figure D.58: Roxithromycin concentration (µg/ml) from niosome vesicle **QC1** in the SCE after tape stripping

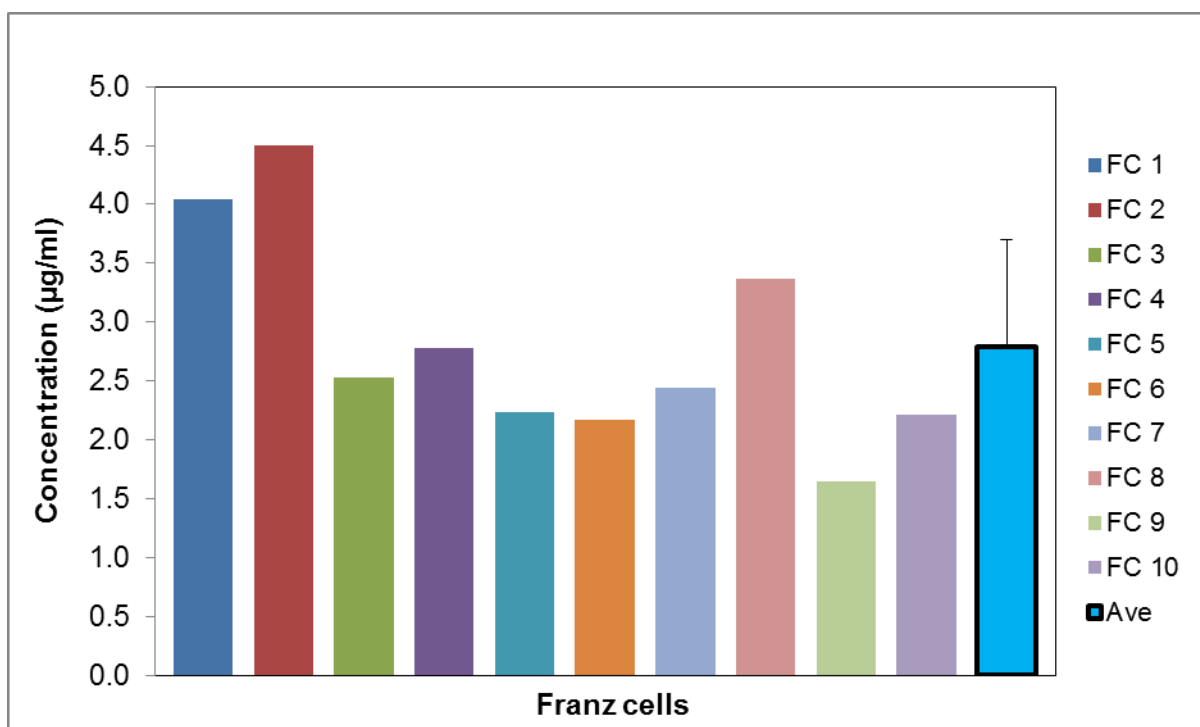


Figure D.59: Roxithromycin concentration (µg/ml) from niosome vesicle **QC2** in the SCE after tape stripping

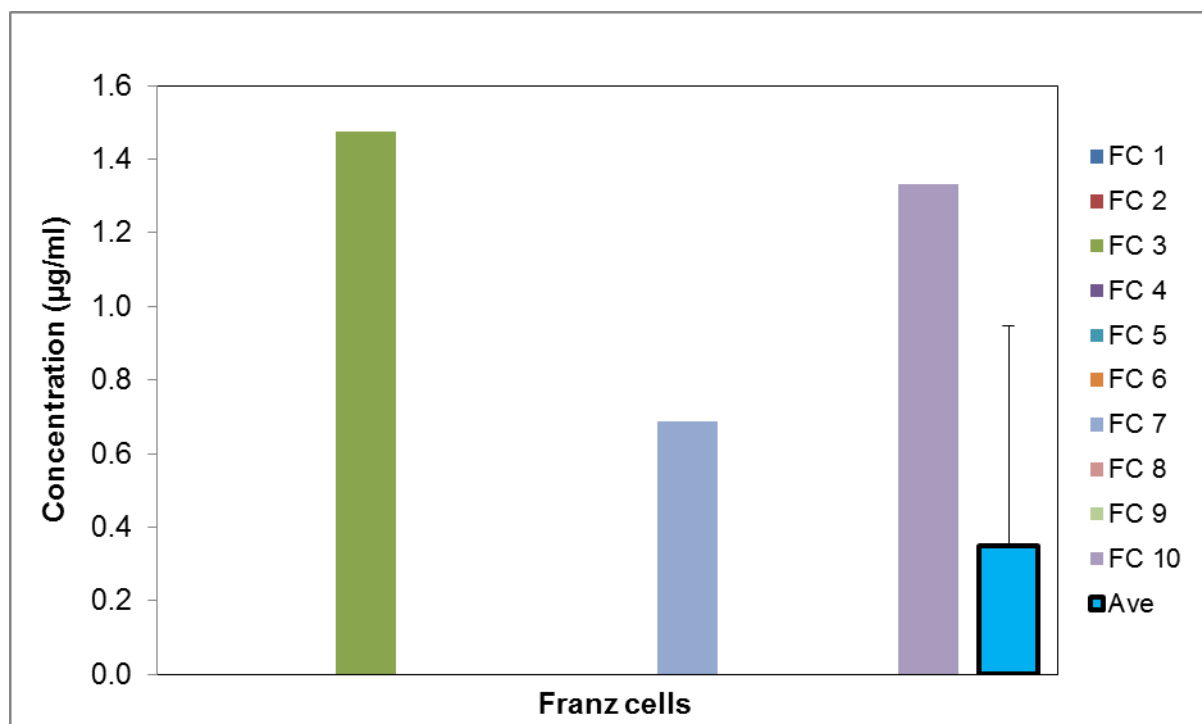


Figure D.60: Roxithromycin concentration (µg/ml) from niosome vesicle **QC3** in the SCE after tape stripping

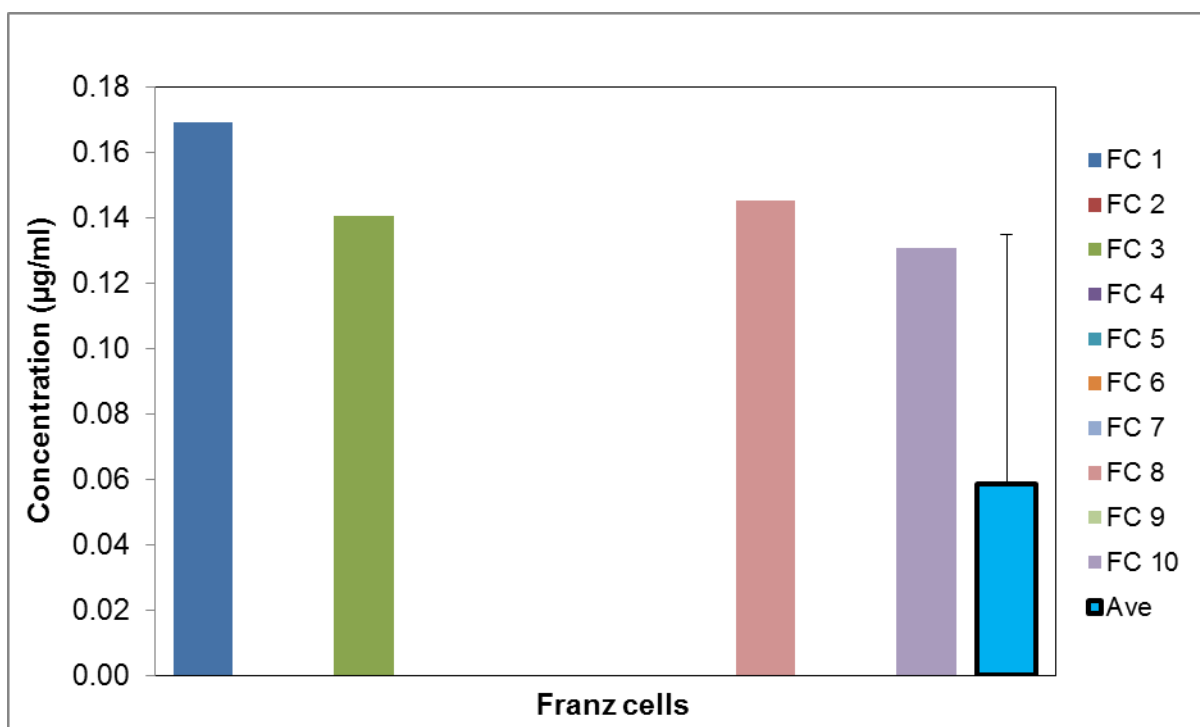


Figure D.61: Roxithromycin concentration (µg/ml) from niosome vesicle **QC4** in the SCE after tape stripping

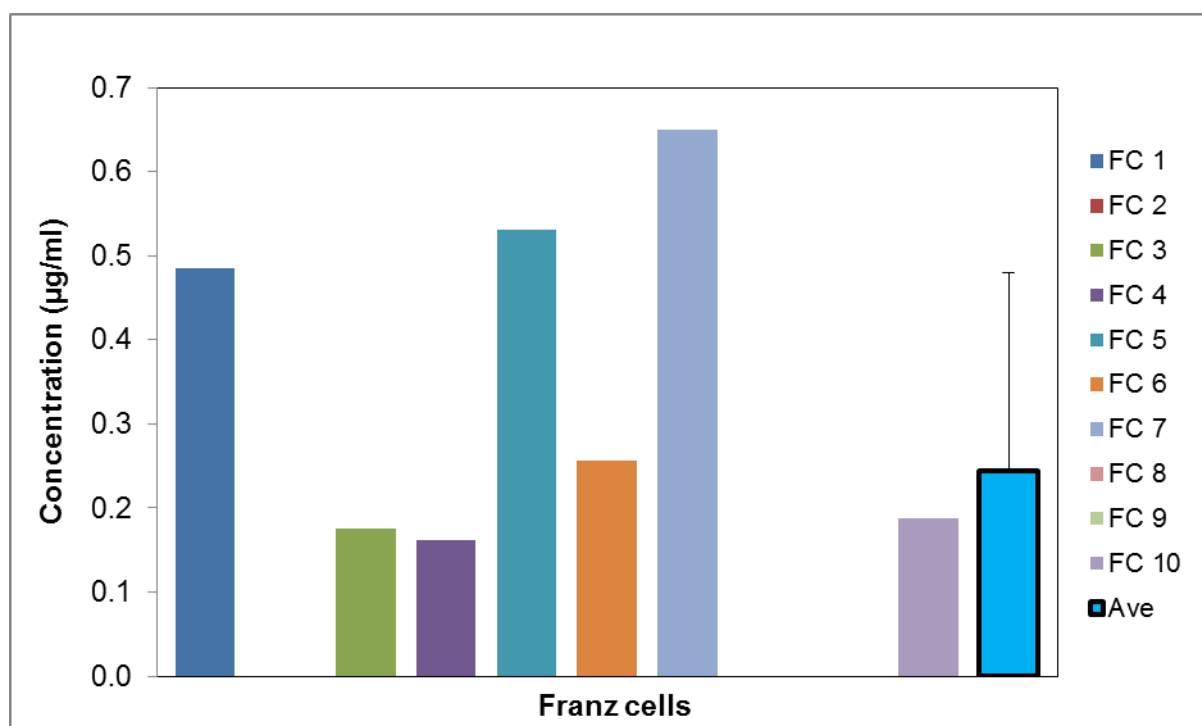


Figure D.62: Roxithromycin concentration (µg/ml) from niosome vesicle **QC5** in the SCE after tape stripping

D.3.4.2.2 Epidermis-dermis

As seen in Table D.3, all fifteen dispersions had diffused into the ED and can be ranked from highest to lowest as follows: **QC2, RM4, RM2, CD3, CD2, RM1, QC1, QC3, RM5, RM2, CD1, CD5, CD4, QC5** and **QC4**. Only 6 of the dispersions had values above the LOD and LLOQ: **QC2, RM4, RM3, CD3, CD2, and RM1**. It is observed that dispersions 2 and 3 were both represented twice, while dispersions 4 and 1 appeared only once.

When dispersions 3 (2.0:1.0) and 4 (2.0:1.5) were compared to the other dispersions, both have a greater ratio of cholesterol to Span[®] 40. Accordingly, cholesterol should have had an influence on the delivery of **RM4, RM3** and **CD3** into the ED. Cholesterol is an amphiphilic substance and has both a hydrophilic and a lipophilic region. It is possible when the concentration of cholesterol is higher than that of Span[®] 40, the hydrophilic rather than the lipophilic properties of cholesterol are favoured and as a result the API may accumulate in the more hydrophilic area of the ED.

Dispersion 2 (1.0:1.0) contained the same amount of cholesterol and Span[®] 40 and in this case it would appear the solid-state form may have a greater influence on the diffusion and/or penetration of the API into the ED rather than the composition of different excipients used during the formulation of the vesicle system (as seen with dispersions 3 and 4).

The dispersions of **RM4, CD3** and **CD4** had no values found in the SCE, only in the ED and it could be said that the use of niosomes as a vesicle system had reached the aim to achieve targeted drug delivery. Targeted drug delivery attempts to concentrate the drug in the target area and reduces the relative amount of drug in the remaining areas (Karim *et al.*, 2010:375). Figures D.63 to D.77 depicts the results from the ED as obtained for all fifteen the dispersions during the skin diffusion studies.

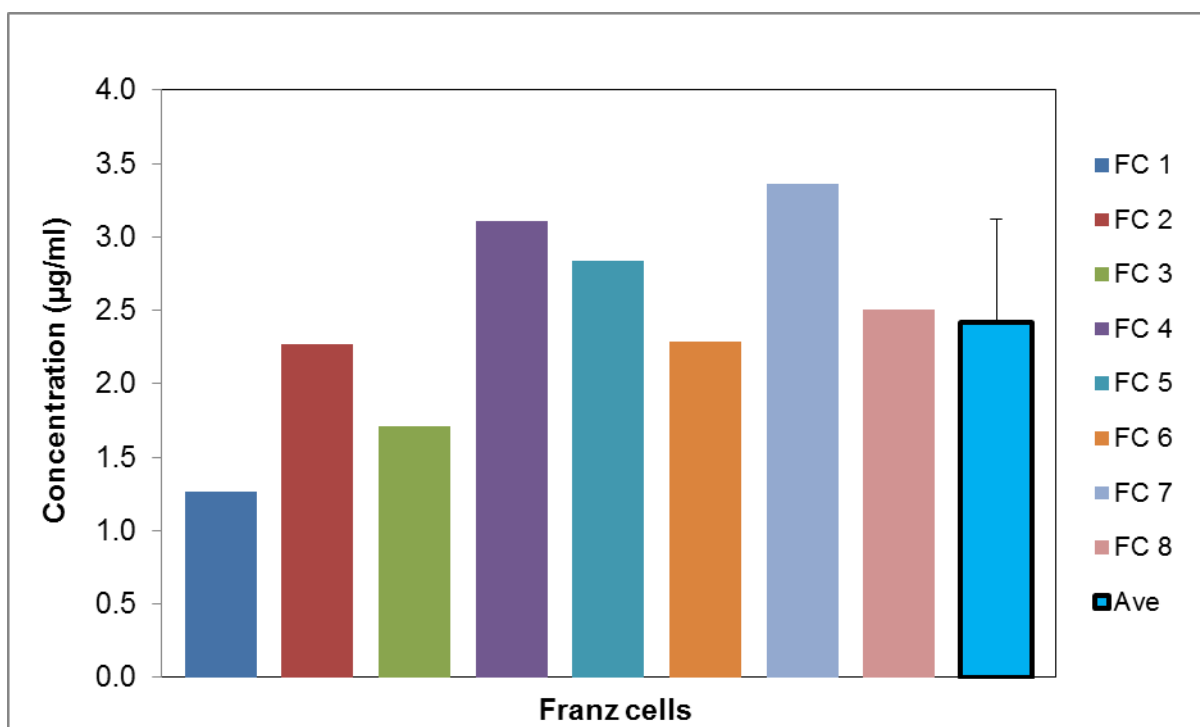


Figure D.63: Roxithromycin concentration (µg/ml) from niosome vesicle **RM1** in the ED after tape stripping

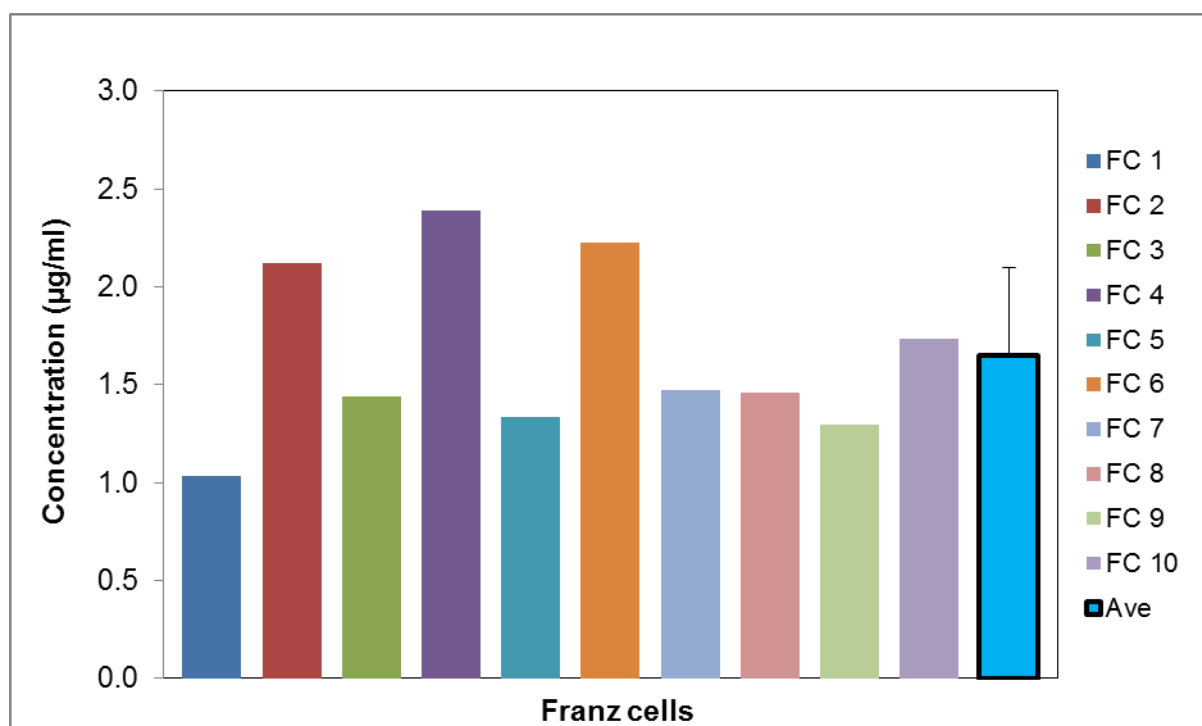


Figure D.64: Roxithromycin concentration (µg/ml) from niosome vesicle **RM2** in the ED after tape stripping

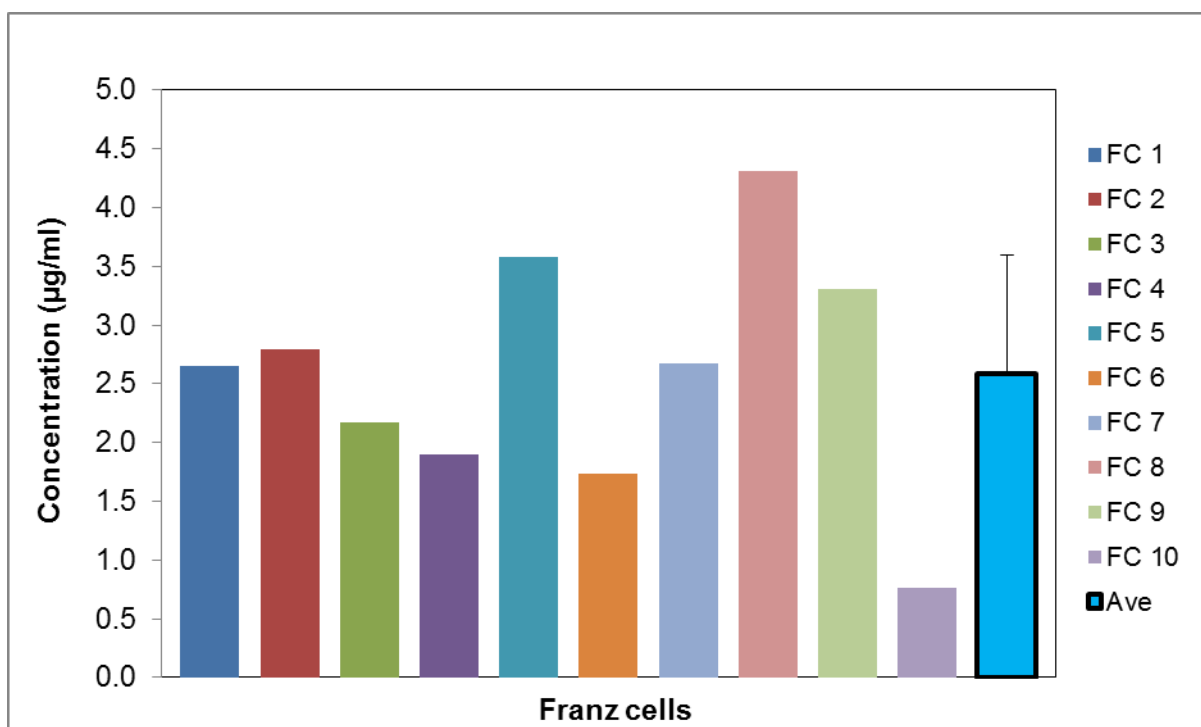


Figure D.65: Roxithromycin concentration (µg/ml) from niosome vesicle **RM3** in the ED after tape stripping

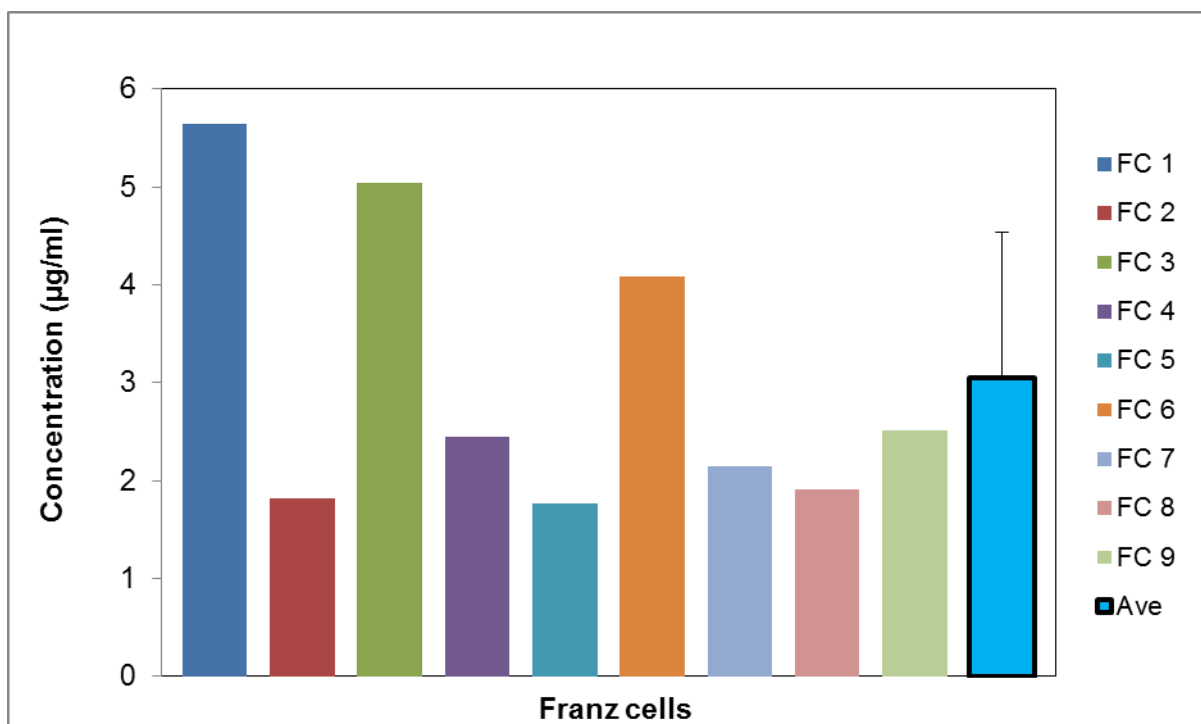


Figure D.66: Roxithromycin concentration (µg/ml) from niosome vesicle **RM4** in the ED after tape stripping

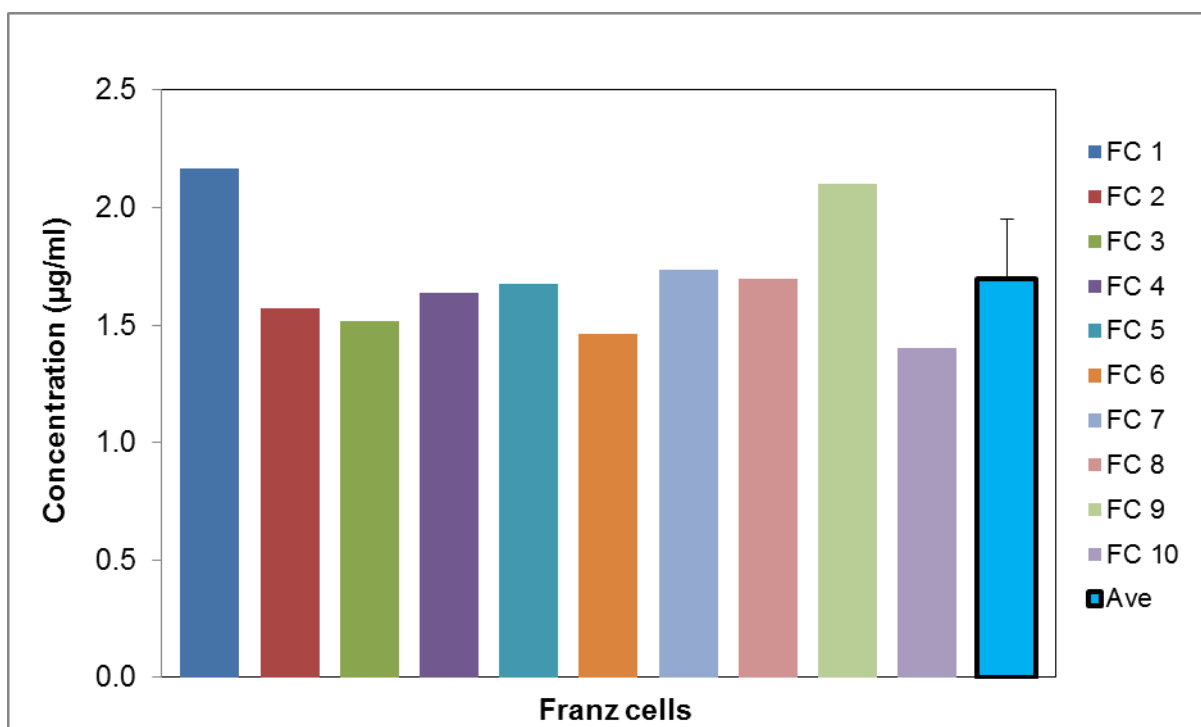


Figure D.67: Roxithromycin concentration (µg/ml) from niosome vesicle **RM5** in the ED after tape stripping

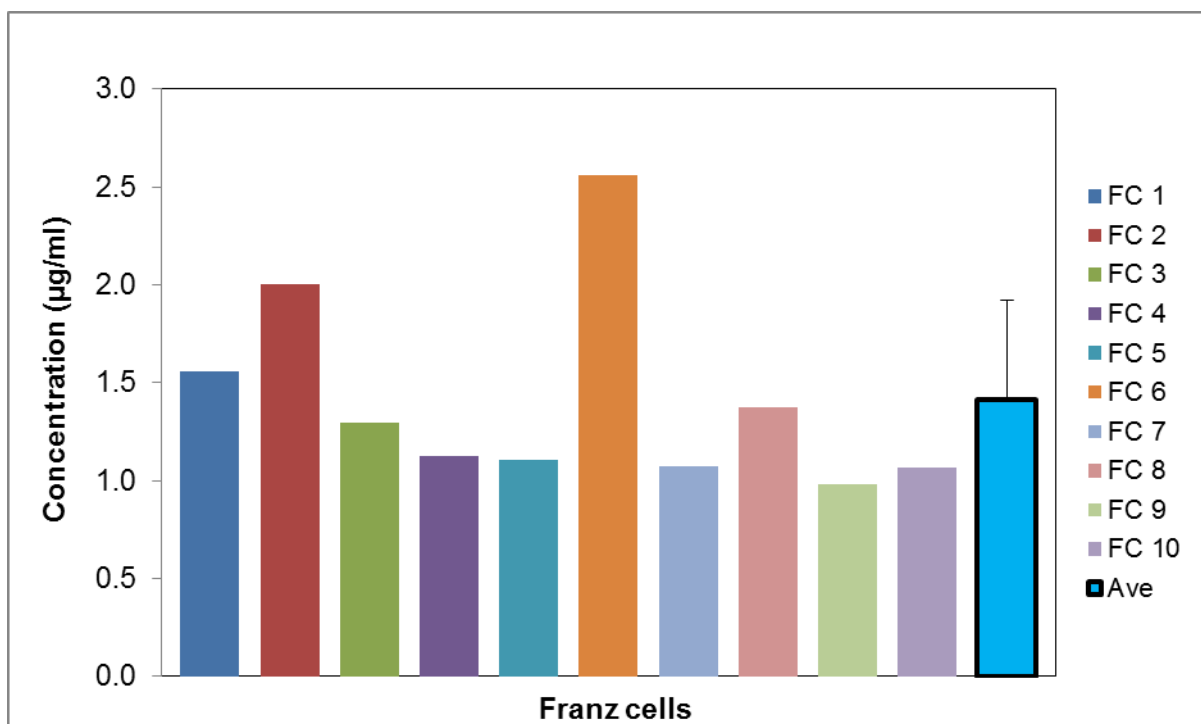


Figure D.68: Roxithromycin concentration (µg/ml) from niosome vesicle **CD1** in the ED after tape stripping

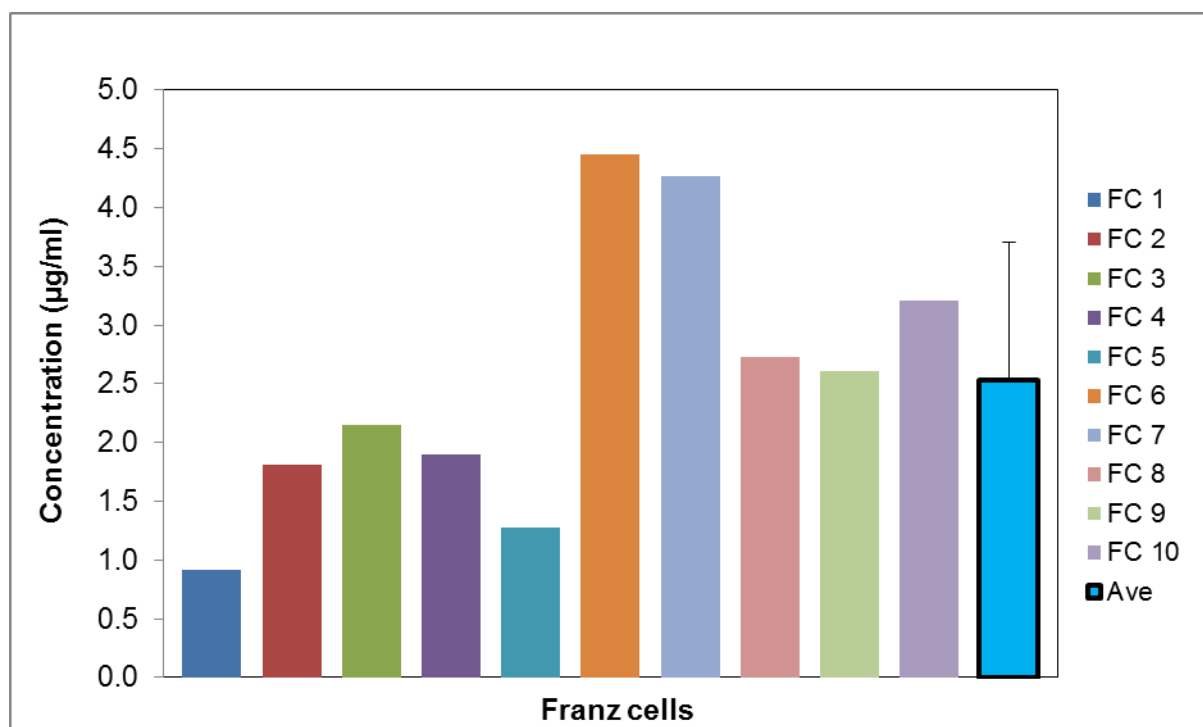


Figure D.69: Roxithromycin concentration (µg/ml) from niosome vesicle **CD2** in the ED after tape stripping

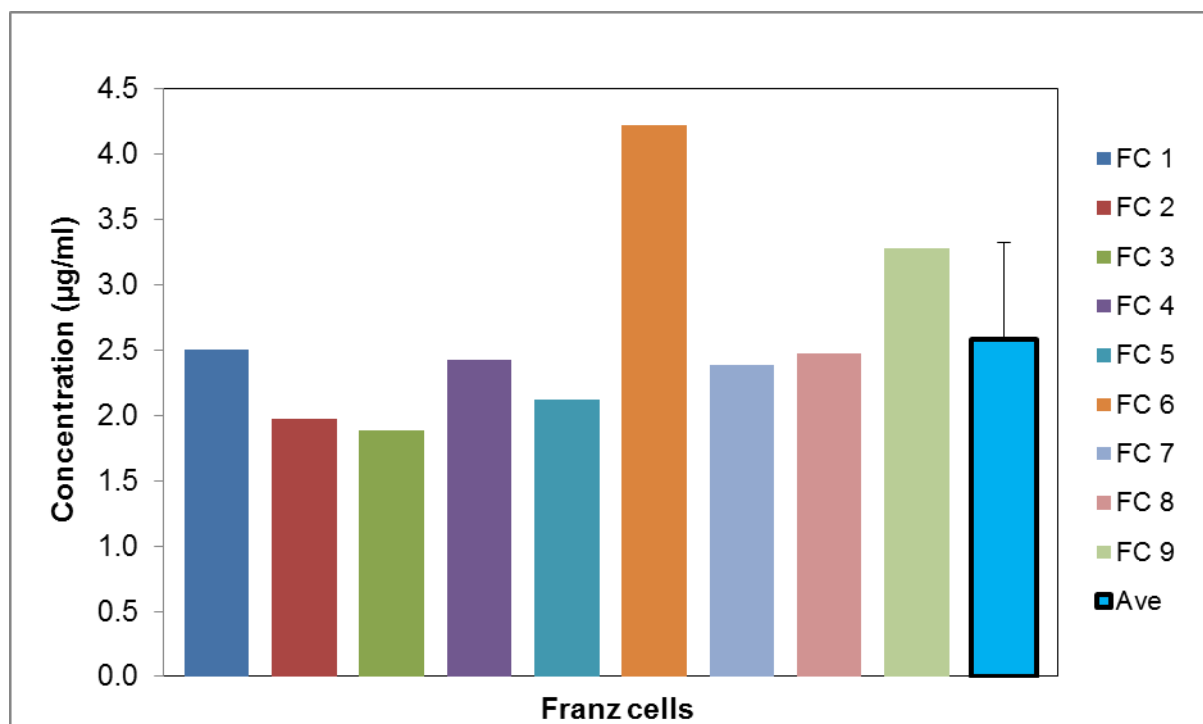


Figure D.70: Roxithromycin concentration (µg/ml) from niosome vesicle **CD3** in the ED after tape stripping

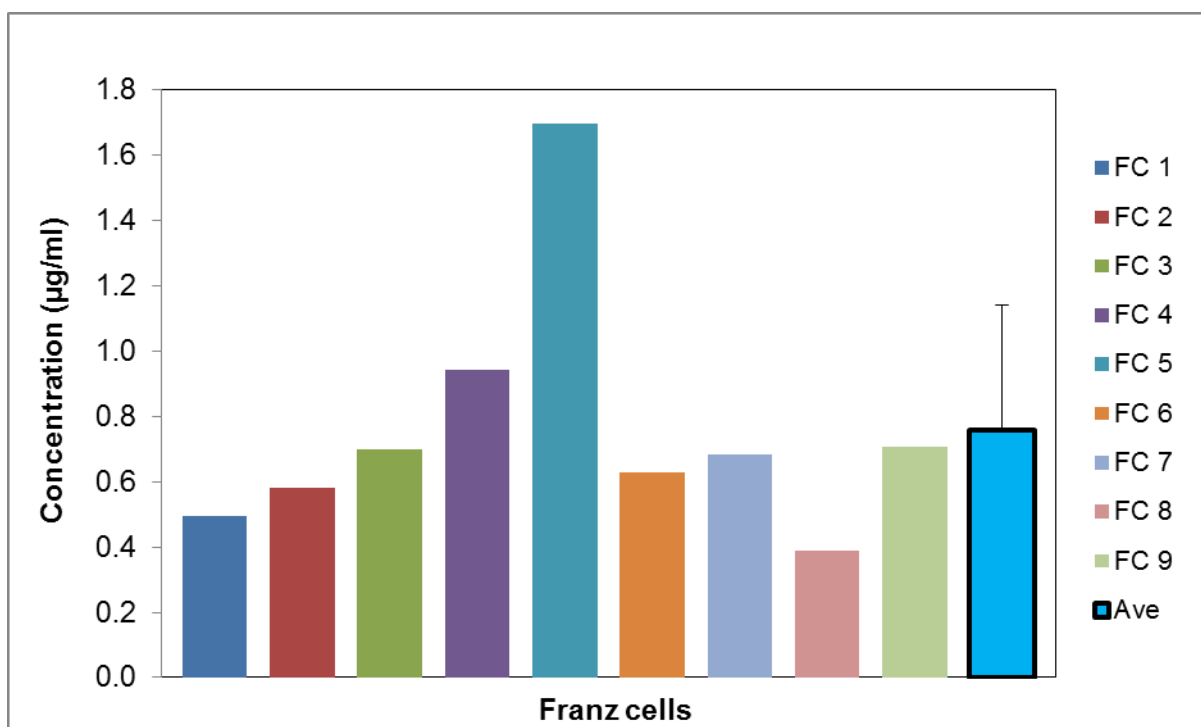


Figure D.71: Roxithromycin concentration (µg/ml) from niosome vesicle **CD4** in the ED after tape stripping

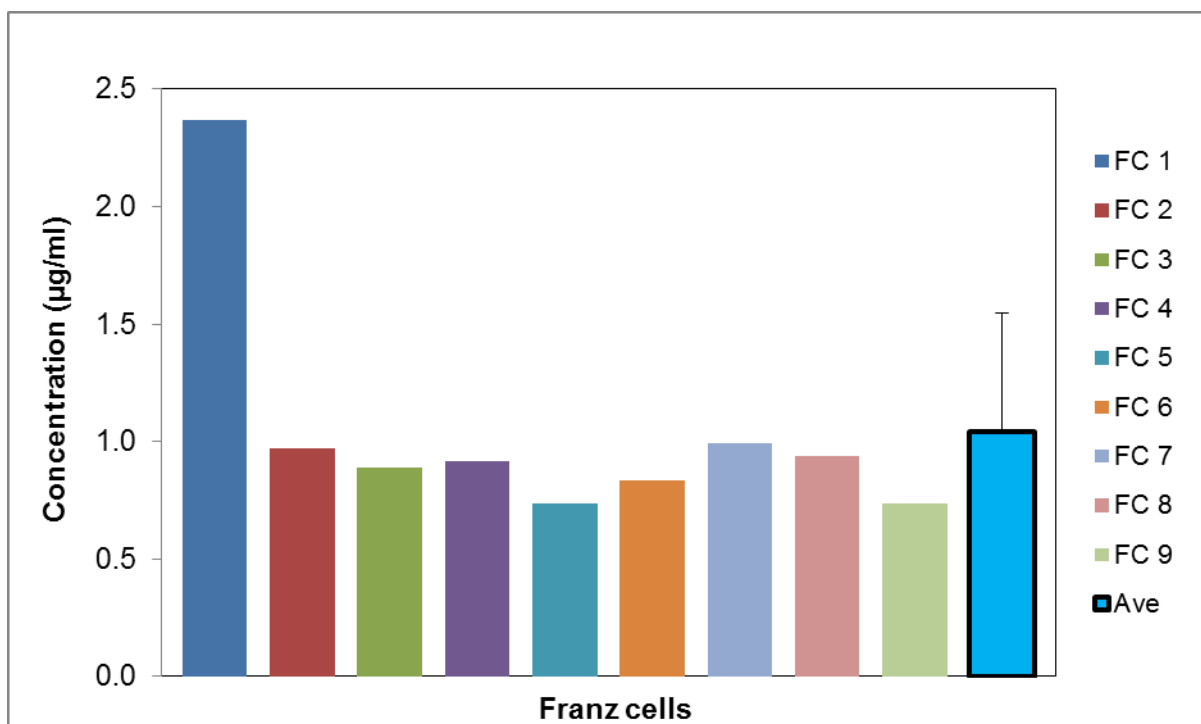


Figure D.72: Roxithromycin concentration (µg/ml) from niosome vesicle **CD5** in the ED after tape stripping

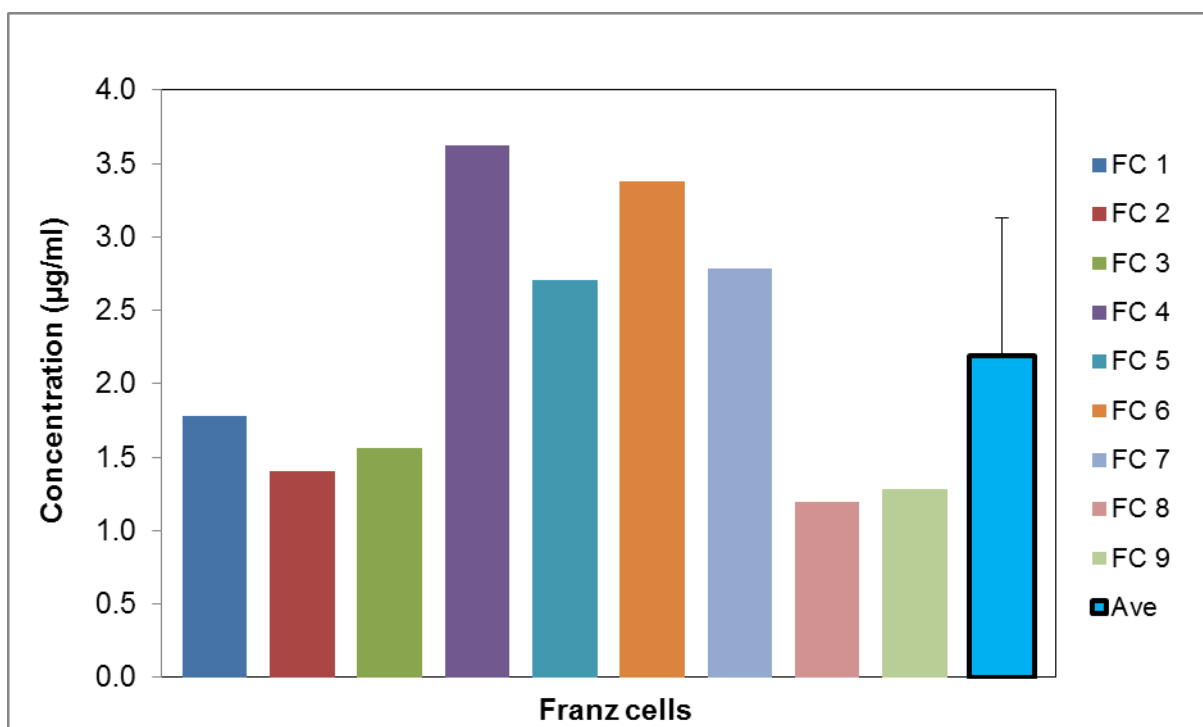


Figure D.73: Roxithromycin concentration (µg/ml) from niosome vesicle **QC1** in the ED after tape stripping

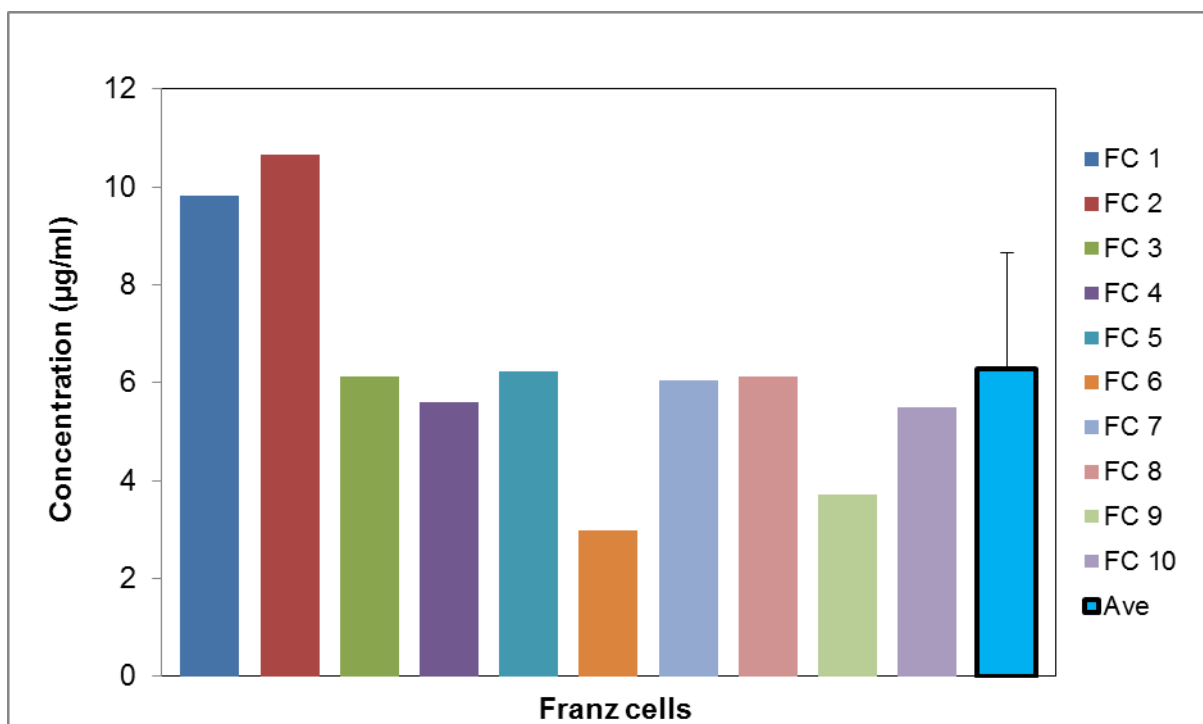


Figure D.74: Roxithromycin concentration (µg/ml) from niosome vesicle **QC2** in the ED after tape stripping

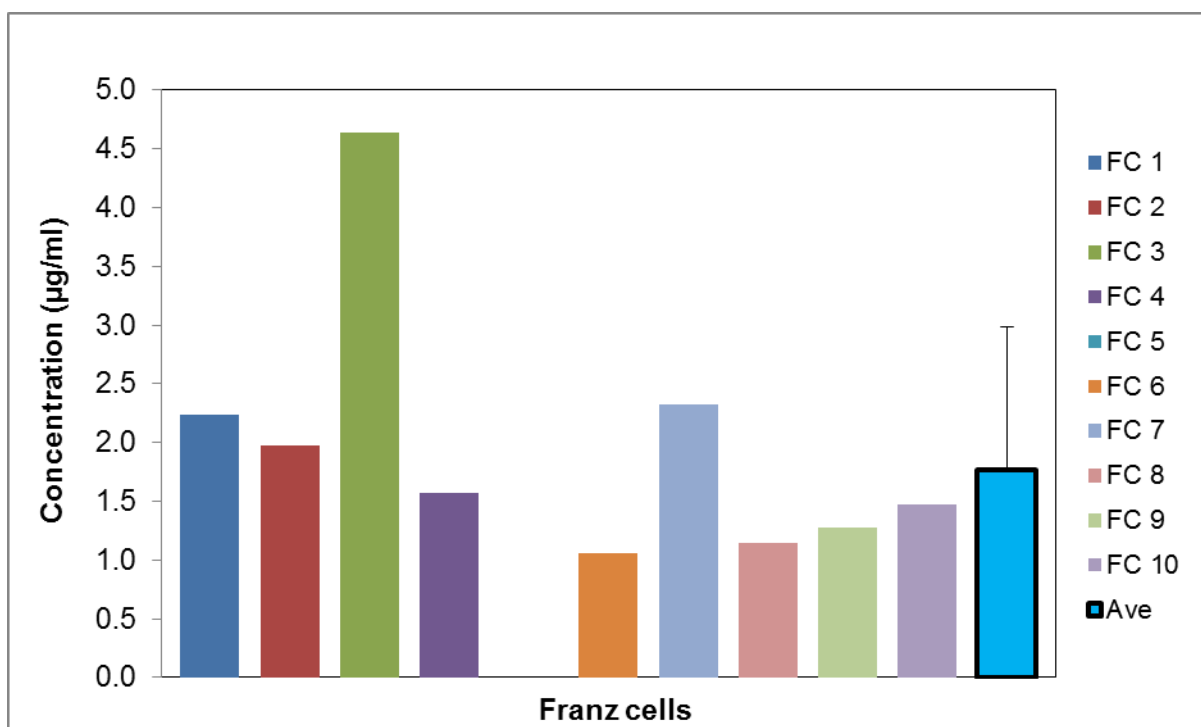


Figure D.75: Roxithromycin concentration (µg/ml) from niosome vesicle **QC3** in the ED after tape stripping

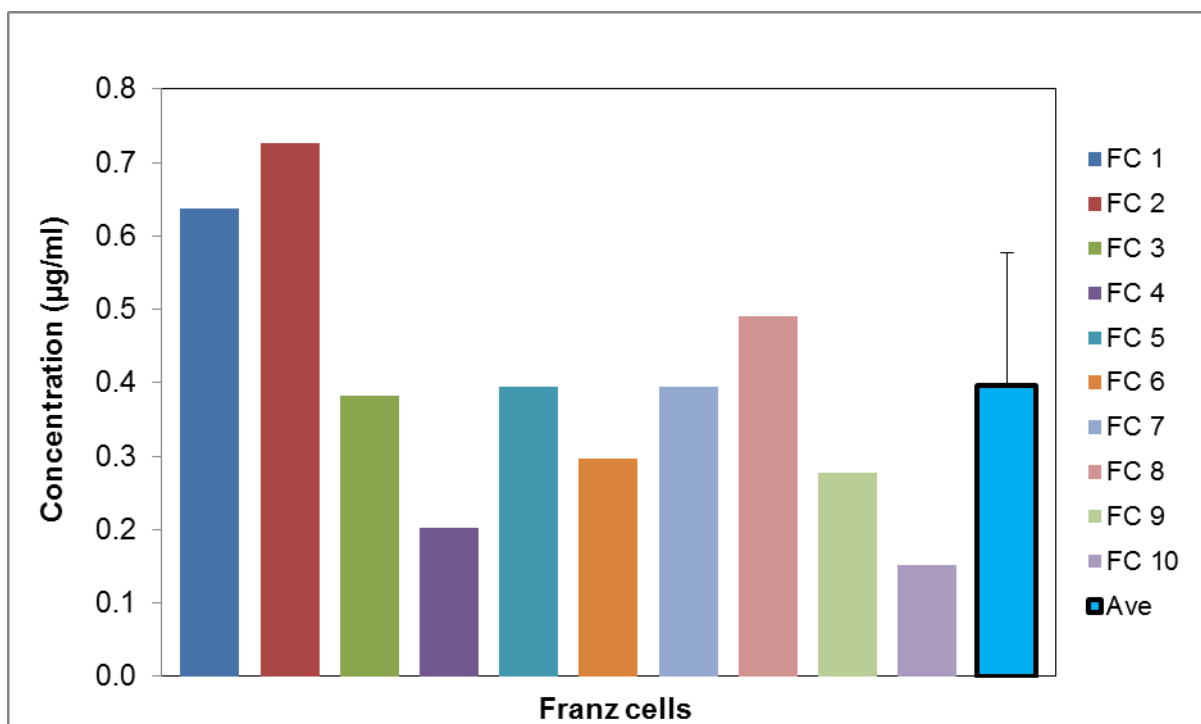


Figure D.76: Roxithromycin concentration (µg/ml) from niosome vesicle **QC4** in the ED after tape stripping

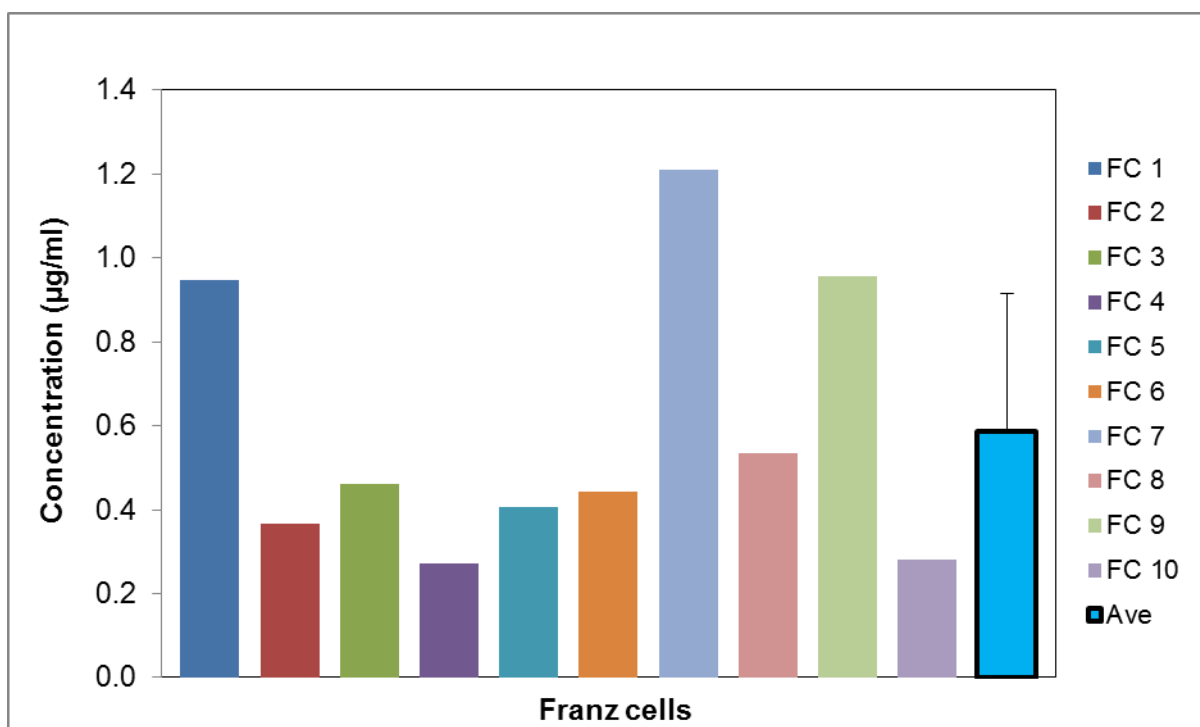


Figure D.77: Roxithromycin concentration (µg/ml) from niosome vesicle **QC5** in the ED after tape stripping

D.4 Conclusion

The aim was to determine which dispersion of the set of fifteen dispersions containing the three solid-state forms of roxithromycin and the five ratios of cholesterol and Span[®] 40 would deliver the best results for topical delivery. Roxithromycin is an antibiotic, used for the treatment of acne that is located in the deep dermal layers of the skin (More *et al.*, 2016:197). It is therefore important for the API to reach the target area and to have a localised effect in order to treat this skin disease. Niosomes are thus an excellent choice as it provides prolonged release of the drug, it increases the time the drug stays in the SCE and ED and it improves skin permeation (Shilakari *et al.*, 2013:80).

The solubility of roxithromycin in PBS (pH 7.4) and the log D values were determined in a previous study by Csongradi (2015:177-178), and both presented favourable for topical drug delivery. Sufficient solubility is required if a substance wants to permeate successfully through the skin (Akhlaq *et al.*, 2014:178). From the log D value the conclusion was made that roxithromycin was a lipophilic drug, an essential fact to know since it could possibly indicate where the API would accumulate and why it acts in a certain way.

Membrane release studies were conducted to determine if the API was released from the niosome vesicle systems into the receptor phase. The results indicated all the dispersions had released the API from the vesicles. The dispersion containing **QC3** had the highest flux and

average percentage released of roxithromycin over the period of 6 h and was ± 2.3 times higher than the nearest competitor. The second and third highest values were that of **QC2** and **QC1**, respectively. The conclusion therefore can be made that the niosome dispersion containing the **QC** amorphous form of roxithromycin displayed the best results during the membrane release studies, followed by the dispersions containing **RM** and lastly, the dispersions containing the amorphous **CD**. It is important to remember that the membrane release studies were performed on a synthetic PVDF membrane and the results when skin diffusions studies were performed could vary considerably from that of the membrane release studies.

Skin diffusion studies followed by tape stripping were performed to see if any transdermal and/or topical delivery of the API had been achieved. Only four of the dispersions (**QC2**, **RM2**, **RM1** and **CD2**) had transdermal delivery and this outcome was favoured, as the aim was to achieve topical delivery of the API into the skin. Three of the dispersions that were delivered transdermally were that of dispersion 2 (1.0:1.0), containing equal amounts of cholesterol and Span[®] 40. It could be assumed they had adequate lipophilic and hydrophilic properties to penetrate through the lipophilic regions of the stratum corneum and then deeper into the aqueous dermal layers to reach the systemic circulation (Perrie *et al.*, 2012:393).

Roxithromycin concentrations were quantified in the SCE and ED with only six dispersions having results above the LOD and LLOQ in both the skin layers. For the SCE, the dispersion which displayed the best average concentration of the API was **QC1**, followed by **RM5**, **QC2**, **RM1**, **CD1** and **CD5**. It was noticed that dispersions containing more Span[®] 40 than cholesterol (dispersions 1 and 5) resided in the SCE. This observation could possibly be ascribed to Span[®] 40 having a HLB value of 6.7, making it more oil soluble, thus it would favour a lipophilic environment (CRODA, 2015:45). The dispersion with the highest average concentration of the API in the ED was **QC2**, followed by **RM4**, **RM3**, **CD3**, **CD2** and **RM1**. It was observed that dispersions 3 and 4, consisting of a greater ratio of cholesterol than Span[®] 40, were found in the ED, which could be attributed to the amphiphilic nature of cholesterol favouring the hydrophilic environment and residing in the ED. Whereas dispersion 2 contained equal amounts of cholesterol and Span[®] 40, therefore it can be assumed the solid-state forms have a greater influence on the penetration into the ED rather than the ratio of the excipients used. From the concentrations obtained in the SCE and ED, the dispersions containing the amorphous **QC** form of roxithromycin had the highest concentration of roxithromycin in the skin followed by **RM** and lastly, **CD**. Although, a few dispersions with **CD** showed values above the LOD and LLOQ, it was surprising; since the membrane release studies had very poor results.

A possible reason why all the dispersions did not diffuse into the skin during this study could be due to the initial concentration of the API in the dispersion. Passive diffusion regulates the absorption of the API through the skin, consequently, the API travels according to the

concentration gradient, i.e. a high to low concentration (Jhawar *et al.*, 2013:49). Hence, a higher concentration of the API could therefore result in better permeation into the skin.

Nonetheless, the aim of this study was reached as quantifiable concentrations of the API were found in the ED and therefore the topical delivery of roxithromycin was successful. It was found that the excipients and the solid-state forms had an influence on the diffusion and/or penetration into and through the skin. Depending on the target area, certain excipients could be used to reach the specific target areas, i.e. in this study, a higher ratio of cholesterol than Span[®] 40 was required to target the ED. It should also be noted that this may not be true in all cases and could be investigated in the future.

The following conclusions can thus be made:

- Roxithromycin in its different solid-state forms was successfully delivered topically.
- The dispersion of **QC3** displayed the highest drug concentration during the membrane release studies.
- The dispersion of **QC1** displayed the highest drug concentration in the SCE.
- The dispersion of **QC2** displayed the highest drug concentration in the ED.
- The dispersions **QC2** and **RM1** were the only two dispersions that delivered the API transdermally and topically (SCE and ED) and also had concentration values higher than the LLOQ.
- Of the three solid-state forms, the dispersions containing the amorphous **QC** form showed the best results followed by **RM** and lastly, the amorphous **CD** form. This is probably due to the fact that the higher free energy of **QC** resulted in enhanced API entrapment into the niosome bilayer. It is unclear why the amorphous **CD** form displayed results inferior to those of the crystalline **RM** form.
- It is possible to deliver the API to the systemic circulation using the skin as an entry point.

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ICH **see** International Conference on Harmonisation.

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Appendix E

Author guidelines: The European Journal of Pharmaceutical Sciences

INTRODUCTION

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

Types of Paper

Research articles

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

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

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

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

Review articles

The manuscript of a review article should be arranged as described for research articles but according to the following sections: title page, abstract and keywords (indexing terms, normally 3 – 6 items), Introduction, Specific sections determined by the author, Conclusions, Acknowledgements, References, Figure legends and Figures, Tables. Sections ranging from the Introduction to the Conclusions should be numbered. Subdivisions within a section should

also be numbered within that section: 2.1., 2.2., 2.3. etc. All pages should be numbered consecutively, the title page being p.1.

Commentaries and Mini-reviews

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

Commentaries (Guidance)

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

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

The journal is looking for a stimulating and provoking essays, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to preferably recent peer-reviewed material. The word count should be approximately 2,000 words maximum.

The commentary should have a short abstract summary of 150 to 200 words and 4 – 5 key words should be included. The text should be broken down into 4 – 5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes.

Mini-review (Guidance)

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

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

The structure of the mini-review is as follows: a title page followed by a 200 – 300 word abstract with 4 – 5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-

reviewed material. There is a combined maximum of 5 figures/tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

Submission checklist

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

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address

All necessary files have been uploaded:

Manuscript:

- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
- Indicate clearly if color should be used for any figures in print

Graphical Abstracts/Highlights files (where applicable)

Supplemental files (where applicable)

Further considerations

- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)
- A competing interests statement is provided, even if the authors have no competing interests to declare
- Journal policies detailed in this guide have been reviewed
- Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing

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

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milligram	mg	nanomole	nmol
microgram	μg	picomole	pmol
nanogram	ng	femtomole	fmol
mole (gram-molecule)	mol	equivalent	eq

Units of time

hour	h	millisecond	ms
minute	min	microsecond	μs
second	s		

Units of volume

litre	l
millilitre	ml
microlitre	μl

Units of length

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centimetre	cm	nanometre	nm
millimetre	mm		

Units of concentration

molar (mol/l)	M	nanomolar	nM
millimolar	mM	picomolar	pM
micromolar	μM		

Units of heat, energy, electricity

joule	J	volt	V
degree Celsius (centigrade)	°C	ohm	Ω
coulomb	C	siemens	S
ampere	A		

Units of radiation

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

Miscellaneous

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dissociation constant	K_d
median doses	LD ₅₀ , ED ₅₀
probability	P
routes of drug administration	i.v., i.p., s.c., i.m.
square centimetre	cm ²
standard deviation	S.D.
standard error of the mean	S.E.M.
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